Cannabinoid Regulation of Serotonin 2A ($5\text{-}HT_{2A}$) Receptor Signaling

By

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Cannabinoid Regulation of Serotonin 2A (5-HT_{2A}) Receptor Signaling

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Abstract

Accumulating evidence indicates that sustained cannabinoid agonist exposure may precipitate the onset of some neuropsychiatric disorders that are associated with dysfunction of serotonin 2A (5-HT_{2A}) receptor neurotransmission in the brain. Recent behavioral evidence suggests that non-selective cannabinoid agonists can regulate 5-HT_{2A} receptor signaling in the brain. However, the molecular mechanisms of this cannabinoid-induced change in 5-HT_{2A} receptor signaling are unknown. Here we present experimental evidence that repeated treatment with a non-selective cannabinoid agonist, CP55940, can enhance 5-HT_{2A} receptor activity and expression in the rodent prefrontal cortex (PFCx) and two neuronal cell culture models, CLU13 and A1A1v cells. Cannabinoids mediate many of their physiological effects through two cannabinoid receptors, cannabinoid type 1 (CB_1) and cannabinoid type 2 (CB_2), which are expressed in the brain. Our evidence indicates that CB_2 receptors would mediate this phenomenon because cannabinoid-induced upregulation and enhanced activity of 5-HT_{2A} receptors was: (1) induced by selective CB_2 receptor but not selective CB_1 receptor agonists and (2) inhibited by CB_2 but not CB_1 shRNA lentiviral particles in neuronal cells. CB_2 receptors are positively coupled to the Extracellular Regulated Kinase 1/2 (ERK1/2) signaling cascade which has been shown to regulate transcription factors, such as cyclic AMP response binding protein (CREB) and activator protein 1 (AP-1), which have consensus sequences in the rat 5-HT_{2A} receptor promoter. Interestingly, we found that cannabinoid-induced upregulation of 5-HT_{2A} receptors was inhibited by ERK1/2 inhibitors and AP-1, but not CREB, inhibitor. Studies show that G-protein receptor kinases (GRKs), such as GRK5, can phosphorylate G-protein coupled receptors (GPCRs) to regulate the formation of the Beta (β)-Arrestin 2/ERK scaffolding complex that can modulate the long term activation of ERK1/2 signaling. Indeed, we found that β-

Arrestin2 shRNA lentiviral treatment prevents cannabinoid-induced upregulation of 5-HT_{2A} receptor and significantly reduces cannabinoid activation of ERK1/2 signaling. Moreover, GRK5 shRNA lentiviral particle treatment inhibits cannabinoid-induced: (1) enhanced CB_2 receptor phosphorylation (2) enhanced co-immunopreciptiation of β-Arrestin 2 and ERK1/2 and (3) upregulation and enhanced activity of 5-HT_{2A} receptors. The enhanced function of 5-HT_{2A} receptors could also involve cannabinoid-induced enhanced interaction between 5-HT_{2A} and dopamine D2 (D_2) receptors. We found that Sprague-Dawley rats treated with a non-selective cannabinoid receptor agonist (CP55940) showed enhanced co-immunoprecipitation of 5-HT_{2A} and D_2 receptors. Formation of the functional 5-HT_{2A} and D_2 receptor heteromer in the PFCx is suggested to contribute to the pathophysiology of neuropsychiatric disorders such as schizophrenia. Furthermore, enhanced activity of cortical 5-HT_{2A} receptors is associated with several physiological functions and neuropsychiatric disorders such as stress response, anxiety, depression, and schizophrenia. Therefore, these cannabinoid-regulated molecular mechanisms may be relevant to some neuropsychiatric disorders in humans.

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List of Abbreviations

2-AG: 2-arachidonoylglycerol

5-HT: 5-hydroxytrptamine (serotonin)

β-Arr2: Beta-Arrestin 2

Δ9-THC: Δ9-tetrahydrocannabinol

ACEA: Arachidonyl-2'-chloroethylamide

AP-1: Activator protein 1

CREB: Cyclic AMP response binding protein

DAG: 1,2 Diacylglycerol

DOI: (-)-1-(2,5-Dimethoxy-4-lodophenyl)-2-aminopropane HCl

ERK: Extracellular signal-regulated kinase

GAPDH: Glyceraldehyde3-phosphate dehydrogenase

GPCR: G-protein coupled receptor

GRK: G-protein coupled receptor kinase

IP_3: Inositol trisphosphate

MAPK: Mitogen-activated protein kinases

qRT- PCR: Quantitative real time-polymerase chain reaction

PFCx: Prefrontal cortex

PI: Phosphoinositol

PKC: Protein kinase C

PLCβ: Phospholipase C β

Chapter 1: Introduction

Cannabinoids are a diverse group of compounds found in the plant *Cannabis sativa* L., that occur naturally in the human body, and which are produced synthetically in a laboratory setting [1]. These compounds are being shown to have diverse therapeutic applications in the treatment of a variety of conditions including neuropathic pain, neurodegenerative diseases, stroke, cocaine addiction, and glaucoma [2-6]. Cannabinoids are also used recreationally as cannabis users comprise the largest number of illicit drug users worldwide [7-9]. Furthermore, recreational use of synthetic cannabinoids are becoming more prevalent [9]. However, a continuously growing number of independent clinical studies are providing strong evidence that repeated exposure to some cannabinoid receptor agonists can have adverse effects on mental health that are associated with dysfunction of serotonin neurotransmission in the brain [10-14]. Currently, the precise molecular mechanisms by which repeated exposure to cannabinoids may contribute to neuropsychiatric disorders such as schizophrenia and anxiety are unknown.

Cannabinoids can produce many of their physiological effects through binding two G-protein coupled cannabinoid receptors, CB_1 receptor and CB_2 receptor, with high affinity in the brain [15]. These cannabinoid receptors couple the G-protein Gαi/o, can regulate the activation of ERK1/2 signaling cascade, and are found throughout the forebrain including the hippocampus, nucleus accumbens, and PFCx [15-19]. CB_1 receptors are predominately expressed presynaptically, while recent studies suggest that CB_2 receptors are located postsynaptically on pyramidal neurons in areas of the brain such as the PFCx and hippocampus [16;20;21]. Activation of presynaptically located CB_1 receptors has been shown to have inhibitory effects on

neurotransmission [22] while the molecular mechanism of postsynaptically located CB_2 receptors is not well known.

5-HT_{2A} receptors have been implicated in regulation of stress, mood and impulse control [23;24]. The 5-HT_{2A} receptors are the most abundant serotonin receptors expressed in the PFCx and are most highly expressed in the pyramidal neurons [25]. Impaired function of cortical 5-HT_{2A} receptors has been linked with neurological and psychiatric disorders such as schizophrenia, Alzheimer's disease, depression, and anxiety [24;26;27]. Furthermore, the therapeutic effects of atypical antipsychotics are proposed to be mediated by the desensitization and down-regulation of 5-HT_{2A} receptors in the pyramidal neurons in the PFCx [28-30]. Moreover, Magalhaes et al. have provided evidence indicating that over-expression and enhanced function of 5-HT_{2A} receptors in the PFCx can modulate anxiety-like behaviors [31].

Interestingly, behavioral evidence suggests that repeated cannabinoid exposure can enhance the activity of 5-HT_{2A} receptors [32]. Specifically, Hill et al. found that repeated treatment with non-selective cannabinoid receptor agonist, HU210, led to a significant enhancement of 5-HT_{2A} receptor mediated head-shake responses [32]. This behavioral test is commonly used as a marker of 5-HT_{2A} receptor function *in vivo* [33-35]. This behavior is prevented by pretreatment with selective 5-HT_{2A} receptor antagonists and is absent in 5-HT_{2A} receptor knock out animals [33-35]. However, the detailed molecular mechanism by which cannabinoid receptor agonists regulate 5-HT_{2A} receptor signaling was unknown.

Since cannabinoids and 5-HT_{2A} receptors are implicated in many of the same neuropsychiatric disorders, our **overall main objective** was to examine the effects of repeated cannabinoid treatment on expression and function of 5-HT_{2A} receptors in rat PFCx. After

establishing that chronic treatment with a non-selective CB_1/CB_2 receptor agonist can upregulate and enhance activity of 5-HT_{2A} receptors, we moved forward to elucidate the molecular mechanisms underlying this cannabinoid-induce upregulation and enhanced function of 5-HT_{2A} receptors in neuronal cultured cells.

This dissertation is divided into the following objectives:

1. Determine effect of repeated cannabinoid agonist treatment on 5-HT_{2A} receptor expression and function in rat PFCx.
2. Study the role of ERK1/2 in the cannabinoid-induced upregulation of 5-HT_{2A} receptor signaling.
3. Examine the relative roles of CB_1 and/or CB_2 receptors in the cannabinoid-induced upregulation and enhanced activity of 5-HT_{2A} receptors.
4. Determine the role of β-Arrestin 2 in the cannabinoid-induced upregulation of 5-HT_{2A} receptors and the effects of repeated cannabinoid treatment on the β-Arrestin 2/ERK scaffolding complex formation.
5. Identify whether GRK5 is necessary for the cannabinoid-induced upregulation and enhanced activity of 5-HT_{2A} receptors.

Chapter 2: Review of Literature

2.1 Cannabinoids

Cannabinoids are a diverse group of highly lipophilic compounds that bind cannabinoid receptors with high affinity [15]. Three different types of cannabinoid ligands have been identified which include: endocannabinoids that are produced endogenously in humans and animals, phytocannabinoids in the *Cannabis sativa* L. plant, and synthetic cannabinoids which are generated in a laboratory [1]. Cannabinoid agonists produce their effects mainly through the activation of two GPCRs in the brain, the CB_1 and CB_2 receptors [36-38]. These receptors bind endocannabinoids, phytocannabinoids, and synthetic cannabinoids with high affinity (Table 1) [36-38].

2.1.1 Endocannabinoids

Endocannabinoids are a family of neuromodulatory lipids that are produced endogenously in mammals. Two main endogenous lipids that have received the greatest amount of study include, anandamide (N-arachidonoylethanolamide, AEA) [39] and 2-arachidonoylglycerol (2-AG) [40]. Other compounds that are considered to be endocannabinoids include: 2-arachidonylglyceryl ether (noladin ether), O-arachidonoylethanolamine (virodhamine) and N-arachidonoyldopamine [41]. However, controversy remains over classification of these latter substances as endocannabinoids because several groups have failed to detect these substances at any "appreciable amount" in the mammalian brain.

In contrast to classical neurotransmitters and neuropeptides, which are produced and stored in synaptic vesicles for future release, endocannabinoids are produced at the postsynaptic

membrane through a phospholipase enzyme that cleaves them from a phospholipid to which they are attached [42-44]. The endocannabinoids produced by the postsynaptic neuron can then activate cannabinoid receptors in the presynaptic neuron through retrograde signaling [45]. Indeed, activation of presynaptically located CB_1 receptors by endocannabinoids at the presynapse can then modulate signaling producing either a transient or prolonged reduction in release of neurotransmitters such as GABA and/or Glutamate [45]. Both Anandamide and 2-AG have higher affinity for CB_1 receptors compared to CB_2 receptors (Table 1) and are agonists for both of these receptors [41].

Anandamide formation mainly occurs through phosphodiesterase-mediated cleavage of N-arachidonyl phosphatidylethanolamine (NAPE) [43]. 2-AG is synthesized through phospholipase C (PLC) mediated hydrolysis of phosphatidyl inositol biphosphate (PIP2) which leads to formation of inositol- (1,4,5)-tripphosphate (IP_3) and 1,2 diacylglycerol (DAG). DAG is then converted to 2-AG by diacylglycerol lipase (DAGL) [44]. Endocannabinoid signaling is terminated via uptake of endocannabinoid by a membrane transporter and then intracellular hydrolysis [46]. Two endocannabinoid metabolizing enzymes have been identified: fatty acid amine hydrolase (FAAH) and monoacylglycerol lipase (MGL). Anandamide is metabolized by fatty acid amine hydrolase [42] while 2-AG is metabolized by monoacylglycerol lipase [47].

Table 1: CB_1 and CB_2 receptor Ki values of various cannabinoids

Classification	Cannabinoid	CB_1 (nM)	CB_2 (nM)	Reference
Endocannabinoids	Anandamide	52	1930	[48]
	2-AG	58.3	>3,000	[48]
Phytocannabinoids	Δ9-THC	40.7	36.4	[49]
	CBD	308	96.3	[50]
Synthetic Cannabinoids				
Non-selective agonists	WIN55,212-2	3.3	62.3	[51]
	JWH073	8.9	38	[52]
	HU210	0.061	0.52	[53]
	CP55940	0.6	0.7	[54]
Selective CB_1 agonist	ACEA	1.4	3,100	[55]
Selective CB_1 antagonists	SR 141716 A	1.98	>1,000	[56]
	PF514273	1	>10,000	[57]
	AM 251	7.49	>2,000	[58]
Selective CB_2 agonists	JWH133	677	3.4	[59]
	GP1a	353	0.037	[60]
Selective CB_2 antagonists	AM630	5148	31.2	[61]
	JTE907	1050	0.38	[62]

2.1.2 Phytocannabinoids

Phytocannabinoids are produced by the *Cannabis sativa* L. plant and are concentrated in a viscous resin in the glandular trichomes of the plant [63]. There are over 80 cannabinoids produced by the *Cannabis sativa* L. plant [64]. Phytocannabinoids are grouped into the following main classes: cannabigerol (CBG), cannabichromene (CBC), cannabicyclol (CBL), cannabivarin (CBV), tetrahydrocannabivarin (THCV), cannabidivarin (CBDV), cannabichromevarin (CBCV), cannabigerovarin (CBGV), and cannabigerol monomethyl ether (CBGM). All classes are from cannabigerol-type compounds and differ primarily through the way the precursor is cyclized [63].

The most notable phytocannabinoids are Δ^9-tetrahydrocannabinol (Δ^9-THC) and Cannibidiol (CBD) which are the most prevalent bioactive constituents found in *Cannabis sativa* L. and have received the most study [63]. Δ^9-THC was first isolated from the plant *Cannabis sativa* L. in pure form in 1964 and has been shown to have analgesic and neuroprotective antioxidant activities [65]. Δ^9-THC binds cannabinoid receptors at submicromolar concentrations with similar affinities for CB_1 and CB_2 receptors and acts as a partial CB_1 and CB_2 receptor agonist (Table 1) [41]. Cannabidiol has been shown to relieve nausea, inflammation, anxiety, and to be effective in the treatment of schizophrenia and multiple sclerosis [66-69]. Cannabidiol has greater affinity for the CB_1 receptor than CB_2 receptor (Table 1) and acts as a CB_1 and CB_2 receptor antagonist [68].

2.1.3 Synthetic Cannabinoids

Synthetic cannabinoids were first synthesized to further examine the therapeutic applications of cannabinoids and signaling mechanisms of cannabinoid receptors. Synthetic

cannabinoids are grouped into a variety of distinct chemical classes including: classical cannabinoids, non-classical cannabinoids, aminoalkylindoles, and eicosanoids [64]. Classical and non-classical cannabinoids are structurally related to Δ^9-THC but have variable side chain modifications that can increase receptor affinity [41]. Classical cannabinoids are ABC-tricyclic dibenzopyran derivatives and include cannabinoids such as HU210, a highly potent non-selective cannabinoid receptor agonist that is widely used in *in vivo* and *in vitro* studies [41]. HU210 has similar affinities for CB_1 and CB_2 receptors (Table 1) and mediates long lasting pharmacological effects [41]. Non-classical cannabinoids are characterized by the opening of the dihydropyran ring and include cannabinoids such as CP55940 [41]. CP55940, which has been widely used to study cannabinoid receptor signaling and has long lasting effects, binds CB_1 and CB_2 receptors with similar affinity (Table 1) and is a full agonist for both receptors [41]. Aminoalkylindoles are structurally different from the cannabinoid agonists previously described and include cannabinoids such as WIN 55,212-2 and JWH-073 [70]. WIN 55,212-2 and JWH-073 display greater affinity for CB_1 receptors than CB_2 receptors and are full agonists for CB_1 and CB_2 receptors (Table 1)[51;52]. Eicosanoids are related to endocannabinoids and include synthetic cannabinoids such as arachidonyl-2'-chloroethylamide (ACEA) [64].

2.1.4 Selective Cannabinoid Receptor Agonists and Antagonists

Selective cannabinoid receptor agonists or antagonists generally have 100-1,000 fold selectivity over CB_1 or CB_2 receptors. The observation that structural modification of anandamide resulted in greater selectivity for the CB_1 receptor led to the development of selective CB_1 receptor agonists [55]. Examples of selective CB_1 receptor agonists include ACEA (Table 1) and ACPA [55;71]. ACEA, which is more selective than ACPA, is a potent and highly selective CB_1 receptor agonist that displays 1,400-fold selectivity over CB_2 receptors (Table 1)

[55]. ACEA has been shown to have antidepressant-like properties [72], while selective CB_1 receptor antagonists, such as SR141716A (Table 1), have been shown to depress food consumption and promote weight loss [73]. Other selective CB_1 receptor antagonists include PF514273 and AM251 (Table 1) [41;57]. Selective CB_2 receptor agonists are being shown to have wide therapeutic applications in the treatment of conditions such as stroke, neuropathic pain, neurodegenerative diseases, and cocaine addiction [2-5]. JWH133 and GP1a are two selective CB_2 receptor agonists (Table 1) [59;60]. Compounds such as AM630 and JTE907 bind CB_2 receptors with higher affinity than CB_1 receptors (Table 1) and exhibit marked potency as CB_2 receptor antagonists [61;62].

2.2 Cannabinoid Receptors

Currently, two high affinity cannabinoid receptors have been cloned and well-characterized pharmacologically: the CB_1 and CB_2 receptors [36-38]. Both CB_1 and CB_2 receptors are prototypical G-protein coupled receptors that belong to family 1a of the GPCR superfamily and couple to the $G\alpha_{i/o}$, G-protein signal transduction pathway. These cannabinoid receptors share structural characteristics with other GPCRs including: an extracellular N-terminus, seven transmembrane α-helices with extracellular and intracellular loops, and an intracellular C-terminus [74].

2.2.1 CB_1 Receptor Gene and Structural Characteristics

The CB_1 receptor was first identified in 1988 in the rat [38] but since has been identified in other mammals as well as birds, fish, and reptiles [75]. The gene for the CB_1 receptor (CNR1) is located on mouse chromosome 4, rat chromosome 5, and human chromosome 6 [75]. The translated regions of the rodent CB_1 receptor are intronless while two splice variants of the

human CB_1 receptor have been identified but their abundance is low and their physiological significance has yet to be identified [76;77].

The human CB_1 receptor shares 94% amino acid sequence identity with rodent CB_1 receptors [75]. Compared to members of the family 1a GPCR superfamily, the CB_1 receptor has an exceptionally long extracellular N-terminal domain (N-tail) of 116 amino acids which impairs the translocation of the receptor across ER membrane, resulting in rapid degradation of the CB_1 receptor by proteasomes and leads to low expression of this receptor at the plasma membrane [78]. Currently, several residues of the CB_1 receptor have been identified as important for binding of CB_1 agonists and antagonists, such as lysine 192 and a cluster of hydrophobic amino acids in the transmembrene domains 3, 5, and 6 which have been shown to be critical for the binding of cannabinoid agonists and antagonists [70;79]. Cysteine residues 257 and 264 are required for a functional CB_1 receptor [80] while several residues located within the C-terminus of the CB_1 receptor have been identified as important for regulation of CB_1 receptor signaling including: serine 317, serine 426, and serine 430 [81;82]. Indeed, posttranslational modifications of these amino acid residues by specific kinases might contribute to the regulation of the activity of CB_1 receptors. For instance, protein kinase C mediated phosphorylation of serine 317 modulates uncoupling of G-protein signaling [81] and G-protein receptor kinase 3 (GRK3) mediated phosphorylation of serine 426 and serine 430 are involved in desensitization of CB_1 receptor activation of ERK1/2 [82].

2.2.2 CB_2 Receptor Gene and Structural Characteristics

The CB_2 receptor was first identified in 1992 in human myeloid cells [36] but has also been identified in mice, rats, bovine, and zebra fish [75]. The genes for mouse, rat, and human

CB_2 receptors (CNR2) are located on chromosomes 4, 5, and 1, respectively [83]. The rodent CB_2 genes have been reported to be intronless [83] while the human CB_2 gene has been reported to have two splice variants: CB_2a and CB_2b [84]. CB_2a is mostly expressed in the testis and brain including the caudate, amygdala, PFCx, hippocampus, cerebellum, and nucleus accumbens.while CB_2b is mostly expressed in spleen and leukocytes [84].

The CB_2 receptor shares 44% amino acid sequence identity with the CB_1 receptor [84]. Humans and mice share 82% identity in amino acid sequence of CB_2 receptors whereas mice and rat share 93% identity [75]. Human, rat, and mouse sequences vary at the C-terminus with the mouse and rat CB_2 receptor C-terminus being 13 amino acids shorter or 50 amino acids longer, respectively, compared to the human CB_2 receptor C-terminus [64]. Unlike the CB_1 receptor, in which lysine 192 plays a critical role in binding of most cannabinoid ligands, the homologous residue lysine 109 in CB_2 transmembrane domain does not contribute to binding of cannabinoid ligands [85;86]. Currently, aspartate 130 in the aspartic acid–arginine–tyrosine motif, alanine 244, and serine 112 have been identified as being important for binding of CB_2 agonists [85;87]. Additionally, CB_2 helix III has been shown to play an important role in the regulation of cannabinoid activities [86]. Cysteines 313 and 320 are required for a functional CB_2 receptor, but not for ligand binding [87]. Similarly to CB_1 receptors, posttranslational modifications of specific amino acid residues by protein kinases such as GRK might contribute to regulate the activity of CB_2 receptors. For instance, GRK5 and/or GRK6 phosphorylation of serine 352 in the C-terminus of the CB_2 receptor has been identified as important for regulation of CB_2 receptor signaling including ERK1/2[88].

2.2.3 CB_1 and CB_2 Receptor Localization

The CB_1 receptor has been found to be widely distributed throughout the brain including the PFCx, hippocampus, striatum, amygdala, hypothalamus, cerebellum, pons and medulla [16;17;89]. Several studies have identified that the majority of high CB_1-expressing cells are GABAergic neurons belonging to cholecystokinin-positive and parvalbumin-negative types of interneurons [16;20;90]. Low CB_1 receptor expression has also been detected in dopaminergic, adrenergic, glutamatergic, and cholinergic neurons [90]. Of note, several studies have reported an absence of CB_1 receptor expression in pyramidal neurons and that CB_1 receptors are primarily located in presynaptic terminals of GABAergic and glutamatergic neurons within regions of the brain such as the PFCx and hippocampus [16;20;91].

While CB_1 receptors were initially identified in the brain, early reports identified CB_2 receptors only in immune cells [36;92]. However, later studies established the expression of CB_2 receptors in neurons in the PFCx, amygdala, hypothalamus, substantia nigra, striatum, and hippocampus of the healthy brain [18;19;93]. Furthermore, recent studies reported that there are functional CB_2 receptors in the medial PFCx including pyramidal neurons and that CB_2 receptors are mainly localized in the post-synaptic neurons [18;21;94].

2.2.4 Cellular Signaling of CB_1 and CB_2 Receptors

Similar to other GPCRs, CB_1 and CB_2 receptors in their inactive state are associated with inactive heterodimeric G-proteins which consist of GDP-bound α subunit with β and γ subunits. Stimulation by an agonist induces conformation changes that initiate a series of intracellular signal cascades, in part, through catalyzing an exchange of GTP for GDP on the α subunit and

triggers the disassociation of the α subunit from the βγ subunits. Signal transduction is terminated by the hydrolysis of GTP to GDP [95]. The classical paradigm of agonist-induced GPCR mediated signal transduction involves agonist-induced dissociation of the G-proteins from the GPCR and subsequent G-protein regulation of secondary messengers [96;97].

Both CB_1 and CB_2 receptors are coupled to the $G\alpha_{i/o}$ G-protein. Activation of these cannabinoid receptors leads to inhibition of adenylate cyclase and activation of mitogen-activated protein kinases (MAPK) such as ERK1/2, c-Jun N-terminal kinase (JNK), and p38 MAPK [98]. Recent evidence has highlighted that neuronal CB_1 receptors can modulate ERK1/2 signaling through $G_{i/o}$ and multiple tyrosine kinase receptors [99] and neuronal CB_2 receptors can modulate ERK1/2 signaling through β-Arrestin 2 [100]. Indeed, recent evidence has identified that β-Arrestins can recruit proteins such as ERK1/2 to the GPCR to form scaffolding complexes that can regulate the activation of signaling cascades [96;97]**.** CB_2 receptor transactivation of tyrosine kinase receptors has not been reported. CB_1 receptors can inhibit N- and P/R- type calcium channels, stimulate G-protein coupled inward rectifying potassium (GIRK) channels and enhance activation of A-type potassium channels (Figure 1) [98].

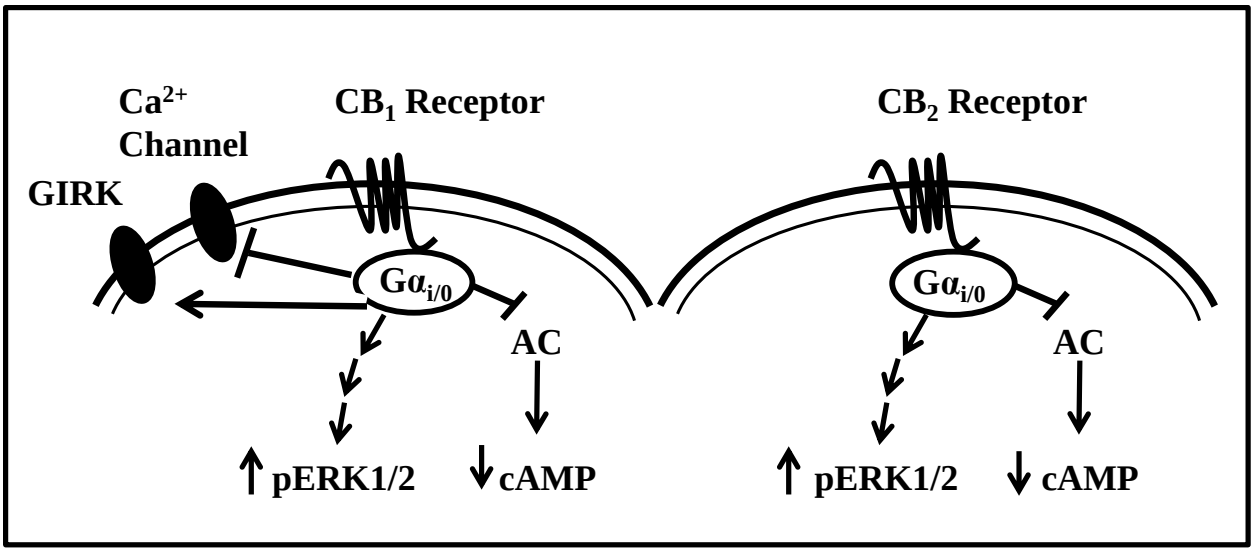

Figure 1: Signaling mechanisms associated with CB_1 and CB_2 receptors

2.3 Additional Cannabinoid Targets

Emerging evidence indicates that some, but not all, ligands for CB_1 and/or CB_2 receptors may target additional receptors that are either established or putative [101]. Recent pharmacological and molecular evidence suggests that the orphan receptor, GPR55, should be characterized as a cannabinoid receptor [102]. GPR55 has been detected in the vasculature and other peripheral tissues, couples to the $G\alpha_{13}$ G-protein, and can modulate the activation of rhoA, cdc42, and rac1 [103]. Additionally, other studies have suggested that GPR18 and GPR119 may be cannabinoid receptors as well; however, only a few ligands for GPR18 or GPR119 receptors have been identified and little is known about the signaling mechanisms of these receptors [104;105]. Some endogenous ligands for CB_1 and CB_2 receptors have also been shown to bind and activate transient receptor potential vanilloid receptor 1 (TRPV1) and peroxisome proliferator-activated receptors γ (PPARγ) [106;107].

2.4 Cannabinoids, Cannabinoid Receptors, and Neuropsychiatric Disorders

Repeated exposure to cannabinoids has been associated with the pathophysiology of neuropsychiatric disorders such as anxiety, depression, and schizophrenia [11;12;14]. While a causal link has not been identified between chronic cannabis use and the etiology of these neuropsychiatric disorders, recent evidence indicates that chronic use of cannabis may precipitate these disorders in individuals who are predisposed to developing them [12-14]. Yet a mechanism by which cannabinoids may precipitate these disorders has not been identified. The long-term effects of chronic synthetic cannabinoid agonist use, which are many times more potent than Δ9-THC, have yet to be addressed.

Recent reports have provided new and strong evidence linking chronic use of cannabis to an earlier onset of psychosis and schizophrenia [11-13]. Henquet et al. 2005, found a strong correlation between cannabis use and subsequent development of psychotic symptoms in a four year follow-up despite controlling for pre-existing psychotic symptoms. Another meta-analysis study found that the onset of psychosis was 2.7 years younger in cannabis users compared to non-users, suggesting that cannabis might precipitate the onset of neuropsychiartic disorders in individuals predisposed to developing them [13]. Accordingly, Kuepper et al. 2011, conducted a cohort study over 10 years and found that chronic cannabis use significantly increased the risk of incident psychotic experiences in adolescents despite controlling for age, sex, socioeconomic status, use of other drugs, urban/rural environment, and childhood trauma.

Preclinical evidence indicates that repeated exposure to non-selective cannabinoid agonists, Δ^9-THC, CP55940 and HU210 and selective CB_2 receptor agonists, such as JWH133, induces anxiety-like behaviors in rodents [93;108;109]. Furthermore, Reilly et al. 1998, reported that chronic cannabinoid users had higher levels of anxiety compared to those who did not use cannabis [110]. Accordingly, other studies have identified that the severity of anxiety symptoms positively correlates with the level of cannabis use [111;112]. Additionally, a cohort study over six years found that daily use of cannabis in young adolescence was associated with an over five-fold increase in the odds of reporting a state of anxiety or depression [10]. Preclinical studies also highlighted chronic, but not acute, exposure to non-selective CB_1/CB_2 (CP55940) and selective CB_2 receptor agonists (JWH133) induced anxiety-like behaviors in young adult [93] and adolescent rodents [108], respectively.

2.5 Serotonergic System in the Brain

Serotonin, also known as 5-hydroxytryptamine (5-HT), is a monoamine neurotransmitter and one of the most important brain neurotransmitters [113]. Serotonergic neurotransmission regulates a broad range of physiological and behavioral processes including thermoregulation, respiration, cognition, aggression, endocrine regulation, feeding, sleep-wake cycle, pain sensitivity, vascular function, and emesis [114;115]. Dysregulation of brain serotoninergic systems contributes to neuropsychiatric disorders such as depression, anxiety, and schizophrenia [115-117].

2.5.1 Serotonin Neurons

Brain 5-HT is synthesized by neurons of the raphe nuclei in the midbrain [113]. The raphe nuclei contains the majority of 5-HT neurons that innervate forebrain [118-120]. This region of the midbrain is divided into the dorsal raphe nucleus (DRN) and median raphe nucleus (MRN) [121;122]. The dorsal raphe nucleus is located on the midline of the brainstem and is subdivided into rostral and caudal regions [123] while the median raphe nucleus (MRN) is located rostral to the nucleus raphe pontis. Serotonergic neurons originating in the dorsal raphe nucleus and/or median raphe nucleus innervate the forebrain areas sending projections to the ventral tegmental area (VTA) and substantia nigra (SN) as well as throughout the forebrain including the PFCx, nucleus accumbens, amygdala, hippocampus, hypothalamus, and striatum [118-121].

5-HT is synthesized from the amino acid L-tryptophan in the raphe nuclei [124]. Once synthesized, 5-HT is stored in presynaptic vesicles or metabolized to 5-hydroxyindoleacetic acid in a process that involves oxidation by monoamine oxidase [113]. Once 5-HT is released by

depolarization of the presynaptic neuron, it produces its effects by activation of pre- and post-synaptic receptors [121;122]. Serotonergic action can be terminated by uptake through the serotonin transporter located on presynaptic neurons [113].

2.5.2 Serotonin Receptors

5-HT receptors are divided into 7 families (5-HT_{1-7}), with a total of 14-16 distinct receptor subtypes recognized [125-127]. With the exception of the 5-HT_3 receptor, a ligand-gated ion channel, all serotonin receptors are classical 7-transmembrane GPCRs that mediate their effects on different secondary messenger enzymes via activation of distinct G-proteins. The 5-HT receptors are found in a variety of difference species including Caenorhabditis elegans, Drosophila melanogaster, and mammals. All brain regions express various serotonin receptors and individual neurons may express multiple serotonin receptors [24].

The 5-HT_1 receptor subtype family is comprised of five receptor subtypes: 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E} and 5-HT_{1F}. These receptors share 40-63% overall sequence identity and are negatively coupled to adenylyl cyclase via the $G\alpha_{i/o}$ family of G-proteins [127]. The 5-HT_{1C} designation is vacant because this receptor was reclassified to 5-HT_{2C} due to structural and transductional similarities with the 5-HT_2 receptor subclass [127]. 5-HT_4-5-HT_7 are positively coupled to adenylyl cyclase via the $G\alpha_s$ family of G-proteins and mediate excitatory neurotransmission [127].

The 5-HT_2 receptor family consists of the 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors. Human 5-HT_{2A} shares 43% and 54% amino acid sequence homology with human 5-HT_{2B} and 5-HT_{2C}, respectively [128;129]. 5-HT_2 receptors are coupled to $G\alpha_q$ and $G\alpha_{11}$ G-proteins and can mediate excitatory neurotransmission [127]. 5-HT_2 receptors have been identified as important

therapeutic targets for the treatment of psychosis, depression, anorexia, insomnia, and anxiety [130-134]. The main focus of this dissertation will be on 5-HT_{2A} receptors, which will be described in more detail.

2.6 5-HT_{2A} Receptors

The 5-HT_{2A} is one of the most studied 5-HT receptors and was first identified as the "D" receptor in 1957 prior to molecular cloning [135;136]. 5-HT_{2A} receptors have been shown to play a role in feeding, temperature and blood pressure regulation, neuroendocrine function and behavior [137-142]. Enhanced 5-HT_{2A} receptor function and/or expression in the PFCx has been linked with the pathophysiology of neuropsychiatric disorders such as anxiety, depression or schizophrenia [31;143-145].

2.6.1 Gene and Structural Characteristics of 5-HT_{2A} Receptors

The rat 5-HT_{2A} receptor was first cloned in 1988 [146] and has been identified in humans and other mammals [147].The gene for the 5-HT_{2A} receptor (HTR2A) is located on human chromosome 13, rat chromosome 15, and mouse chromosome 14 [148;149]. The 5-HT_{2A} receptor gene has three exons separated by two introns which spans over 20 kb [149].

The human 5-HT_{2A} receptor shares approximately 90-92% amino acid sequence identity with rodent 5-HT_{2A} receptors [129;147;150]. Aspartate 155, aspartate 120, and serine 159 in transmembrane III have been shown to be important for agonist and/or antagonist binding to the 5-HT_{2A} receptor [151;152]. The third interacellular loop (ic3) is important for Gαq coupling [153] and aspartate 120 (D120), which is located in transmembrane helix II, is important for the modulation of PI hydrolysis [151]. The 5-HT_{2A} receptor has a canonical Type 1 PDZ-binding

domain (*X*-Ser/Thr-*X*-Φ) at its c-terminus that can interact with PDZ domain-containing proteins such as post-synaptic density protein-95 (PSD-95) [154]. The 5-HT_{2A} can also undergo posttranslational modifications that can modulate the function of the 5-HT_{2A} receptor. Indeed, RSK2 (p90 ribosomal S6 kinase 2) has been shown to phosphorylate serine residue 314 in the third intracellular loop of the 5-HT_{2A} receptor which can have inhibitory effects on 5-HT_{2A} receptor mediated phosphoinositide hydrolysis [155].

2.6.2 Localization of 5-HT_{2A} Receptors

5-HT_{2A} receptors are located in post-synaptic membranes of 5-HT target cells and are widely distributed in many brain areas, including limbic regions such as the hypothalamus, amygdala, nucleus accumbens, striatum, hypothalamus, and PFCx [156;157]. 5-HT_{2A} receptors are most highly expressed in the deep layers of the human and rodent cerebral cortex, especially in Layer IV and V of the pyramidal neurons [157]. 5-HT_{2A} receptors have also been found to be expressed at much lower levels in parvalbumin-expressing GABAergic interneurons [25;158].

2.6.3 Cellular Signaling of 5-HT_{2A} Receptors

As stated above, 5-HT_{2A} receptors are mainly coupled to $G\alpha_q$ and/or $G\alpha_{11}$ G-proteins [159;160]. After receptor activation by an agonist, $G\alpha_q$ or $G\alpha_{11}$ and βγ subunits disassociate to initiate downstream effector pathways. $G\alpha_{q/11}$-GTP activates the effector enzyme phospholipase Cβ (PLCβ) to produce inositol triphosphate (IP_3) and diacyl glycerol (DAG) [161]. IP_3 can then enhance Ca^{2+} release which activates calcium dependent kinases such as protein kinase C (PKC) [159;160]. The 5-HT_{2A} receptor can also regulate the activation of phospholipase D, Rho/Rho kinase, ERK1/2 signaling pathway, and enhance the formation of arachadonic acid through phospholipase A2 (PLA2) activation [162;163]. The 5-HT_{2A} receptor can regulate the activation

of phospholipase A2 through either Gαi/o dependent activation of the ERK1/2 signaling or $G\alpha_{12/13}$ dependent activation p38 MAPK signaling [164;165].

2.6.4 Regulation of 5-HT_{2A} Receptors

Classically, most GPCRs will be desensitized and down-regulated by overexposure to an agonist and will become supersensitive when treated with antagonists for a sustained period of time. 5-HT_{2A} receptors are unusual in their regulation because sustained treatment with agonists or antagonists will induce their desensitization and down-regulation [150;166;167]. Desensitization can occur through uncoupling of the receptor from the G-protein and internalization of the $5HT_{2A}$ receptor. The classical paradigm of agonist-induced GPCR desensitization involves agonist-induced dissociation of the G-proteins from the GPCR and subsequent G-protein regulation of secondary messengers [96;97]. Phosphorylation of the GPCR by either GRKs or secondary messenger-dependent protein kinases such as cAMP-dependent protein kinase A (PKA) or protein kinase C (PKC) then plays an important role in the short-term desensitization of GPCRs [167;168]. These intracellular kinases can phosphorylate the serine or threonine residues within the intracellular loops and C-terminus to uncouple the receptor from the G-protein, and hence, their effectors. GRKs only phosphorylate the activated or agonist-occupied forms of GPCRS, initiating homologous desensitization [169;170]. GRKs, such as GRK5 and GRK2, have been implicated in phosphorylation and homologous desensitization of 5-HT_{2A} receptors [167]. GRK-mediated phosphorylation of the receptor triggers the binding of β-Arrestins to the receptor preventing further G-protein mediated activation of the secondary messengers [171]. β-Arrestins can bind the third intracellular loop of the 5-HT_{2A} receptor and are co-expressed with 5-HT_{2A} receptor in cortical pyramidal neurons [167]. In contrast, secondary messenger-dependent kinases such as protein kinase C (PKC) and

protein kinase A (PKA) can phosporylate both agonist-activated receptors and receptors that have not been exposed to an agonist [172]. Hence secondary messenger-dependent kinases initiate both homologous and heterologous desensitization.

2.7 Neuropsychiatric Disorders and 5-HT_{2A} Receptors

5-HT_{2A} receptors have been shown to regulate the dopamine mesoaccumbens pathway as well as stress, mood, impulse control, and the behavior effects of drugs of abuse [31;137;173]. Dysfunction of 5-HT_{2A} receptor signaling in the PFCx has been associated with several physiological functions and neuropsychiatric disorders such as stress response, anxiety, depression, and schizophrenia [28;137;174;175]. Specifically, increases in 5-HT_{2A} receptor function in this limbic region may be clinically relevant to the pathophysiology of mood and cognitive disorders. Indeed, Weisstaub et al. have shown that cortical 5-HT_{2A} receptor signaling modulates anxiety-like behaviors in mice as anxiety-related behaviors are prevented in 5-HT_{2A} knockout animals [143]. Moreover, enhanced 5-HT_{2A} receptor function and expression in the PFCx has been linked to modulating anxiety-like behaviors in rodents [31;143]. Magalhaes et al. found that CRF treatment enhanced anxiety-like behaviors in rodents were associated with enhanced 5-HT_{2A} receptor-mediated inositol phosphate formation and cell surface expression of 5-HT_{2A} receptors in the PFCx [31]. Interestingly, selective 5-HT_{2A} receptor antagonist treatment prevented the CRF induced increases in anxiety-like behavior and 5-HT_{2A} receptor activity the PFCx indicating that the 5-HT_{2A} receptor was modulating the CRF-induced enhanced anxiety-like behavior [31].

5-HT_{2A} receptors are molecular targets in the treatment of neuropsychiatric disorders such as depression, anxiety, and schizophrenia [28;137;176-178]. Indeed, atypical antipsychotics, which show high affinity for the 5-HT_{2A} receptor and are potent 5-HT_{2A} receptor

antagonists, are proposed to mediate and maintain their therapeutic effects through desensitization of 5-HT_{2A} receptor signaling [28;179]. Interestingly, evidence indicates that blockade of pyramidal neurons in PFCx, which are particularly enriched in 5-HT_{2A} receptors, may underlie the beneficial effects of atypical antipsychotic drugs [28;29].

2.8 5-HT_{2A} Receptors and Cannabinoids

The serotonergic system shares a high level of overlap with the endocannabinoid system in respect to physiological processes that both systems regulate. Indeed, both the endocannabinoid system and the serotonergic system regulate body temperature, behavior, feeding, sleep, and emotional processes [180-185]. Accumulating evidence suggests that there are functional interactions that occur between the cannabinoid system and the serotonergic system [32-35]. For instance, activation of presynaptically localized CB_1 receptors has been shown to have inhibitory effects on 5-HT release [22]. Furthermore, behavioral evidence has suggested that cannabinoids can have differential effects on various 5-HT receptors including the 5-HT_{2A} receptor [32;34;35].

Behavioral reports have suggested that cannabinoid receptor agonists can regulate the activity of 5-HT_{2A} receptors [32;34;35]. Acute cannabinoid agonist treatment has been linked with inhibition of 5-HT_{2A} receptor-mediated behavioral responses [34;35] while chronic cannabinoid treatment has been linked with enhancement of 5-HT_{2A} receptor mediated behavioral responses [32]. Specifically, Darmini et al. showed that a single treatment with non-selective cannabinoid agonists, such as HU-210, CP55940, or Δ9-THC, 20 minutes prior to treatment with the selective 5-HT_{2A} receptor agonist (-)DOI prevented (-)DOI-induced increases in the head shake response [34]. Additionally, Gorzalka et al. have shown that a single treatment

with an anandamide reuptake inhibitor (AM404) prevents (-)DOI-induced increases in wet dog shakes [35]. In contrast, Hill et al. have reported that chronic treatment with the non-selective cannabinoid agonist, HU-210, led to a significant enhancement of the 5-HT_{2A} receptor-mediated head shake responses [32]. These behavioral tests are used as a measurement of 5-HT_{2A} receptor function *in vivo* [186-188]. 5-HT_{2A} receptors agonists induced this behavior and selective 5-HT_{2A} receptor antagonists block this behavioral response [34;189;190] while selective 5-HT_{2C} receptor antagonists do not block these behavioral responses [191]. Furthermore, 5-HT_{2A} receptor knockout animals do not produce the head shake or wet dog shake response after 5-HT_{2A} receptor agonist treatment [192;193].

Purpose of the Present Study

Accumulating evidence is indicating that chronic exposure to some cannabinoid agonists is linked with neuropsychiatric conditions such as anxiety, depression, and schizophrenia [10-14]. Currently, the exact molecular mechanism by which repeated exposure to some cannabinoid agonists may contribute to the pathophysiology of these neuropsychiatric disorders is currently unknown. Impaired function of 5-HT_{2A} receptors has been linked with various neuropsychiatric disorders including schizophrenia, depression, and anxiety [24;26;27]. Specifically studies indicate that increased function and/or expression of 5-HT_{2A} receptors in areas of the brain such as the PFCx may be associated with anxiety and schizophrenia [31;144;197]. Interestingly, behavioral evidence has indicated that repeated treatment with non-selective cannabinoid agonists can enhance the function of 5-HT_{2A} receptors [32].

Since behavioral evidence suggests that chronic cannabinoid agonist treatment can modify the function of 5-HT_{2A} receptors in the brain, it is therefore critical to define whether repeated cannabinoid treatment can alter function and expression of 5-HT_{2A} receptors in areas of the brain such as the PFCx. Our ***overall hypothesis*** is that repeated exposure to cannabinoid agonists upregulates and enhances the expression of 5-HT_{2A} receptor in rat PFCx. Our studies might provide a molecular mechanism by which repeated cannabinoid agonist exposure contributes to cannabinoid-related neuropsychiatric disorders.

Cannabinoids are being shown to have wide therapeutic application and their recreational use is rapidly growing, it is therefore critical to identify the molecular mechanisms by which cannabinoids can elicit adverse effects on mental health. Indeed, the molecular mechanisms defined in this dissertation could provide insight into mechanisms that can be targeted to prevent the potential adverse effects while deriving the therapeutic benefits of cannabinoids.

Chapter 3: Cannabinoid-Induced Enhanced Interaction and Protein Levels of Serotonin 2A and Dopamine D2 Receptors in Rat Prefrontal Cortex

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3.1 Abstract

Recent evidence suggests that non-selective cannabinoid receptor agonists may regulate 5-HT_{2A} receptor neurotransmission in brain. The molecular mechanisms of this regulation are unknown but could involve cannabinoid-induced enhanced interaction between 5-HT_{2A} and D_2 receptors. Here, we present experimental evidence that in Sprague-Dawley rats treated with a non-selective cannabinoid receptor agonist (CP55940) there was enhanced co-immunoprecipitation of 5-HT_{2A} and D_2 receptors and enhanced membrane-associated expression of D_2 and 5-HT_{2A} receptors in PFCx. Furthermore, 5-HT_{2A} receptor mRNA levels were increased in PFCx suggesting a cannabinoid-induced upregulation of 5-HT_{2A} receptors. To date, two cannabinoids receptors have been found in brain, CB_1 and CB_2 receptors. We used selective cannabinoid agonists in a neuronal cell line to study mechanisms that could mediate this 5-HT_{2A} receptor upregulation. We found that selective CB_2 receptor agonists upregulate 5-HT_{2A} receptors by a mechanism that seems to involve activation of Gαi G-proteins, ERK1/2, and AP-1 transcription factor. We hypothesize that the enhanced cannabinoid-induced interaction between 5-HT_{2A} and D_2 receptors and in 5-HT_{2A} and D_2 receptors protein levels in the PFCx might provide a molecular mechanism by which activation of cannabinoid receptors might be contribute to the pathophysiology of some cognitive and mood disorders.

3.2 Introduction

5-HT_{2A} and D_2 receptors are molecular targets in the treatment of various neuropsychiatric disorders such as depression, anxiety, and schizophrenia [28;137;143;176-178]. For instance, the therapeutic benefits of atypical antipsychotics (which are more potent 5-HT_{2A} receptor antagonists than D_2 receptor antagonists) and antidepressants are proposed to be mediated by antagonism and subsequent desensitization of 5-HT_{2A} and D_2 receptors signaling in several brain areas, including PFCx [28;194]. Noteworthy, recent evidence indicates that post-synaptically located 5-HT_{2A} and D_2 receptors can assemble into functionally interacting heteromers in PFCx [195;196]. Although the molecular mechanisms that regulate this 5-HT_{2A} and D_2 receptor interaction have not been clearly established, this 5-HT_{2A}-D_2 receptor complex might have a key significance in understanding the pathophysiology of several neuropsychiatric disorders and the mechanism of action of drugs used to treat them. Indeed, atypical antipsychotics target this heteromer decreasing its formation [197].

The clinical implications of the formation of a 5-HT_{2A}-D_2 receptor complex in PFCx have not been identified. However, dimerization of 5-HT_{2A} and D_2 receptors may provide a mechanism by which these receptors might regulate each other's activity. Indeed, activation of D_2 receptors would enhance the affinity of 5-HT_{2A} receptors to specific agonists and could modify the signaling of 5-HT_{2A} receptors, as it has been recently suggested [195]. Specifically, recent studies reported that the activity of 5-HT_{2A} receptors in PFCx would be synergistically enhanced by the formation of this 5-HT_{2A}-D_2 receptor complex [196;198]. Therefore, it is possible that drug-treatments that modify the expression of either 5-HT_{2A} or D_2 receptors could modify the formation of this 5-HT_{2A}-D_2 receptor complex.

Recent behavioral studies suggest that chronic exposure to a non-selective cannabinoid agonist is associated with enhanced activity of 5-HT_{2A} receptors in brain [32]. It was reported that rats treated with HU-210, a non-selective cannabinoid receptor agonist, exhibited enhanced 5-HT_{2A} receptor mediated-head twitches [32]. This behavioral test has been widely used as a model of activity of 5-HT_{2A} receptors in PFCx [190;199]. If exposure to cannabinoids modifies the expression of cortical 5-HT_{2A} receptors in PFCx, it could also modify the 5-HT_{2A}-D_2 heteromer formation in this brain area. Here, we focus on determining the effect of exposure to cannabinoid agonists on the interaction between 5-HT_{2A} and D_2 receptors and the expression of 5-HT_{2A} and D_2 receptors in rat PFCx.

The biological effects of cannabinoids in the brain are produced mainly through G-protein coupled cannabinoid receptors, CB_1 and CB_2 receptors [98]. While CB_1 receptors were initially identified in the brain, early reports identified CB_2 receptors only in immune cells [36;92;200;201]. However, recent studies have established the expression of CB_2 receptors in neurons in cortex, amygdala, hypothalamus, and hippocampus of the healthy brain [18;19;19;200-202]. CB_1 and CB_2 receptors couple to $G\alpha_{i/o}$ G-proteins [15;18;203] and could activate ERK in a protein kinase C (PKC)-dependent manner [19;88]. Here, we also used cultured cells to explore some molecular mechanisms that could contribute to the cannabinoid-induced upregulation of 5-HT_{2A} receptors.

Our results suggest that chronic cannabinoid exposure could enhance the formation and activity of 5-HT_{2A}-D_2 receptor heteromers in rat PFCx. This could provide a molecular mechanism by which chronic use of cannabinoids might contribute to the pathophysiology of some neuropsychiatric disorders associated with dysfunction of 5-HT_{2A} and D_2 neurotransmission in brain limbic areas such as PFCx.

3.3 Materials and Methods

Drugs

(-)-*cis*-3-[2-Hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol (CP55940), a CB_1 and CB_2 receptor agonist, N-(Piperidin-1-yl)-1-(2,4-dichlorophenyl)-1,4-dihydro-6-methylindeno[1,2-c]pyrazole-3-carboxamide (GP1a), a highly selective CB_2 receptor agonist; 3-(1,1-Dimethylbutyl)-1-deoxy-Δ^8-tetrahydrocannabinol (JWH133), a selective CB_2 receptor agonist; [6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl](4-methoxyphenyl)-methanone (AM630), a selective CB_2 receptor antagonist; Pertussis Toxin (PTX); *N*,*N*-Dimethyl-(3*R*,4a*R*,5*S*,6a*S*,10*S*,10a*R*,10b*S*)-5-(acetyloxy)-3-ethenyldodecahydro-10,10b-dihydroxy-3,4a,7,7,10a-pentamethyl-1-oxo-1*H*-naphtho[2,1-*b*]pyran-6-yl ester β-alanine hydrochloride (NKH477)**,** a potent activator of adenyl cyclase; 2-[1-(3-Dimethylaminopropyl)indol-3-yl]-3-(indol-3-yl) maleimide (GF109203X)**,** a very potent and selective inhibitor of protein kinase c; 5,6,7,13-Tetrahydro-13-methyl-5-oxo-12*H*-indolo[2,3-*a*]pyrrolo[3,4-*c*]carbazole-12-propanenitrile (Go6967)**,** a potent protein kinase c inhibitor; (1a*R*,1b*S*,4a*R*,7a*S*,7b*S*,8*R*,9*R*,9a*S*)-1a,1b,4,4a,5,7a,7b,8,9,9a-Decahydro-4a,7b-dihydroxy-3-(hydroxymethyl)-1,1,6,8-tetramethyl-5-oxo-1*H*-cyclopropa[3,4]benz[1,2-*e*]azulen-9,9a-diyl butanoic acid ester (Phorbol 12,13-dibutylrate, PDBu**),** a protein kinase c activator; (*E*,*E*,*Z*,*E*)-3-Methyl-7-(4-methylphenyl)-9-(2,6,6-trimethyl-1-cyclohexen-1-yl)-2,4,6,8-nonatetraenoic acid (SR11302), an inhibitor of activating protein-1 transcription factor activity and N-(2-Chloroethyl)-5Z,8Z,11Z,14Z-eicosatetraenamide (ACEA), a highly selective CB_1 receptor agonist, were purchased from Tocris (Ellisville, MO). Naphthol AS-E phosphate, a CREB inhibitor, was purchased from Sigma-Aldrich Inc. (St. Louis, MO).

Animal Experimental Protocol

Male Sprague-Dawley rats (225-275 g; Harlan Laboratories, Indianapolis, IN) were housed two per cage in a temperature-, humidity-, and light-controlled room (12 hr light/dark cycle, lights on 7:00 AM-19:00 PM). Food and water were available *ad libitum*. All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals as approved by the University of Kansas Institutional Animal Care and Use Committee (IACUC).

After arrival, the rats were allowed to acclimate to their environment for at least 4 days prior to the start of the treatment period. Eight rats were randomly assigned to each group, cage mates were assigned to the same treatment group. All solutions were made fresh before administration and rats were injected with either vehicle (Tween-80/ethanol/saline (1:1:18); 1ml/kg, i.p.) or CP55940 (0.05 mg/kg, i.p.) once a day for 7 days. Rats were sacrificed by decapitation 48 hrs after the last CP55940 injection. The brains were immediately removed and the PFCx was dissected and frozen in dry ice.

Co-Immunoprecipitation

Co-immunoprecipitation (co-IP) was done using the Thermo Scientific Pierce co-IP kit following manufacturer's protocol. 5-HT_{2A} receptor antibody was a generous gift from Dr. Nancy A. Muma and the D_2 receptor antibody was purchased from Santa Cruz, CA. Briefly, 5-HT_{2A} receptor antibody or D_2 receptor antibody was first immobilized for 2 hours using AminoLink Plus coupling resin. The resin was washed and incubated with pre-cleared PFCx cortex lysate (300 µg) from vehicle and CP55940 treated rats overnight. A negative control in this assay included a non-reactive resin that was also incubated with either 5-HT_{2A} or D_2 receptor antibodies. In this control, the coupling resin is not amine-reactive preventing covalent

immobilization of the primary antibody onto the resin. This inactive resin was provided with the IP kit to assess non-specific binding in samples that received the same treatment as the co-IP samples, including 5-HT_{2A} receptor antibody or D_2 receptor antibody. After the overnight incubation of all the PFCx lysates from vehicle- and CP55940-treated samples with either active or inactive resins, the resins were washed (3x) and the protein eluted using elution buffer. Samples were analyzed by Western blot using 5-HT_{2A} receptor antibody or D_2 receptor antibody. The specificity of the 5-HT_{2A} and D_2 receptor antibody has been verified in the literature [194;204;205].

Western Blot

Membrane-associated proteins were isolated using the ProteoExtract^{TM} Native Membrane Protein Extraction kit (Calbiochem, La Jolla, CA). Nuclear-associated proteins were isolated using NE-PER ® Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, IL). Samples containing 5 µg of protein were separated by sodium dodecyl-polyacrylamide gel electrophoresis containing 0.1% SDS, 12.5% acrylamide/bisacrylamide (30:0.2), 4.6 M urea, and 275 mM Tris, pH 8.7. Gels were transferred electrophoretically by semi-dry blot to nitrocellulose membranes. After incubation with a blocking buffer (phosphate buffered saline containing 0.2% casein and 0.1% Tween 20), immunodetection was performed at 4ºC overnight using primary antibody. c-Fos antibody was purchased from Santa Cruz, CA. The anti-dopamine D_2 receptor, cytoplasmic domain, long form antibody was purchased from Millipore (Billerica, MA) and the Dopamine D_2 Receptor (Short Isoform 239-246) antibody was purchased from Acris Antibodies GmbH (Germany). The specificity of the antibodies has been verified in the literature [194;204-207]. Antibodies were used at the following dilutions: c-Fos (1:1,000), D_2L (1:1,000), D_2S (1:1,000), 5-HT_{2A} (1:5,000) and D_2 (1:1,000). The overnight incubation was followed by

incubation with peroxidase-labeled secondary antibody for 1 hour at room temperature. The membranes were incubated with enhanced chemiluminescence substrate solution (Amersham Biosciences Inc., Piscataway, NJ). Protein loading for each lane was verified using an anti-actin antibody (Santa Cruz Biotechnology, Inc.). Negative controls included either the omission of primary antibody or addition of preimmune rabbit immunoglobulins.

Film Analysis

Films were analyzed densitometrically with values calculated from the integrated optical density (IOD) of each band using Scion Image software (Scion Corporation, Frederick, MD, USA). The gray scale density readings were calibrated using a transmission step-wedge standard. The integrated optical density (IOD) of each band was calculated as the sum of the optical densities of all the pixels within the area of the band outlined. An adjacent area was used to calculate the background optical density of the film. The IOD for the film background was subtracted from the IOD for each band. The resulting IOD for each protein was then divided by the amount of protein loaded on the corresponding lane, and each sample was expressed as IOD per microgram of protein. Each sample was measured on three independent gels. All samples were standardized to controls and normalized to their respective actin levels.

Quantitative Real-Time PCR

Total RNA was isolated from either cell culture or PFCx tissue using the RNeasy Mini Kit (Qiagen, Valencia, CA) protocol as described by the manufacturer. Total mRNA was reverse transcribed to generate cDNA. Quantitative real time PCR reactions were prepared using QuantiFast SYBR Green PCR Kit (Qiagen, Valencia, CA), a 4% (v/v) concentration of cDNA product, and forward and reverse primers at a final concentration of 0.35 mM. All reactions were performed in triplicate using the ABI 7500 fast real time PCR system (Applied Biosystems,

Foster City, CA). A negative control lacking cDNA or any known DNA template was included for each primer pair. The primers used in this manuscript were: 5-HT_{2A} (F:5'-AACGGTCCATCCACAGAG-3' and R:5'-AACAGGAAGAACACGATGC-3'), D_2 (F:5'-CACCACGGCCTACATAGCAA-3' and R:5'-GGCGTGCCCATTCTTCTCT-3'), and GAPDH (F:5'-TGGAGTCTACTGGCGTCTTCAC-3' and R:5'-GGCATGGACTGTGGTCATGA-3'). These primers have been previously validated in the literature [29;208-210].

In all real-time PCR experiments, measurements were made from the number of cycles required to reach the threshold fluorescence intensity [cycle threshold (Ct)]. Ct values for each reaction were subtracted from Ct values for GADPH and then subtracted from Ct values for vehicle-treated animals that served as a baseline, and the result was referred to as ΔΔCt. Fold changes in gene expression were calculated as $2^{-\Delta\Delta Ct}$ to reflect the fact that, under optimal conditions, the amount of PCR product doubles with each amplification cycle. Results were normalized to those obtained for amplifications of the same cDNA samples using primers designed against GADPH, which acts as an internal standard, and averaged for each treatment group.

Cell Culture Protocol

We purchased CLU213 cells from Cedarlane Laboratories (Burlington, NC). We selected this neuronal cell line because: (1) it coexpresses 5-HT_{2A}, D_2, CB_1, and CB_2 receptors; and (2) the preliminary results in our lab showed that it reproduces the effect of sustained cannabinoid exposure *in vivo* experiments. This was confirmed in experiments reported in this paper (Fig. 3E and 3F). Although many *in vitro* cannabinoid studies use transformed cells that overexpress neurotransmitter receptors, we chose this neuronal cell line because it endogenously expresses 5-

HT_{2A}, D_2, CB_1, and CB_2 receptors. Therefore, we anticipate that the results depicted in this manuscript could be a good model of the mechanisms underlying 5-HT_{2A} upregulation *in vivo*.

CLU213 cells were grown on 100-mm^2 plates treated with polystyrene (Corning Incorporated, Corning, NY) and maintained in 5% CO_2 at 37°C, in Dulbecco's modified eagle medium (DMEM; Mediatech Inc, Manassas, VA) containing 10% fetal bovine serum (FBS; Thermo Scientific, Logan, UT).

Effect of Selective CB_1 and CB_2 Receptor Agonists on 5-HT_{2A} and D_2 Receptor mRNA

CLU213 cells were incubated with either vehicle (ethanol 0.01% final concentration), CP55940 (CB_1 and CB_2 agonist, 1nM) [54;211]; ACEA (CB_1 agonist, 15nM) [55;72]; or GP1a (CB_2 agonist, 1nM) [60;212] for 24 hours. mRNA was isolated and qRT-PCR for 5-HT_{2A} and D_2 mRNA was performed as described above.

Effect of Highly Selective CB_2 Receptor Agonists on 5-HT_{2A} Receptor mRNA

CLU213 cells were pretreated with either vehicle (ethanol 0.01% final concentration) or 1 μM AM630 [200], a highly selective CB_2 receptor antagonist. Twenty minutes later cells were treated with either vehicle or one of the following highly selective CB_2 agonists, 30 nM JWH133 [2;200] or 1nM GP1a [60;212]. 24 hours later mRNA was isolated and qRT-PCR for 5-HT_{2A} mRNA was performed as previously described.

Effect of Pertussis Toxin (PTX) on GP1a-Induced Increases in 5-HT_{2A} Receptor mRNA and Protein Levels

CLU213 cells were treated with either vehicle (PBS) or PTX (100 ng/ml) [213;214]. Twenty minutes later cells were treated with either vehicle (ethanol 0.01% final concentration) or GP1a (1nM) for 24 hours. mRNA was isolated and qRT-PCR for 5-HT_{2A} was performed as described above.

In a different experiment, CLU213 cells were treated with either vehicle (PBS) or PTX (100 ng/ml) for 20 minutes. Cells were then incubated with either vehicle (ethanol 0.01% final concentration) or GP1a (1nM) for 72 hours. Cells were washed (3x) with PBS every 24 hours and fresh vehicle or GP1a was added. Expression of membrane-associated 5-HT_{2A} receptors was determined by Western blot as previously described.

Effect of a Selective ERK1/2 Inhibitor (PD198306) or Adenylyl Cyclase Activator (NKH477) on GP1a-Induced Increases in 5-HT_{2A} Receptor mRNA

CLU213 cells were treated with either vehicle (ethanol 0.01% final concentration), NKH477 (20μM) [215;216] or PD198306 [217;218]. Twenty minutes later cells were incubated with either vehicle (ethanol 0.01% final concentration) or GP1a (1nM) for 24 hours. mRNA was isolated and qRT-PCR for 5-HT_{2A} was performed as described.

Effect of PKC Inhibitors on GP1a-Induced Increases in 5-HT_{2A} Receptor mRNA

CLU213 cells were treated with either vehicle (ethanol 0.01% final concentration), GF109203X (5μM) [219;220], or Go 6967 (10nm) [221] for 20 min. Cells were then treated with either vehicle (ethanol 0.01% final concentration) or GP1a (1nM) for 24 hours. mRNA was isolated and qRT-PCR for 5-HT_{2A} was performed as described above.

Effect of PKC Activator on GP1a-Induced Increases in 5-HT_{2A} Receptor mRNA

CLU213 cells were treated with either vehicle (DMSO 0.01% final concentration), PDBu (1μM), or PDBu (30nM) [222;223] for 20 minutes. Cells were then treated with either vehicle (ethanol 0.01% final concentration) or GP1a (1nM) for 24 hours. mRNA was isolated and qRT-PCR for 5-HT_{2A} was performed as described above.

Effect of Transcription Factor Inhibitors on GP 1a-Induced Upregulation of 5-HT_{2A} Receptors

CLU213 cells were treated with either vehicle (ethanol 0.01%), Naphthol AS-E phosphate (10μM) [224] or SR 11302 (1μM) [225;226] for 20 minutes. Cells were then treated with either vehicle (ethanol 0.01%) or GP 1a (1 nM) for 24 hours. mRNA was isolated and qRT-PCR for 5-HT_{2A} was performed as previously described.

Effect of a Selective ERK1/2 Inhibitor on GP1a-Induced Increases in Nuclear levels of c-Fos Protein

CLU213 cells were treated with either vehicle (ethanol 0.01% final concentration) or PD198306 (200nM) [217;218] for 20 minutes. Cells were then incubated with either vehicle (ethanol 0.01% final concentration) or GP 1a (1 nM) for 15 minutes. After 15 minutes of incubation, cells were collected and nuclear-associated proteins were isolated. Expression of nuclear-associated c-Fos was determined by Western blot as previously described.

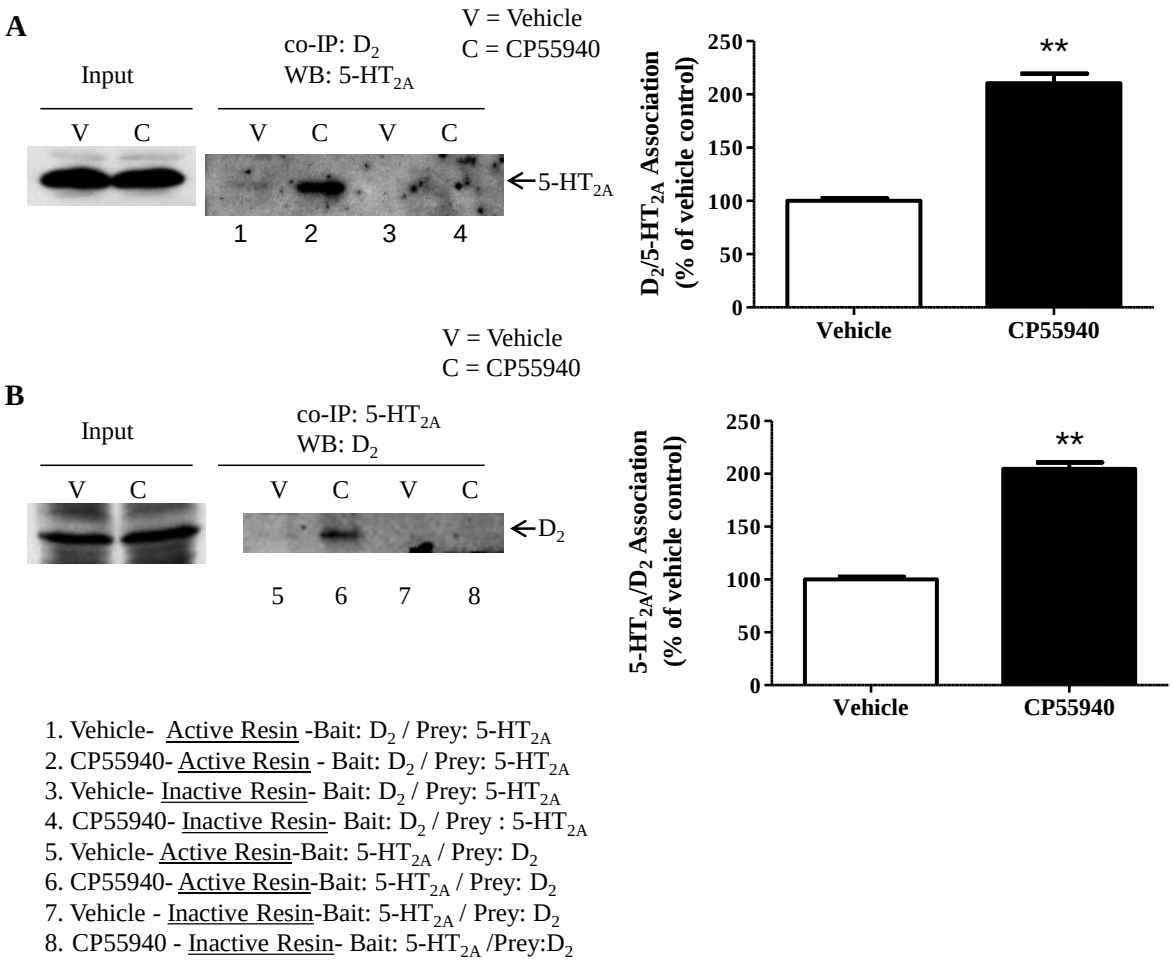
Statistics

All data are expressed as the mean ± S.E.M., where *n* indicates the number of rats or cell culture plates per group. Data was analyzed by an unpaired Student's t-test or ANOVA (Newman-Keuls post-hoc test). GB-STAT software (Dynamic Microsystems, Inc., Silver Spring, MD, USA) was used for all statistical analyses.

3.4 Results

Effect of CP 55940 Treatment on the Co-Immunoprecipitation of 5-HT_{2A} and D_2 Receptors in Rat PFCx

We used co-immunoprecipitation protocols to study the effect of CP55940 on the physical interaction between 5-HT_{2A} and D_2 receptors in rat PFCx (Fig. 2). PFCx lysate of rats treated with either vehicle or CP55940 (a non-selective CB_1/CB_2 receptor agonist) for 7 days was used in this experiment as described in Methods. We used either D_2 or 5-HT_{2A} receptor antibodies as baits in two different co-immunoprecipitation experiments. In the first experiment, we used active columns to precipitate 5-HT_{2A} receptors using D_2 receptors as bait (Fig. 2A, lanes 1 and 2). We also used inactive columns, unable to bind D_2 receptor antibody as control (Fig. 2A, lanes 3 and 4), as described in methods. We found that 5-HT_{2A} receptors co-precipitate with D_2 receptors when we used D_2 receptors as bait. Indeed, we found an enhanced co-immunoprecipitation of 5-HT_{2A} and D_2 receptors in PFCx of CP55940-treated rats compared with vehicle controls (approximate 200% increase, $p<0.01$, t 12.031, df 2, Fig. 2A lanes 1 and 2 for vehicle or CP55940 samples, respectively). No co-precipitation of 5-HT_{2A} and D_2 receptors was detected when using inactive columns (Fig. 2A, lanes 3 and 4). Similarly, we found an approximate two-fold ($p<0.01$, t 15.728, df 2) increased co-precipitation of D_2 receptors with 5-HT_{2A} receptors in PFCx lysate of CP55940-treated rats compared to controls when we used 5-HT_{2A} receptor as bait (Fig. 2B, lanes 5 and 6 for vehicle of CP55940 samples, respectively). No co-precipitation of 5-HT_{2A} and D_2 receptors was detected when using inactive columns (Fig. 2B, lanes 7 and 8). This evidence suggests that CP55940 treatment enhances formation of a 5-HT_{2A}-D_2 receptor heteromer in rat PFCx.



1. Vehicle- Active Resin -Bait: D_2 / Prey: 5-HT_{2A}
2. CP55940- Active Resin - Bait: D_2 / Prey: 5-HT_{2A}
3. Vehicle- Inactive Resin- Bait: D_2 / Prey: 5-HT_{2A}
4. CP55940- Inactive Resin- Bait: D_2 / Prey : 5-HT_{2A}
5. Vehicle- Active Resin-Bait: 5-HT_{2A} / Prey: D_2
6. CP55940- Active Resin-Bait: 5-HT_{2A} / Prey: D_2
7. Vehicle - Inactive Resin-Bait: 5-HT_{2A} / Prey: D_2
8. CP55940 - Inactive Resin- Bait: 5-HT_{2A} /Prey:D_2

Figure 2: CP55940-induced enhanced co-immunoprecipitation of 5-HT_{2A} and D_2 receptors in rat PFCx.

(A) Enhanced immunoprecipitation of the 5-HT_{2A} receptor (Lane 2) compared to vehicle-treated controls (Lane 1). (B) Enhanced immunoprecipitation of the D_2 (Lane 6) receptor compared to vehicle-treated controls (Lane 5). Negative controls (Lanes 3, 4, 7, and 8) received the same concentration of D_2 or 5-HT_{2A} receptor antibody except that the coupling resin was replaced with control agarose resin that is not amine reactive. All columns were incubated with PFCx lysate (300 µg) from vehicle (Lanes 1,3,5, and 7) or CP55940 (2, 4, 6, and 8) treated rats. PFCx lysate

(45 µg of protein) was used as an input control for both immunoprecipitations. The data represent mean ± SEM (n=3).

Effect of Chronic CP55940 Treatment on the Protein Expression of D_2 and 5-HT_{2A} Receptors in Rat PFCx

CP55940 enhanced expression of post-synaptically located D_2 and 5-HT_{2A} receptors could underlie the enhanced co-immunoprecipitation of these receptors detected in Fig. 2. In our next experiments, we studied the effect of CP55940 exposure on the membrane-associated protein levels of 5-HT_{2A} and D_2 receptors. There are two alternatively spliced isoforms of the D_2 receptor that are coded for the same gene [227-229]. These are the dopamine D_2 receptor Long (D_2L) and Short (D_2S) isoforms that differ by a 29 amino acid insert in the third cytoplasmic loop [230]. The D_2S receptor (M.Wt 48 kDa) is mainly presynaptically localized while the D_2L receptor (M.Wt 50 kDa) and the 5-HT_{2A} receptor (M.Wt 42 kDa) are mainly located postsynaptically [227-229].

Chronic administration of CP55940 produced significant increases in membrane-associated levels of D_2S receptors (Fig. 3A), D_2L receptors (Fig. 3B), and 5-HT_{2A} receptors (Fig. 3C) in rat PFCx. Membrane-associated levels of D_2L and 5-HT_{2A} receptors increased between 60% and 100% compared to vehicle-treated animals ($p<0.01$, t 3.264, df 10 and $p<0.05$, t 2.55, df 10, respectively) while D_2S receptor levels increased almost three-fold compared to vehicle treated controls ($p<0.05$, t 2.299, df 10). We also determined the effect of chronic CP55940 treatment on 5-HT_{2A} and D_2 mRNA levels in rat PFCx. 5-HT_{2A} receptor mRNA was significantly ($p<0.05$) increased (approximate 90% increase) in PFCx of CP55940-treated rats compared to vehicle-treated controls (Fig. 3D). Interestingly, D_2 receptor mRNA was significantly ($p<0.05$) reduced (approximate 45% reduction) in PFCx of CP55940 treated rats compared to vehicle-treated controls.

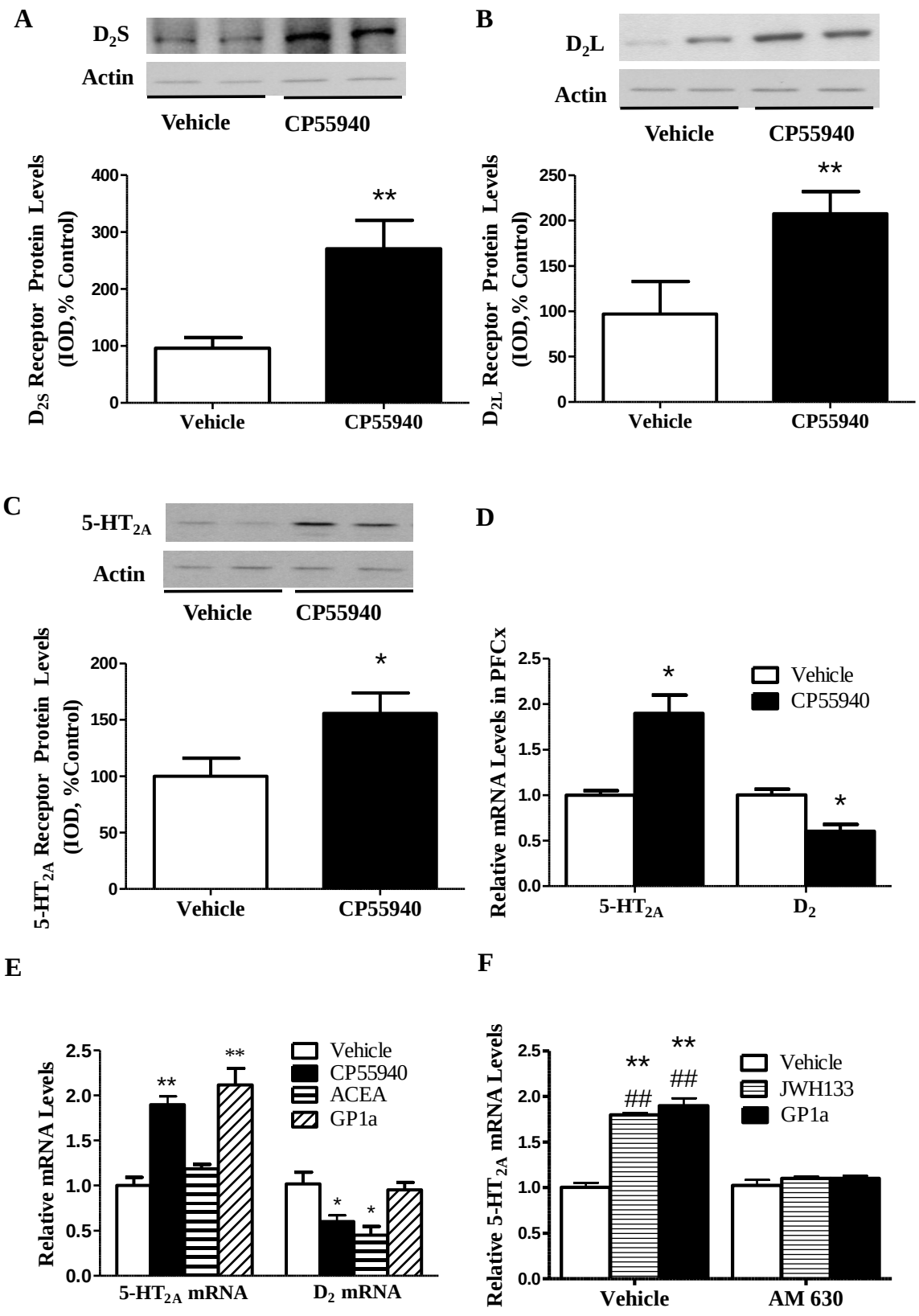
Effect of Non-Selective and Selective Cannabinoid Agonists on the 5-HT_{2A} and D_2 mRNA levels in a Neuronal Cell Line.

We used a neuronal cell line, CLU213 cells, in our next experiments to better examine the mechanisms involved in the cannabinoid-induced upregulation of 5-HT_{2A} receptors. CLU213 cells express 5-HT_{2A}, D_2, $CB_{1,}$ and CB_2 receptors. In these experiments we tested the effect of either a non-selective cannabinoid agonist (CP55940) [54;211]; a selective CB_1 receptor agonist (ACEA) [55;72]; or a selective CB_2 receptor agonist (GP1a) [60;212].

We found that either CP55940 or GP1a produced a significant ($p<0.01$) upregulation of 5-HT_{2A} receptor mRNA levels in CLU213 cells (Fig. 3E). Cells treated with either CP55940 or GP1a exhibited an approximate two-fold increase in 5-HT_{2A} receptor mRNA levels compared to controls. No significant differences ($p>0.05$) in the 5-HT_{2A} receptor mRNA levels were detected between cells treated with either CP55940 or GP1a. The CB_1 agonist ACEA did not have significant effects on 5-HT_{2A} receptor mRNA levels (Fig. 3E). On the other hand, cells treated with either CP55940 or ACEA exhibited a significant ($p<0.05$) downregulation of D_2 mRNA levels in CLU213 cells. Cells treated with CP55940 exhibited an approximate 60% reduction ($P<0.05$) in D_2 mRNA levels while cells treated with ACEA exhibited an approximate 52% reduction ($P<0.05$) in D_2 mRNA levels. No significant differences ($p>0.05$) in D_2 mRNA levels were detected between cells treated with either CP55940 or ACEA.

Since we detected a very strong regulation of 5-HT_{2A} receptor mRNA induced by GP1a, a highly selective CB_2 receptor agonist, we also studied the effect of other selective CB_2 agonist and antagonist on 5-HT_{2A} upregulation. In this experiment cells were pretreated with either vehicle or AM630, a selective CB_2 antagonist. Twenty minutes later the cells were incubated with either vehicle, JWH133 or GP1a as described in Methods. We found that both JWH133 and

GP 1a produced a significant ($p<0.01$) upregulation of 5-HT_{2A} receptor mRNA in CLU213 cells (Fig. 3F). There were no significant ($p>0.05$) differences between the 5-HT_{2A} upregulation induced by JWH133 or GP1a. This strong 5-HT_{2A} mRNA upregulation induced by these CB_2 receptor agonists was significantly ($p<0.01$) inhibited in cells pretreated with a selective CB_2 antagonist, AM630 (Fig. 3F). No significant ($p>0.05$) differences in 5-HT_{2A} mRNA were found between vehicle treated cells and cells pretreated with AM630 and later treated with either vehicle, JWH133, or GP1a (Fig. 3F). The two-way ANOVA for 5-HT_{2A} mRNA showed a significant main effect of AM630 pretreatment ($F_{(1,17)} =134.8$, $p<0.0001$) and CB_2 agonists treatment ($F_{(2,17)} = 65.98$, $p<0.0001$). There was also a significant interaction between AM630 pretreatment and CB_2 agonists treatment ($F_{(2,17)} =40.03$, $p<0.0001$).


A
D2S
Actin
Vehicle
CP55940
D2S Receptor Protein Levels (IOD,% Control)
400
300
200
100
0
**
Vehicle
CP55940
B
D2L
Actin
Vehicle
CP55940
D2L Receptor Protein Levels (IOD,% Control)
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50
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Vehicle
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5-HT2A Receptor Protein Levels (IOD, %Control)
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Relative mRNA Levels in PFCx
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Vehicle
CP55940
5-HT2A
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Relative mRNA Levels
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Vehicle
CP55940
ACEA
GP1a
5-HT2A mRNA
D2 mRNA
F
Relative 5-HT2A mRNA Levels
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2.0
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0.0
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Vehicle
JWH133
GP1a
Vehicle
AM 630

Figure 3. CP55940-induced increased membrane associated expression of 5-HT_{2A} and D_2 receptors in rat PFCx.

(A and B) Increased membrane-associated D_2S or D_2L receptor protein levels in PFCx of CP55940 treated rats. (**p<0.01 significant effect of CP55940 treatment compared to vehicle-treated controls). (C) Increased membrane-associated 5-HT_{2A} receptor protein levels in PFCx of CP55940 treated rats. (*p<0.05 significant effect of CP55940 treatment compared to vehicle-treated controls). (D) Increased 5-HT_{2A} receptor mRNA levels and reduced D_2 receptor mRNA levels in PFCx of CP55940 treat rats. (*p<0.05 significant effect of CP55940 treatment compared to vehicle-treated controls). (E) Increased 5-HT_{2A} receptor mRNA levels in CP 55,940 or GP1a treated cells (**p<0.01 significant effect of CP55490 or GP1a treatment compared to vehicle-treated controls) and reduced D_2 receptor mRNA levels in CP55940 or ACEA treated cells (*p<0.05 significant effect of CP55940 or ACEA treatment compared to vehicle-treated controls). (F) AM630 pretreatment prevents GP1a and JWH133-induced increases in 5-HT_{2A} receptor mRNA. **p<0.01, significant effect of GP1a or JWH133 treatment on 5-HT_{2A} receptor mRNA levels compared to vehicle-treated controls. ##p<0.01, significant effect of AM630 pretreatment on the GP1a or JWH133-induced upregulation of 5-HT_{2A} receptors. Representative Western blots are shown in this figure and IOD was calculated as described in Experimental Procedures. The data represent mean ± SEM (n=6-8).

Effect of G-Protein and ERK1/2 Signaling Inhibitors on the GP1a-Induced Upregulation of 5-HT_{2A} Receptors in CLU213 Cells

Our next experiments were designed to identify some signaling components that would mediate the upregulation of 5-HT_{2A} receptors by the CB_2 receptor agonist GP1a. Previous reports suggested that CB_1 and CB_2 receptors couple to $G\alpha_{i/o}$ G-proteins receptors to inhibit adenylyl cyclase activation and to induce the activation of the ERK1/2 signaling cascade [88]. Here we used PTX to prevent the GP1a-induced activation of $G\alpha_{i/o}$ G-proteins [88]. PTX-induced ADP-ribosylation of $G\alpha_{i/o}$ subunits mediates the inactivation of their signaling by interfering with Gα/receptor coupling [213;214].

Figure 4 illustrates the effect of PTX pretreatment on GP1a-induced upregulation of 5-HT_{2A} receptors in CLU213 cells. CLU213 cells were pretreated with either vehicle or PTX (100 ng/ml) for 20 minutes then vehicle or GP1a (1nM) was added to the media. We found that in vehicle pretreated cells, GP1a significantly ($p<0.01$) increased 5-HT_{2A} mRNA levels (two-fold increase) over controls (Fig. 4A). This effect of GP1a was prevented ($p<0.01$) in cells pretreated with PTX. No significant ($p>0.05$) effect of PTX was found in basal 5-HT_{2A} receptor mRNA levels. The two-way ANOVA for 5-HT_{2A} mRNA showed significant main effects of PTX pretreatment ($F_{(1,15)} =23.52$, $p<0.0004$) and GP1a treatment ($F_{(1,15)} =34.11$, $p<0.0001$). There was a significant interaction between PTX pretreatment and GP1a treatment ($F_{(1,15)} =47.74$, $p<0.0001$).

In Figure 4B, CLU213 cells were treated with either vehicle or PTX (100 ng/ml) then vehicle or GP1a (1nM) was added to the media 20 min later. Membrane-associated 5-HT_{2A} receptor protein expression was measured in these cells after 3 days of incubation with GP1a, as described in Methods. We found that in vehicle pretreated cells, GP1a significantly ($p<0.01$)

increased 5-HT_{2A} receptor protein levels (approximate 60% increase) over controls (Fig. 4B). The effect of GP1a on 5-HT_{2A} receptor protein levels was prevented ($p<0.01$) in cells pretreated with PTX. No significant ($p>0.05$) effect of PTX was found on basal 5-HT_{2A} receptor protein levels. The two-way ANOVA for 5-HT_{2A} receptor protein levels showed significant main effects of PTX pretreatment ($F_{(1,22)} =23.18$, $p<0.0001$) and GP1a treatment ($F_{(1,22)} =19.34$, $p<0.0003$). There was a significant interaction between PTX pretreatment and GP1a treatment ($F_{(1,22)} =7.14$, $p<0.0151$). These data suggest that the GP1a-induced upregulation of 5-HT_{2A} receptors is mediated by a $G\alpha_{i/o}$ G-protein mechanism.

Coupling of CB_2 cannabinoid receptors to $G\alpha_{i/o}$ G-proteins mediates the increases in ERK signaling and also the inhibition of adenylate cyclase that results in reduced cAMP levels [15]. In our next experiment, we studied the effect of an ERK1/2 inhibitor (PD198306) [218] and an adenylyl cyclase activator (NHK477) [215] on the GP1a-induced upregulation of 5-HT_{2A} receptor mRNA. CLU213 cells were treated with either vehicle, PD198306 (200nM) or NKH477 (20µM). Twenty min later cells were treated with either vehicle or GP1a (1nM) for 24 hrs. Consistent with our previous findings, GP1a significantly ($p<0.05$) increased 5-HT_{2A} mRNA levels (approximate two-fold increase) over controls (Fig. 4C). The effect of GP1a was prevented ($p<0.05$) in cells pretreated with either PD198306 or NKH477. No significant ($p>0.05$) effect of PD198306 or NKH477 was found on basal 5-HT_{2A} receptor mRNA levels (Fig. 4C). These results suggest that the GP1a-induced upregulation of 5-HT_{2A} receptors is dependent on ERK1/2 activation and prevented by activation of adenylyl cyclase.

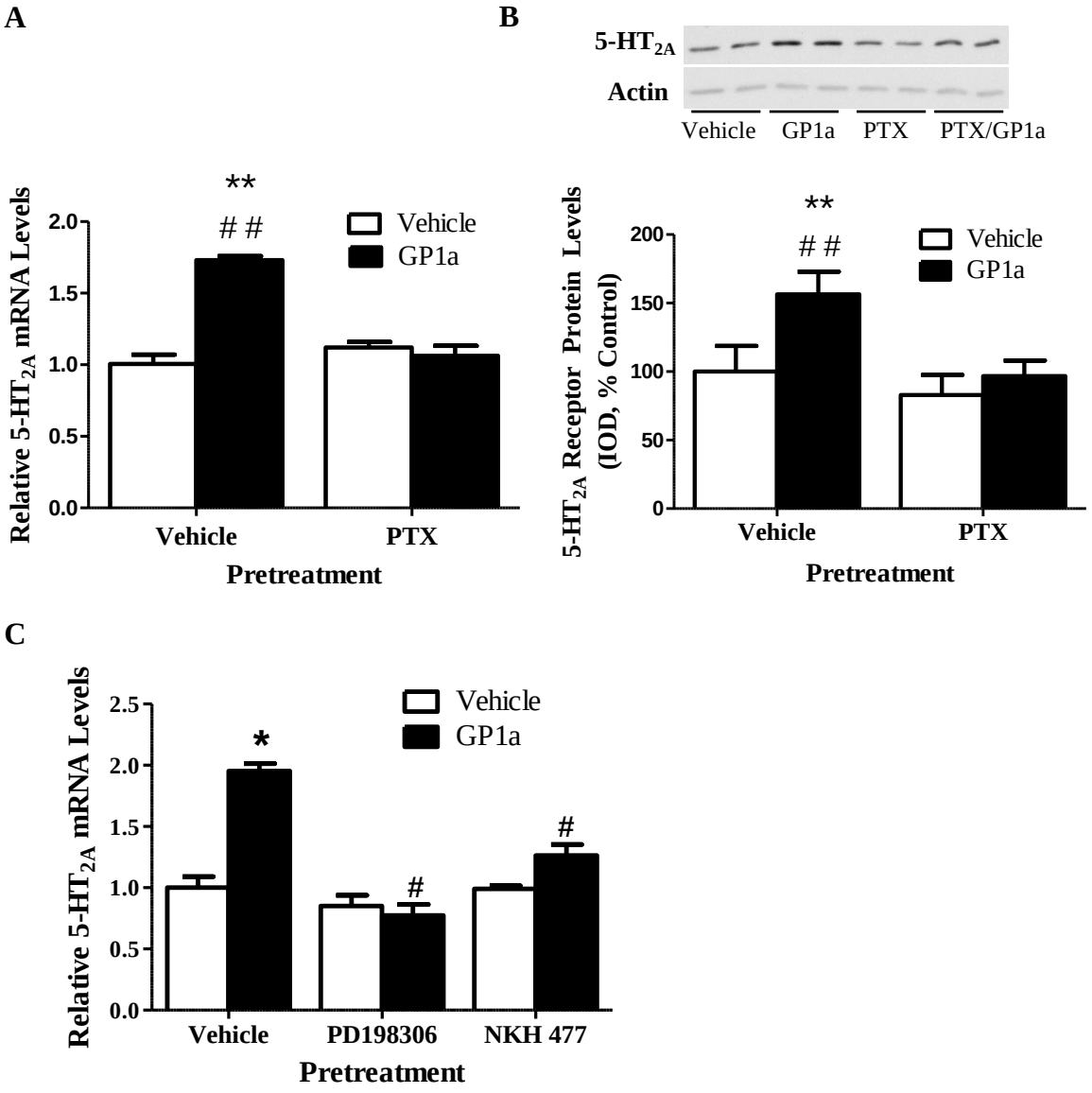

Figure 4. GP1a upregulated 5-HT$_{2A}$ receptors via Gαi-protein in CLU213 cells.

(A) Pertussis toxin (100 ng/ml) prevents GP1a-induced increases in 5-HT$_{2A}$ receptor mRNA. **p<0.01, significant effect of GP1a treatment on 5-HT$_{2A}$ receptor mRNA levels compared to vehicle-treated controls. ##p<0.01, significant effect of pertussis toxin pretreatment on the GP1a-induced upregulation of 5-HT$_{2A}$ receptors. (B) Pertussis toxin (100 ng/ml) prevents GP1a-induced increases in membrane-associated 5-HT$_{2A}$ receptor protein expression. **p<0.01,

significant effect of GP1a treatment on 5-HT_{2A} receptor protein levels compared to vehicle-treated controls. ##p<0.01, significant effect of pertussis toxin pretreatment on the GP1a-induced increases in membrane-associated 5-HT_{2A} receptor protein levels. (C) An inhibitor of ERK1/2 (PD198306) prevents GP1a-induced increases in 5-HT_{2A} receptor mRNA and an activator of adenylyl cyclase (NHK477) prevents GP1a-induced increases in 5-HT_{2A} receptor mRNA. *p<0.05, significant effect of GP1a treatment on 5-HT_{2A} receptor mRNA levels compared to vehicle-treated controls. #p<0.05, significant effect of PD198306 or NKH477 pretreatment on the GP1a-induced increases in 5-HT_{2A} receptor mRNA. The data represent mean ± SEM (n=3-6).

Effect of PKC on GP1a-Induced Upregulation of 5-HT_{2A} Receptor mRNA

Figures 5A and 5B illustrate the effect of PKC inhibition on GP1a-induced 5-HT_{2A} receptor upregulation. Bouaboula et al. proposed that cannabinoid receptors activate the ERK1/2 signaling cascade through PKC activation [88]. Additionally, they reported evidence to suggest that Ca^{2+-} dependent PKC isoforms could be involved in CB_2 signal transduction, which are not involved in CB_1 signal transduction [88]. Here we studied the effect of two different PKC inhibitors (GF109203X and Go6967) on GP1a-induced increases in 5-HT_{2A} receptor mRNA levels. GF109203X does not discriminate between Ca^{2+} -dependent and -independent isoforms of PKC (IC_{50} values are 0.0084, 0.0180, 0.210, 0.132, and 5.8 μM for α, β1, δ, ε and ζ isoforms, respectively) [220] while Go6967 selectively inhibits Ca^{2+} -dependent isoforms PKCα and PKCβ1 (IC_{50} values are 2.3 and 6.2 nM, respectively) [221].

In Figure 5A, CLU213 cells were pretreated with either vehicle or GF109203X (5μM) for 20 minutes and then treated with vehicle or GP1a (1nM). In this experiment, 5μM GF109203X should produce a substantial inhibition of most PKC isoforms. We found that GP1a significantly ($p<0.05$) increased 5-HT_{2A} receptor mRNA levels (two-fold increase) in vehicle pretreated cells compared to vehicle treated controls (Fig. 5A). Furthermore, GF109203X pretreatment significantly ($p<0.05$) increased basal 5-HT_{2A} receptor mRNA levels (two-fold increase) over vehicle treated controls and had no significant effect ($p>0.05$) on GP1a-induced increases in 5-HT_{2A} receptor mRNA levels. The two-way ANOVA for 5-HT_{2A} mRNA showed significant main effects of GF109203X pretreatment ($F_{(1,11)}$ =6.68, $p<0.0324$) and GP1a treatment ($F_{(1,11)}$ =10.82, $p<0.011$). There was no significant interaction between GF109203X pretreatment and GP1a treatment ($F_{(1,11)}$ =2.17, $p>0.05$).

In order to address the role of PKC Ca^{2+}-dependent isoforms on the regulation of 5-HT_{2A} receptor mRNA, CLU213 cells were pretreated with either vehicle or Go6967 (10nM) for 20 minutes then treated with either vehicle or GP1a (1nm) for 24 hrs. GP1a significantly ($p<0.01$) increased 5-HT_{2A} receptor mRNA levels (two-fold increase) compared to vehicle pretreated controls (Fig. 5B). Pretreatment with Go6967 significantly ($p <0.01$) increased basal 5-HT_{2A} receptor mRNA levels (58% increase) over vehicle pretreated controls while Go6967 pretreatment did not have a significant effect ($p>0.05$) on GP1a-induced upregulation of 5-HT_{2A} receptors mRNA. The two-way ANOVA for 5-HT_{2A} receptor mRNA showed a main effect of Go6967 pretreatment ($F_{(1,11)} =5.85$, $p<0.0418$) and a main effect of GP1a treatment ($F_{(1,11)} =16.15$, $p<0.0038$). There was no significant interaction between Go6967 pretreatment and GP1a treatment ($F_{(1,11)} =2.03$, $p>0.05$).

Next we examined the effect a PKC activator, PDBu (K_d values are 1 μM, 0.98 μM, 26 nM, 11 nM, and 9 nM for ε, δ, β1, α, and ζ isoforms, respectively) [222], has on GP1a-induced increases in 5-HT_{2A} receptor mRNA. We used two doses of PDBu in our experiments, 1 μM and 30 nM. We expect to activate all the different isoforms with the 1 μM dose and selectively activate the β1, α, and ζ isoforms (Ca^{2+}-dependent isoforms) with the lowest dose (30nM) [222].

CLU213 cells were pretreated with either vehicle or PDBu (1μM) for 20 minutes .Cells were then treated with either vehicle or GP1a (1nM). GP1a significantly ($p<0.05$) increased 5-HT_{2A} receptor mRNA levels (two-fold increase, Fig. 5C). This effect of GP1a was prevented ($p<0.05$) in cells pretreated with PDBu. No significant ($p>0.05$) effect of PDBu was found on basal 5-HT_{2A} receptor mRNA levels. The two-way ANOVA for 5-HT_{2A} receptor mRNA showed significant main effects of PDBu pretreatment ($F_{(1,11)} =6.12$, $p<0.0385$) and GP1a treatment

($F_{(1,11)}$ =6.10, p<0.0375). There was a significant interaction between PDBu pretreatment and GP1a treatment ($F_{(1,11)}$ =5.38, p<0.0489).

In order to examine the effect Ca^{2+}-dependent PKC isoforms on GP1a-induced increases in 5-HT_{2A} receptor mRNA, we used a concentration of PDBu (30nM) that activated the Ca^{2+}-dependent isoforms [222]. GP1a significantly (p<0.01) increased 5-HT_{2A} receptor mRNA levels (two-fold increase, Fig. 5D). There was no significant (p>0.05) effect of PDBu 30 nM found on basal 5-HT_{2A} receptor mRNA levels and PDBu pretreatment significantly reduced (approximate 20% decrease, p<0.05) the GP1a-induced upregulation of 5-HT_{2A} receptors. The two-way ANOVA for 5-HT_{2A} receptor mRNA showed significant main effects of PDBu pretreatment ($F_{(1,11)}$ =6.26, p<0.0368) and GP1a treatment ($F_{(1,11)}$ =79.39, p<0.0001). There was no significant interaction between PDBu pretreatment and GP1a treatment ($F_{(1,11)}$ =1.62, p<0.2385). The use of PKC activators seems to suggest that both Ca^{2+}-dependent and Ca^{2+}-independent PKC isoforms play a role preventing the GP1a-induced upregulation of 5-HT_{2A} receptor mRNA.

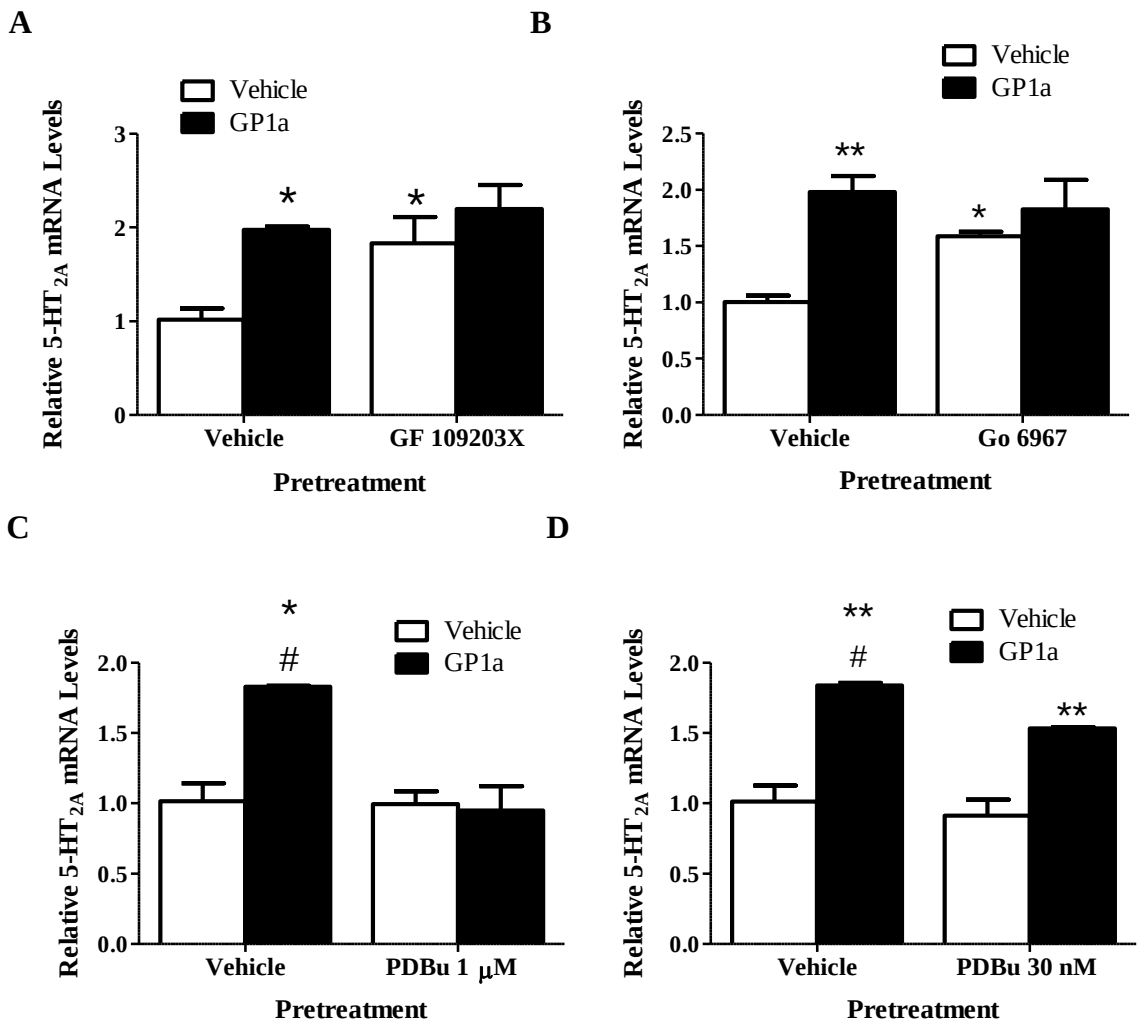

Figure 5. Ca^{2+} -independent and dependent isoforms of PKC regulate 5-HT_{2A} receptor mRNA levels in CLU213 cells.

(A) Inhibition of Ca^{2+} -independent and –dependent isoforms of PKC (GF109203X) enhanced basal levels of 5-HT_{2A} receptor mRNA. *$p<0.05$, significant effect of GP1a treatment, GF109203X pretreatment, and GP 1a/GF109203X treatment compared to vehicle-treated controls. (B) Inhibition of Ca^{2+} -dependent isoforms of PKC (Go6967) enhanced basal levels of 5-HT_{2A} receptor mRNA. **$p<0.01$, significant effect of GP 1a treatment, Go6967 pretreatment,

and GP1a/Go 6967 treatment compared to vehicle-treated controls. (C) Activation of Ca^{2+} -independent and -dependent isoforms of PKC (PDBu) prevented GP1a-induced increases in 5-HT_{2A} receptor mRNA. *$p<0.05$, significant effect of GP1a treatment compared to vehicle-treated controls. #$p<0.05$, significant effect of PDBu pretreatment on GP1a-induced increases in 5-HT_{2A} receptor mRNA. (D) Activation of Ca^{2+} -dependent isoforms of PKC did not prevent GP1a-induced increases in 5-HT_{2A} receptor mRNA. **$p<0.01$, significant effect of GP1a treatment compared to vehicle-treated controls. The data represent mean ± SEM (n=3).

Effect of CREB and AP-1 Transcription Factors Inhibitors on the GP1a- Induced Upregulation of 5-HT_{2A} Receptor mRNA

Figure 6 illustrates the effect of CREB or AP-1 transcription factor inhibitor pretreatment on GP1a-induced upregulation of 5-HT_{2A} receptor mRNA in CLU213 cells. Here we wanted to identify possible transcription factor(s) that would contribute to GP1a-induced increases of 5-HT_{2A} receptor mRNA. In our previous experiments we showed that the GP1a-induced upregulation of 5-HT_{2A} receptors is prevented by PD198306, an inhibitor of ERK1/2 activation. Activation of ERK involved the phosphorylation of this protein in the cytoplasm and its translocation to the nucleus [231-233]. In the nucleus, phosphorylated ERK (pERK) can activate several transcription factors such as CREB, c-Fos, ELK-1, SP-1, and EGR-1 [231-233]. The transcription factors CREB and AP-1 have consensus sequences within the promoter region of the rat 5-HT_{2A} receptor gene [234-236]. Therefore, we decided to test the effects of inhibitors of these transcription factors on the GP1a-induced upregulation of 5-HT_{2A} receptor mRNA.

CREB is a transcription factor that binds to certain DNA sequences called cAMP response elements (CRE), thereby increasing or decreasing transcription of downstream genes [237;238]. c-Fos belongs to the immediate early gene family of transcription factors. Members of the Fos family dimerize with c-jun to form the AP-1 transcription factors, which can upregulate transcription of various genes [239]. In our first experiment, we studied the effect of CREB inhibitor pretreatment on the GP1a-induced upregulation of 5-HT_{2A} receptors.

CLU213 cells were treated with either vehicle or Naphthol AS-E phosphate (10μM) for 20 minutes and then treated with vehicle or GP1a (1nM). Naphthol AS-E phosphate blocks cAMP-induction of CREB-dependent gene transcription (K_i 10μM) [224]. We found that Naphthol AS-E phosphate did not inhibit or decrease GP1a-induced increases in 5-HT_{2A} receptor

mRNA (Fig. 6A). No significant ($p>0.05$) effect of Naphthol AS-E phosphate was found on basal 5-HT_{2A} mRNA levels. The two-way ANOVA for 5-HT_{2A} receptor mRNA showed no significant main effect of Naphthol AS-E phosphate pretreatment ($F_{(1,11)}$ =0.006, $p>0.9384$) and a significant main effect of GP 1a treatment ($F_{(1,11)}$ =28.91,$p<0.0007$). There was no significant interaction between Naphthol AS-E pretreatment and GP1a treatment ($F_{(1,11)}$ =0.23, $p>0.6453$). These data indicate that CREB is not involved in GP1a-induced 5-HT_{2A} receptor upregulation.

We then studied the effect of AP-1 inhibition on GP1a-induced increases in 5-HT_{2A} receptor mRNA. CLU213 cells were treated with either vehicle or SR11302 (1μM) for 20 min then vehicle or GP1a (1nM) was added to the incubation media. SR11302 is retinoid that transrepresses AP-1 without transactivating the retinoic acid response element (E_{max} 1 μM) [225]. As expected, GP1a induced a significant ($p<0.05$) increase in 5-HT_{2A} mRNA levels (approximate two fold increase in 5-HT_{2A} mRNA) (Fig. 6B). SR11302 pretreatment significantly reduced (approximately 55% decrease, $p<0.05$) the GP1a-induced upregulation of the 5-HT_{2A} receptor mRNA (Fig. 6B). No significant ($p>0.05$) effect of SR11302 was found in basal 5-HT_{2A} mRNA levels. The two-way ANOVA for 5-HT_{2A} mRNA did not show a significant main effects of SR11302 pretreatment ($F_{(1,11)}$ =2.89, $p>0.1271$) and did show a significant effect of GP1a treatment ($F_{(1,11)}$ =32.80, $p<0.0004$). There was a significant interaction between SR11302 pretreatment and GP1a treatment ($F_{(1,11)}$ =7.48, $p<0.0256$).

Our data seems to indicate that GP1a-induced upregulation of 5-HT_{2A} receptors would be mediated, at least in part, by ERK1/2 and AP-1 activation. Here we examined whether inhibition of ERK1/2 can prevent the GP1a-induced increases in the nuclear-associated protein levels of c-fos. CLU213 cells were treated with either vehicle or PD198306 (200 nm) for 20 minutes and then treated with either vehicle or GP1a (1nM) for 15 minutes. As mentioned above, PD198306

is a potent inhibitor of ERK1/2 (IC_{50} 100 nM) [218]. We found that in vehicle pretreated cells GP1a significantly ($p<0.05$) increased c-Fos levels over controls (Fig. 6C). Indeed, GP1a induced an approximate 40% increase in the nuclear-associated protein levels of c-Fos. This effect of GP1a was prevented ($p<0.01$) in cells pretreated with PD198306 (Fig. 6C). No significant ($p>0.05$) effect of PD198306 was found on basal 5-HT_{2A} mRNA levels. The two-way ANOVA for 5-HT_{2A} mRNA showed significant main effects of PD198306 pretreatment ($F_{(1,23)}$ =15.74, $p<0.0008$) and GP1a treatment ($F_{(1,23)}$ =6.09, $p<0.0147$). There was a significant interaction between PD198306 pretreatment and GP1a treatment ($F_{(1,23)}$ =5.95, $p<0.0241$).

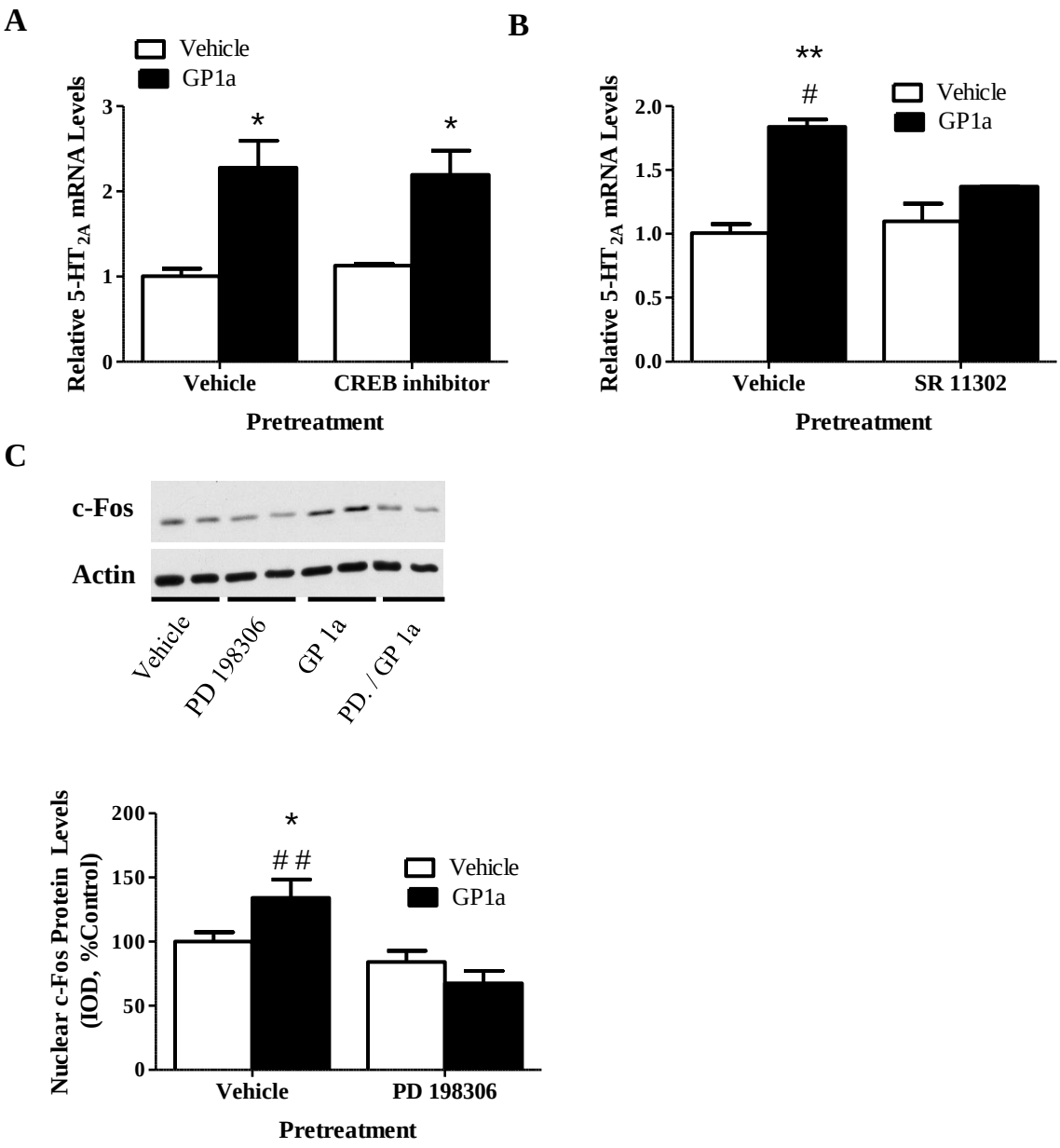

Figure 6. CB_2 receptor-induced upregulation of 5-HT_{2A} receptor involves AP-1 and c-Fos, but not CREB activation.

(A) Inhibition of CREB activation did not prevent or significantly reduce GP1a-induced increases in 5-HT_{2A} receptor mRNA. *$p<0.05$, significant effect of GP1a and CREB/GP1a treatment on 5-HT_{2A} receptor mRNA levels compared to vehicle-treated controls. (B) CB_2

receptor-mediated upregulation of 5-HT_{2A} receptor involves AP-1 transcription factor activity. **$p<0.01$, significant effect of GP1a treatment compared to vehicle-treated controls. #$p<0.05$, significant effect of AP-1 transcription factor inhibitor pretreatment on GP1a-induced increases in 5-HT_{2A} receptor mRNA. (C) Inhibition of GP1a mediated increases in nuclear c-fos protein levels via a selective ERK1/2 inhibitor (PD198306). *$p<0.05$, significant effect of GP1a treatment on nuclear c-fos levels compared to vehicle treated controls. ##$p<0.01$, significant effect of PD198306 pretreatment on GP1a-induced increases in nuclear c-fos levels. The data represent mean ± SEM (n=3-6).

3.5 Discussion

GPCRs can exist as dimers or part of larger oligomeric complexes [240;241]. Interestingly, recent reports from several independent groups suggest that 5-HT_{2A} and D_2 receptors co-expressed in the same cells could form 5-HT_{2A}-D_2 receptor heterodimers [145;195-197]. This 5-HT_{2A}-D_2 receptor complex would be found in cultured cells that co-express these monoamine receptors such as CLU213 cells and in several brain areas such as PFCx and substantia nigra [145]. Our results suggest that exposure to CP55940, a non-selective cannabinoid CB_1/CB_2 receptor agonist [54;211], increases the interaction between 5-HT_{2A} and D_2 receptors in rat PFCx (Fig. 2). Indeed, we found increased co-immunoprecipitation of 5-HT_{2A} and D_2 receptors in PFCx samples of CP55940 treated rats compared to vehicle controls. Co-immunoprecipitation has been successfully used by some groups to demonstrate the interaction between these two monoamine receptors in cultured cells and *in vivo* [145;195]. The nature of this interaction between 5-HT_{2A} and D_2 receptors in PFCx is still not well defined but it could be favored by the high degree of co-localization of 5-HT_{2A} and D_2 receptors in this brain area and by specific domains in the third intracellular loop and the C-tail of the D_2 and 5-HT_{2A} receptors, respectively [145].

The CP55940-enhanced co-immunoprecipitation between 5-HT_{2A} and D_2 receptors in PFCx seems to be mediated by increased protein levels of membrane-associated levels of 5-HT_{2A} and D_2 receptors in this area of the limbic brain. This was shown by increased membrane-associated protein levels of D_2L, D_2S, and 5-HT_{2A} receptors in PFCx of CP55940 treated rats compared to control (Fig. 3A, 3B and 3C). Moreover, we found increased 5-HT_{2A} receptor mRNA in PFCx of CP55940 treated rats compared to controls (Fig. 3D) suggesting that increases in 5-HT_{2A} receptor expression most likely occurs through cannabinoid-mediated

enhanced transcription of the 5-HT_{2A} receptor gene. Our evidence also indicates that exposure to CP55940 induced decreases in D_2 mRNA levels in PFCx (Fig. 3D). This latter study suggests that the CP55940-mediated increases in D_2 receptor protein in rat PFCx may be occurring through mechanisms such as increased trafficking of D_2 receptors from the cytosol to the membrane and/or through decreased degradation of D_2 receptors. Noteworthy, typical antipsychotics such as haloperidol increase D_2 receptor protein levels independently of D_2 mRNA levels even after several days of treatment [242;243]. These latter studies suggest that trafficking of D_2 receptors might play an important role in the regulation of membrane-associated levels of this monoamine receptor. More importantly, recent studies in human tissue using nonselective cannabinoid agonists also support the hypothesis that activation of cannabinoid receptors downregulate D_2 mRNA expression [244]. Δ^9-THC, the main psychoactive component of cannabis sativa (marijuana) is a nonselective CB_1 and CB_2 receptor agonist [245]. Wang et al (2004) reported that expression of D_2 receptor mRNA is decreased in several brain areas of human fetal specimens from mothers with documented evidence of cannabis use during pregnancy suggesting that stimulation of cannabinoid receptors mediates a reduction in D_2 mRNA levels in mesocorticolimbic neural systems [244].

In this study, we used a neuronal cell line and selective CB_1 and CB_2 receptor agonists to determine the contribution of these receptors to the regulation of D_2 and 5-HT_{2A} receptor mRNA levels in cultured cells (Fig. 3E). D_2 receptor mRNA levels were decreased in neuronal cells treated with either CP55940, a nonselective CB_1/CB_2 agonist, or ACEA, a selective CB_1 agonist [55;72] (Fig. 3E). GP1a, a selective CB_2 agonist [60;212], did not modify D_2 mRNA levels in cultured cells (Fig. 3E). These studies suggest that the effect of CP55940 on D_2 mRNA would be mediated by activation of CB_1 receptors. On the other hand, activation of CB_2 receptors seems to

mediate the CP55940-mediated upregulation of 5-HT_{2A} receptor mRNA (Fig. 3E and 3F). Cells treated with either of the following highly selective CB_2 agonists, JWH133 [2;200] or GP1a [60;212], upregulated 5-HT_{2A} receptor mRNA levels in cultured cells compared to vehicle controls. ACEA did not modify 5-HT_{2A} mRNA levels in this cell line. Supporting these results, AM630 a highly selective CB_2 antagonist [200] prevented the JWH 133- or the GP1a-induced upregulation of 5-HT_{2A} mRNA in CLU213 cells. AM630 shows an approximate 165-fold selectivity over CB_2 receptors compared to CB_1 receptors [200].

The results presented here suggest that CB_2, but not CB_1, receptor agonists mediate the upregulation of 5-HT_{2A} receptors. Interestingly, there has been some controversy regarding the expression of CB_2 receptors in the brain. Indeed, CB_2 receptors were initially identified in the periphery but not in the brain [246;247]. Brain expression of CB_2 receptors has been much less well established and characterized in comparison to the expression of brain CB_1 receptors. Later studies have identified CB_2 receptors in several brain areas including: cortex, hippocampus, amygdala, substantia nigra, and cerebellum [18;202]. Furthermore, recent studies reported that there are functional CB_2 receptors in the medial PFCx and that CB_2 receptors are mainly localized in post-synaptic neurons [18;21;94]. These findings have led to a re-evaluation of the possible roles that CB_2 receptors may play in the brain. Interestingly, deletion of the CB_2 receptor induces schizophrenia-related behaviors in mice and chronic treatment with a selective CB_2 agonist (JWH133) increases anxiety in mice [93;248;249]. Here we found that a selective CB_2 receptor agonist induced increases in 5-HT_{2A} receptor mRNA and protein expression in a neuronal cell model. It is possible that CB_2 receptors that are co-localized with 5-HT_{2A} receptors in the PFCx could be driving the upregulation of 5-HT_{2A} receptors in the PFCx of animals

chronically treated with CP55940. However, it is currently unknown whether CB_2 receptors co-localize with 5-HT_{2A} receptors in PFCx.

GP1a and JWH133, two CB_2 receptor agonists, induce an approximate two-fold increase in 5-HT_{2A} receptor mRNA and protein (Fig. 3F). Similar increases in expression of 5-HT_{2A} receptor protein levels have been associated with exposure to drugs of abuse and estrogen [250-252]. In our next experiments we used GP1a to study some of the molecular mechanisms involved in the upregulation of 5-HT_{2A} receptors by CB_2 receptor agonists. For these experiments, we selected GP1a because it shows higher CB_2/CB_1 receptor selectivity compared to JWH133 (>5,000- and 165-fold CB_2/CB_1 selectivity, respectively) [60;212]. First, we examined the role of $G\alpha_i$ G-protein and PKC in the upregulation of 5-HT_{2A} receptors in a neuronal cell line. CB_2 receptors couple to PTX-sensitive Gαi G-proteins to mediate: (1) the inhibition of adenylyl cyclase; and (2) the activation of ERK1/2 signaling pathway [88]. PTX catalyses the ADP-ribosylation of specific $G\alpha_i$ subunits preventing the receptor–G-protein interaction [88]. Our results indicate that PTX prevented the GP1a-induced increases in 5-HT_{2A} mRNA and protein levels (Fig. 4A and 4B). Additionally, we found that the GP1a-induced increases of 5-HT_{2A} receptor mRNA levels are prevented by: (1) inhibition of ERK1/2 activation by PD198306; and by (2) activation of adenylyl cyclase by NKH477 (Fig. 4C) in cultured cells. Our results seem to indicate that the GP1a-induced 5-HT_{2A} upregulation would involve the ERK1/2 activation by PTX-sensitive Gαi G-proteins.

While our results point to the role of Gαi G-proteins and ERK1/2 in the GP1a-induced upregulation of 5-HT_{2A} receptors, the role of PKC is not clear. Based on previous reports we expected a main role of PKC in mediating the GP1a-induced activation of ERK signaling [88]. We found that non-selective PKC inhibitors and selective calcium-dependent PKC inhibitors did

not prevent or significantly reduce the GP1a-induced 5-HT_{2A} upregulation (Fig. 5A and 5B). Moreover, 5-HT_{2A} mRNA basal levels were increased by exposure to either of these PKC inhibitors (calcium dependent and independent inhibitors). Furthermore, activation of calcium dependent and independent isoforms with 1μM PDBu or the selective activation of calcium dependent PKC isoforms with 30 nM PDBu [222] (Fig. 5C and 5D) significantly reduced GP1a-induced increases in 5-HT_{2A} mRNA levels. Specifically, activation of both calcium dependent and independent isoforms completely inhibited the GP1a-induced upregulation of 5-HT_{2A} mRNA, while inhibition of selective calcium dependent isoforms partially prevented it. Hence, these findings do not support a role for different isoforms of PKC as a signaling component in the GP1a-induced upregulation of 5-HT_{2A} receptor signaling but rather they point to a regulatory role of PKC in this signaling pathway. This could be because PKC isoforms are expressed in a tissue-specific manner and individual isoforms play cell-type specific roles in cellular responses as reported [253]. Moreover, activation of certain PKC isoforms inhibits gene transcription [254-256] and that could prevent the GP1a-induced increases in 5-HT_{2A} receptor mRNA levels. In summary, it appears that the GP1a-mediated activation of ERK1/2 would not be mediated by PKC isoforms but it could involve the direct activation of the ERK signaling pathway by scaffold proteins such as β-arrestins [257].

Activation of ERK signaling stimulates several transcription factors such as CREB, c-Fos, ELK-1, SP-1, and EGR-1 [231-233]. AP-1 is a heterodimeric protein composed of proteins belonging to the c-Fos and c-Jun family. Interestingly, CREB and AP-1 have consensus sequences within the promoter region of the rat 5-HT_{2A} receptor gene [234-236]. Therefore, we tested the effects of inhibitors of these transcription factors on the GP1a-induced upregulation of 5-HT_{2A} receptor mRNA. Our results suggest that inhibition of AP-1, but not the CREB,

activation significantly decreased the GP1a-induced upregulation of 5-HT_{2A} receptors (Fig. 6A and 6B). The partial inhibition of the GP1a-induced increases in 5-HT_{2A} mRNA levels by SR11302 suggests that other transcription factors yet to be identified could also contribute to this upregulation. Supporting this hypothesis, we also found that inhibition of ERK1/2 by PD198306 prevented the GP1a-induced activation of c-Fos (Fig. 6C). Although further research is needed, SP-1 could also mediate the GP1a-induced upregulation of 5-HT_{2A} mRNA. This transcription factor is also activated by the ERK signaling cascade and has a consensus sequence within the rat 5-HT_{2A} receptor promoter region [233;258].

Exposure to cannabinoids has been associated in the pathophysiology of several neuropsychiatric disorders such as anxiety, depression and schizophrenia [11-14]. As stated above, these diseases have been also associated with dysregulation of 5-HT_{2A} and D_2 receptor signaling. A causal link has not been found between chronic cannabis use and the etiology of these neuropsychiatric disorders. Recent evidence suggests that chronic use of cannabis may precipitate these disorders in individuals who are prone to developing them [12-14]. Yet a mechanism by which chronic use of cannabis may precipitate these disorders has not been identified. Furthermore, the long term effects of chronic synthetic cannabinoid agonist use, which are now commonly included in herbal incenses and are many times more potent than Δ^9-THC [245], have yet to be addressed. We provide evidence here that exposure to cannabinoids might enhance the formation and activity of 5-HT_{2A}-D_2 receptor heterodimers in PFCx. This would involve increases in membrane-associated levels of 5-HT_{2A} and D_2 receptors in this brain area. In a neuronal cell line we also found that CB_2, but not CB_1 agonists, seems to mediate this increase in 5-HT_{2A} mRNA. We hypothesize that this CB_2 receptor agonist-induced upregulation of 5-HT_{2A} receptors could provide a molecular mechanism by which chronic use of cannabinoids

might precipitate the onset of some cognitive and mood disorders in individuals predisposed to developing them.

Chapter 4: Cannabinoid Receptor Agonists Upregulate and Enhance Serotonin 2A (5-HT_{2A}) Receptor Activity via ERK1/2 Signaling

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4.1 Abstract

Recent behavioral studies suggest that non-selective agonists of cannabinoid receptors may regulate 5-HT_{2A} receptor neurotransmission. Two cannabinoids receptors are found in brain, CB_1 and CB_2 receptors, but the molecular mechanism by which cannabinoid receptors would regulate 5-HT_{2A} receptor neurotransmission remains unknown. Interestingly, we have recently found that certain cannabinoid receptor agonists can upregulate 5-HT_{2A} receptors. Here, we present experimental evidence that rats treated with a non-selective cannabinoid receptor agonist (CP55940) showed increases in 5-HT_{2A} receptor protein levels, 5-HT_{2A} receptor mRNA levels, and 5-HT_{2A} receptor-mediated phospholipase C Beta (PLCβ) activity in PFCx. Similar effects were found in neuronal cultured cells treated with CP55940 but these effects were prevented by selective CB_2, but not selective CB_1, receptor antagonists. CB_2 receptors couple to the ERK signaling pathway by $G\alpha_{i/o}$ class of G-proteins. Noteworthy, GP1a (selective CB_2 receptor agonist) produced a strong upregulation of 5-HT_{2A} receptor mRNA and protein, an effect that was prevented by selective CB_2 receptor antagonists and by an ERK1/2 inhibitor, PD 198306. In summary, our results identified a strong cannabinoid-induced upregulation of 5-HT_{2A} receptor signaling in rat PFCx. Our cultured cell studies suggest that selective CB_2 receptor agonists upregulate 5-HT_{2A} receptor signaling by activation of the ERK1/2 signaling pathway. Activity of cortical 5-HT_{2A} receptors has been associated with several physiological functions and

neuropsychiatric disorders such as stress response, anxiety, depression, and schizophrenia. Therefore, these results might provide a molecular mechanism by which activation of cannabinoid receptors might be relevant to the pathophysiology of some cognitive and mood disorders in humans.

4.2 Introduction

5-HT_{2A} receptors play an important role in the regulation of stress, mood and impulse control [23;24] and the behavioral effects of several drugs of abuse [173;259;260]. 5-HT_{2A} receptors are the most abundant serotonin receptor in PFCx and are predominantly expressed in pyramidal neurons [261]. Impaired function of cortical 5-HT_{2A} receptors has been identified in several neurological and psychiatric disorders such as schizophrenia, Alzheimer's disease, depression, and anxiety [24;26;27].

A recent behavioral report has suggested that repeat exposure to cannabinoid agonists is associated with enhanced activity of 5-HT_{2A} receptors in adult rats [32]. Specifically, Hill et al. (2006) reported that chronic treatment with HU-210, a CB_1/CB_2 receptor agonist, led to a significant enhancement of 5-HT_{2A} receptor mediated head-shake responses [32]. This behavioral test has been widely used as a marker of 5-HT_{2A} receptor function *in vivo* as this behavior is prevented by pretreatment with selective 5-HT_{2A} receptor antagonists and is absent in 5-HT_{2A} receptor knockout animals [33-35]. The detailed molecular mechanism by which cannabinoid receptor agonists regulate 5-HT_{2A} receptor signaling in brain remains unknown; however, we have recently reported that selective cannabinoid agonists can upregulate 5-HT_{2A} receptors [262;263]. Nevertheless in those manuscripts we did not assess the effect of cannabinoid agonists on the activity of 5-HT_{2A} receptors *in vivo* or *in vitro*.

Two cannabinoid receptors have been identified in the brain, CB_1 and CB_2 receptors [18;19;21;202;264]. Endogenous cannabinoids (endocannabinoids), synthetic cannabinoids, and cannabinoids found in nature (such as Δ^9-THC) bind to these receptors with high affinity [15;265]. CB_1 and CB_2 receptors couple to $G\alpha_{i/o}$ class of G-proteins and to the ERK signaling pathway [15;265]. These CB_2 receptors have been identified at postsynaptic terminals while CB_1

receptors are located at presynaptic terminals [19;94;266]. While the activation of presynaptic cannabinoid receptors inhibits the release of several neurotransmitters such as serotonin (5-HT) [267], activation of postsynaptic cannabinoid receptors might modulate the activity of several postsynaptic receptors, including serotonin and dopamine receptors[247;268].

The objectives of the present study were to identify whether repeat exposure to a non-selective cannabinoid agonist can modify the activity of 5-HT_{2A} receptors in rat PFCx. Also two neuronal cell lines were used to better study the mechanisms of cannabinoid-induced upregulation of 5-HT_{2A} receptors after a single and repeated exposure to cannabinoid agonists. These two independent cell lines were utilized to address whether the cannabinoid-induced regulation of serotonin receptors is a generalized phenomenon and not exclusive to a single cell line. Additionally, we investigated the effect of single or repeated exposure to cannabinoids on the ERK1/2 signaling pathway and the role of this signaling protein in the cannabinoid-mediated increases in 5-HT_{2A} receptor protein levels. As 5-HT_{2A} receptors in PFCx have been associated with several physiological functions and neuropsychiatric disorders such as stress response, anxiety, depression, and schizophrenia [28;137;174;175], increases in 5-HT_{2A} receptor function in this limbic region may be clinically relevant to the pathophysiology of mood and cognitive disorders.

4.3 Materials and Methods

Drugs

(-)-*cis*-3-[2-Hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)cyclo-hexanol (CP55940), a CB_1 and CB_2 agonist; N-(Piperidin-1-yl)-1-(2,4-dichlorophenyl)-1,4-dihydro-6-methylindeno[1,2-c]pyrazole-3-carboxamide (GP1a) a highly selective CB_2 receptor agonist; N-(2-Chloroethyl)-5Z,8Z,11Z,14Z-eicosatetraenamide (ACEA) a highly selective CB_1 receptor agonist; 2-(2-Chlorophenyl)-3-(4-chlorophenyl)-7-(2,2-difluoropropyl)-6,7-dihydro-2*H*-pyrazolo[3,4-*f*][1,4]oxazepin-8(5*H*)-one (PF-514273) a selective and potent CB_1 receptor antagonist; *N*-(1,3-Benzodioxol-5-ylmethyl)-1,2-dihydro-7-methoxy-2-oxo-8-(pentyloxy)-3-quinolinecarboxamide (JTE 907) a selective CB_2 receptor antagonist; [6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl](4-methoxyphenyl)-methanone (AM630) a selective CB_2 receptor antagonist; 6-Chloro-2,3-dihydro-5-methyl-*N*-[6-[(2-methyl-3-pyridinyl)oxy]-3-pyridinyl]-1*H*-indole-1-carboxyamide dihydrochloride (SB 242084) a selective 5-HT_{2C} receptor antagonist; and *N*-(Cyclopropylmethoxy)-3,4,5-trifluoro-2-[(4-iodo-2-methylphenyl)amino]-benzamide (PD 198306) a potent and selective ERK1/2 inhibitor were purchased from Tocris (Ellisville, MO, USA). (-) DOI [(-)-1-(2, 5-dimethoxy-4-iodophenyl)-2-aminopropane HCl] was purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA).

Animal Experimental Protocols

Male Sprague-Dawley rats (225-275 g) were purchased from Harlan (Indianapolis, IN, USA). The rats were housed two per cage in a temperature-, humidity-, and light-controlled room (12 hr light/dark cycle, lights on 7:00 AM-19:00 PM). Food and water were available *ad libitum*. All procedures were conducted in accordance with the National Institutes of Health

Guide for the Care and Use of Laboratory Animals as approved by the University of Kansas Institutional Animal Care and Use Committee (IACUC).

After arrival, the rats were allowed to acclimate to their environment for at least 4 days prior to the start of the treatment period. Eight rats were randomly assigned to each group. Cage-mates were assigned to the same treatment group. Rats were injected with either vehicle (Tween-80/ethanol/saline (1:1:18); 1ml/kg, i.p.) or CP55940 (0.05 mg/kg, i.p.) once a day for 7 days. Rats were sacrificed by decapitation 48 h after the last CP55940 injection. The brains were immediately removed and the PFCx was dissected and frozen in dry ice.

Phospholipase C (PLCβ) Activity assay in rat PFCx

PFCx tissue from treatment groups that received the saline challenge were utilized for measurement of PLCβ activity. PLCβ activity was measured by the amount of inositol 1,4,5 trisphosphate produced by PLCβ in the membrane fraction of the isolated tissue as previously described[269;270]. 5-HT-stimulated PLCβ activity in PFCx is a selective measure of 5-HT_{2A} receptor function as previously demonstrated using selective antagonists[270]. Briefly, membrane protein from PFCx (30 µg) was diluted in 100 µl of buffer (25 mM Hepes-Tris pH 7.4, 3 mM EGTA, 10 mM LiCl, 12 mM $MgCl^2$, 1.44 mM sodium deoxycholate) with 1 µM GTPγS, 300 nM free Ca^{2+}, 0.3 µM 5-HT, and 1 mM unlabeled phosphatidyl inositol. We used 0.3 µM 5-HT to stimulate PLCβ activity because this is the EC_{50} previously described in the literature [269;270]. Samples were kept on ice until the reaction was started with 100 µM [^{3}H] phophatidyl inositol at 37ºC for 20 minutes. This reaction was then stopped through addition of 0.9 ml $CHCl_2$/ MeOH (1:2) and 0.3 ml of chloroform. Samples were shaken for 90 s and centrifuged at 21,000 g for 90 s at room temperature. Finally, 0.3 ml of the upper aqueous phase was mixed with 6 ml of scintillation cocktail and counted by a scintillation counter for 5 minutes.

Western blots

Proteins isolated from cultured cells and PFCx were used in these experiments. Membrane-associated proteins were isolated using the ProteoExtract™ Native Membrane Protein Extraction kit (Calbiochem, La Jolla, CA, USA). Expression of membrane-associated 5-HT_{2A} receptors was determined by Western blot as previously described [271]. The anti-5-HT_{2A} antibody was a generous gift from Dr. Nancy A. Muma and has been previously validated in the literature [194]. ERK1/2 and pERK antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Negative controls included either omission of primary antibody or addition of pre-immune rabbit immunoglobulins. β-actin was used as a control for protein loading (approximate 46 kDa molecular weight).

Film analysis

Films were analyzed densitometrically using Scion Image software (Scion Corporation, Frederick, MD, USA), as previously described [272]. Each sample was measured on three independent gels. All samples were standardized to controls and normalized to their respective actin levels.

qRT-PCR experiments

Total RNA was isolated from cultured cells and PFCx tissue by using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) protocol as described by the manufacturer. Total RNA was reverse transcribed to generate cDNA. Quantitative real time PCR reactions were prepared using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), a 4% (v/v) concentration of cDNA product, and forward and reverse primers at a final concentration of 0.35 mM run as we previously described [29]. All reactions were performed in triplicate using the ABI 7500 fast real time PCR system (Applied Biosystems, Foster City, CA, USA). A negative

control lacking cDNA or any known DNA template was included for each primer pair. The primers used in this manuscript were: 5-HT_{2A} (F:5'-AACGGTCCATCCACAGAG-3' and R:5'-AACAGGAAGAACACGATGC-3'), CB_1 (F:5'-CATCATCATCCACACGTCAGAAG-3' and R:5'-ATCAACACCACCAGGATCAGAAC-3'), CB_2 (F:5'-CCAACATGTAGCCAGCTTGACT-3' and R:5'-TGCAGGAACCAGCATATGA-3'), $G\alpha_q$ (F:5'-AGTTCGAGTCCCCACCACAG-3' and R:5'-CCTCCTACATCGACCATTCTGAA-3'), 5-HT_{1A} (F:5'-CCGCACGCTTCCGAATCC-3' and R:5'-TGTCCGTTCAGGCTCTTCTTC-3'), and GAPDH (F:5'- TGGAGTCTACTGGCGTCTTCAC-3' and R:5'-GGCATGGACTGTGGTCATGA-3'). These primers have been previously validated in the literature [29;208;273;274].

In all real-time PCR experiments, measurements were made from the number of cycles required to reach the threshold fluorescence intensity [cycle threshold (Ct)]. Ct values for each reaction were subtracted from Ct values for GADPH and then subtracted from Ct values for vehicle-treated controls that served as a baseline, and the result was referred to as ΔΔCt. Fold changes in gene expression were calculated as 2-ΔΔCt to reflect the fact that, under optimal conditions, the amount of PCR product doubles with each amplification cycle.

Cell culture protocols

CLU213 and A1A1v cells, neuronal cell lines that endogenously, express CB_1, CB_2 and 5-HT_{2A} receptors, were either purchased from Cedarlane Laboratories (Burlington, NC, USA) or kindly provided by Dr. William Clarke and Kelly Berg (University of Texas Health Science Center, San Antonio, TX, USA), respectively [262;263;275]. We utilized two cells line in our work in order to examine whether our findings could be replicated in two independent neuronal cells lines**.** These two cell lines were grown on 100-mm^2 plates treated with polystyrene (Corning Incorporated, Corning, NY, USA) and maintained in 5% CO_2 at 37°C, in Dulbecco's

modified eagle medium (Mediatech Inc, Manassas, VA, USA) containing 10% fetal bovine serum (Thermo Scientific, Logan, UT, USA).

Determination of the effect of CP55940 on 5-HT_{2A} receptor mRNA

CLU213 cells were incubated with either vehicle (ethanol 0.01% final concentration) or CP55940 (1nM) for 24 hours. Total RNA was isolated and qRT-PCR for 5-HT_{2A}, CB_1, and CB_2 was performed as described above. Samples were run in triplicate.

Regulation of 5-HT_{2A} Receptor Protein Levels by Non-selective Cannabinoid Receptor Agonist

CLU213 cells were incubated with either vehicle (ethanol 0.01% final concentration) or CP55940 (1nM) for 72 hours. Cells were washed with PBS every 24 hours and fresh vehicle or CP55940 (1nM) were added. Expression of membrane-associated 5-HT_{2A} receptors was determined by Western blot as described above.

Phosphoinositol Hydrolysis in cultured cells

CLU213 cells were seeded at a density of 10,000 cells/well in 24-well plates in complete medium (day 1). On day 2, cells were placed in serum-free medium and incubated with CP55940 (1nM) for 3 days. Cells were washed with PBS every 24 hours and vehicle or CP55940 was added. On day 4, Myo-[3H]Inositol (Du Pont NEN, USA) was added to the incubation media[276]. 5-HT_2 receptor-mediated phosphoinositol (PI) hydrolysis assays were performed on day 5 as previously described using (-)DOI (10^{-6} M), a 5-$HT_{2A/2C}$ receptor agonist[276]. In all these experiments, cells were pretreated with SB242084 10nM, a 5-HT_{2C} receptor antagonist [277], prior to (-)DOI treatment.

Effect of selective cannabinoid receptor antagonists on the CP55940-induced upregulation of 5-HT_{2A} receptors

CLU213 cells were pretreated with either vehicle (ethanol 0.01% final concentration), PF-514273 (20nM, CB_1 antagonist)[57], or JTE907 (10nM, a CB_2 receptor antagonist)[61;62]. Twenty min later cells were incubated with either vehicle (ethanol 0.01% final concentration) or CP55940 (1nM) for 24 hours. Total RNA was isolated and qRT-PCR for 5-HT_{2A} was performed as described above.

Regulation of 5-HT_{2A} receptor mRNA transcription by selective CB_1 and CB_2 receptor agonists

Either CLU213 or A1A1v cells were incubated with either vehicle (ethanol 0.01% final concentration), CP55940 1nM (non-selective CB_1/CB_2 agonist K_i: 0.58nM and 0.68nM for CB_1 and CB_2 receptors, respectively)[50], ACEA 15nM (selective CB_1 agonist, K_i: 1.4nM and 3.1μM for CB_1 and CB_2 receptors, respectively)[55;72], or GP1a 1nM (selective CB_2 agonist, Ki: 0.037nM and 35nM for CB_2 and CB_1 receptors, respectively)[60] for 24 hours. Total RNA was isolated and qRT-PCR for 5-HT_{2A} mRNA was performed as described above.

Effect of selective cannabinoid receptor antagonists on the CB_2 receptor agonist-induced upregulation of 5-HT_{2A} receptors

In a different experiment, CLU213 cells were incubated with either vehicle (ethanol 0.01% final concentration) or one of the following CB_2 receptor antagonists, JTE907 (10nM), or AM630 (1μM) [61;62;248]. Twenty minutes later cells were treated with either vehicle or 1nM GP1a, selective CB_2 agonist [60]. Twenty four hours later total RNA was isolated and qRT-PCR for 5-HT_{2A} mRNA was performed as previously described.

Regulation of 5-HT_{2A} receptor protein levels by a CB_2 receptor agonist

CLU213 cells were incubated with either vehicle (ethanol 0.01% final concentration) or GP1a (1nM) for 72 hours. Cells were washed with PBS every 24 hours and fresh vehicle or GP1a was added. Expression of membrane-associated 5-HT_{2A} receptors was determined by Western blot as described above.

Effect of ERK1/2 inhibition on the GP1a-induced upregulation of 5-HT_{2A} receptor mRNA

CLU213 or A1A1v cells were incubated with either vehicle (ethanol 0.01% final concentration) or PD 198306 (200nM) for 20 min. PD 198306 is a potent inhibitor of ERK1/2 activation [278;279]. Cells were then treated with either vehicle (ethanol 0.01% final concentration) or GP1a (1nM) and incubated for 24 hours. Total RNA was isolated and 5-HT_{2A} receptor mRNA levels were determined as described above.

Effect of ERK1/2 inhibition on the GP1a-mediated increases in Nuclear-associated levels of pERK

CLU213 cells were incubated with either vehicle (ethanol 0.01% final concentration) or PD 198306 (200nM) for 20 min. Cells were then treated with either vehicle (ethanol 0.01% final concentration) or GP1a (1nM) and incubated for 24 hours. Cells were then rinsed with PBS, collected and nuclear-associated proteins were isolated using NE-PER ® Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, IL, USA). Expression of nuclear-associated pERK levels were determined by Western blot as previously described.

Effect of ERK1/2 inhibition on the GP1a-induced increase in 5-HT_{2A} receptor protein levels

CLU213 cells were rinsed with PBS (3x) and incubated with either vehicle (ethanol 0.01% final concentration) or PD 198306 (200nM) for 20 min. Cells were then treated with either vehicle (ethanol 0.01% final concentration) or GP1a (1nM) and incubated for 24 hours.

This procedure was repeated over three days. Cells were collected 72 h after the initial treatment. 5-HT_{2A} receptor protein levels were measured in whole cell lysate by Western blot as described above.

Effect of a single or repeated GP1a exposure on ERK1/2 or pERK protein levels in CLU213 cells

Cells were treated with either vehicle (ethanol 0.01% final concentration) or GP1a (1nM) for 24 hours. The next day, cells were rinsed with PBS (3x) and incubated with either vehicle (ethanol 0.01% final concentration) or GP1a (1nM) for 15 minutes. Cells were rinsed with PBS, collected and cytosolic and nuclear-associated proteins were isolated using NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, IL, USA). Expression of either cytosolic ERK1/2 protein or nuclear-associated pERK protein levels were determined by Western blot as previously described.

In a different experiment, CLU213 cells were treated with either vehicle (ethanol 0.01% final concentration) or GP1a (1nM) for 24 hours. The next day, cells were rinsed with PBS (3x) and incubated with either vehicle (ethanol 0.01% final concentration) or GP1a (1nM) for 15 minutes. Total RNA was isolated and 5-HT_{2A} receptor mRNA levels were determined as described above.

Statistics

All data are expressed as the mean ± S.E.M., where *n* indicates the number of rats or cell culture plates per group. Data was analyzed by an unpaired Student's t-test or ANOVA (Newman-Keuls post-hoc test). GB-STAT software (Dynamic Microsystems, Inc., Silver Spring, MD, USA) was used for all statistical analyses.

4.4 Results

CP55940 exposure enhances 5-HT_{2A} receptor signaling and expression in rat PFCx

We first examined the effect of chronic administration of CP55940 (50µg/kg for 7 days), a CB_1/CB_2 receptor agonist [88], on the activity and expression of 5-HT_{2A} receptors in rat PFCx. Previously we have found that chronic CP55940 treatment increases 5-HT_{2A} receptor expression but the effect on 5-HT_{2A} receptor signaling is unknown [263]. Initially, we measured activity of 5-HT_{2A} receptors in PFCx because 5-HT-stimulated phosphoinositol hydrolysis in this brain area has been reported to be mediated primarily by activation of 5-HT_{2A} receptors [269;270]. 5-HT_{2A} receptor stimulated PLCβ activity was significantly ($p<0.01$) greater in CP55940-treated rats compared with controls (578 ± 44 and 397 ± 34 pmoles/mg protein/min for CP55940 and vehicle-treated rats, respectively) (Fig. 7A). The two-way ANOVA detected a main effect of treatment with CP55940 ($F_{(1,17)}$ =58.18, $p<0.0001$) and 5-HT ($F_{(1,17)}$ =1000.48, $p<0.0001$) on the PLCβ activity and a main interaction between these two factors ($F_{(1,17)}$ =58.45, $p<0.0001$). Noteworthy, this CP55940-induced enhanced PLCβ activity was associated with a significant ($p<0.05$) two-fold increase in the membrane-associated levels of 5-HT_{2A} receptors in PFCx compared to controls (Fig. 7B). Here, 5-HT_{2A} receptors were identified as a single and prominent band with a molecular mass of approximately 42-43 kDa as previously described [194].

5-HT_{2A} receptor mRNA was significantly ($p<0.05$) increased in PFCx of CP55940-treated rats compared to vehicle-treated controls (approximate 90% increase, Fig. 7C). No significant changes in the levels of 5-HT_{1A} receptor or $G\alpha_q$ G-protein mRNAs were detected in PFCx of CP55940-treated animals. This highlights the specificity of the effect of CP55940 on 5-HT_{2A} receptor signaling. Also, we found a significant ($p<0.05$) downregulation of CB_1 and CB_2 receptor mRNA in PFCx of CP55940-treated rats compared to vehicle controls (Fig. 7C). CB_1

and CB_2 mRNA levels were reduced by 35% and 60% in PFCx of CP55940-treated animals, respectively.

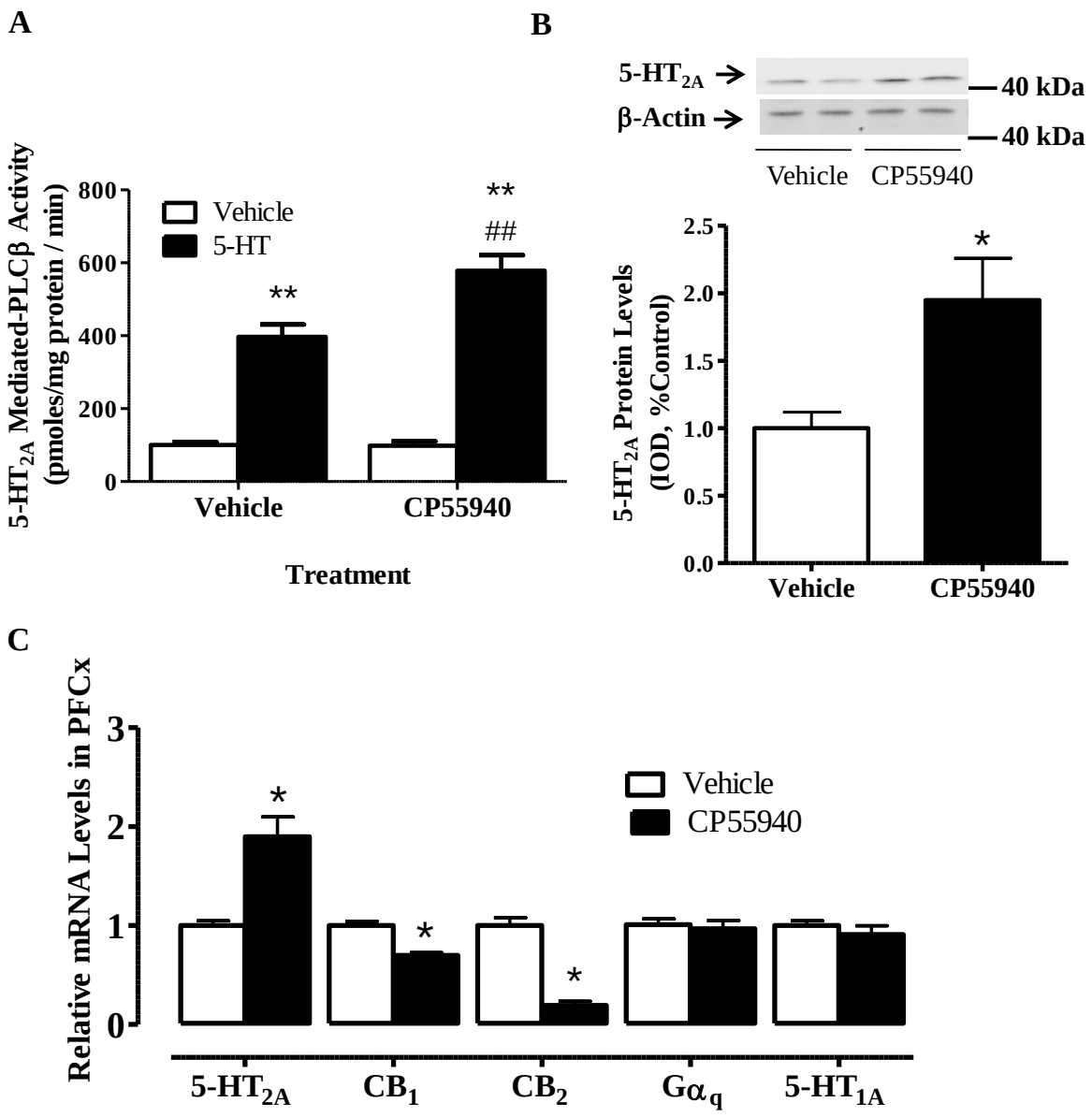


Figure 7. CP55940-induced enhance activity and upregulation of 5-HT$_{2A}$ receptors in PFCx. (A) 5-HT$_{2A}$ receptor stimulated PLCβ activity in PFCx of rats treated with either vehicle or CP55940 (50μg/kg, i.p.) for 7 days and withdrawn for 48 hours. We detected an increased 5-HT-stimulated PLCβ activity in rats treated with CP55940 compared to control rats (**p <0.01, significant effect compared to vehicle-treated rats; $^{\#\#}$ p <0.01, significant effect of 5-HT-stimulated PLCβ activity compared to vehicle-treated rats). (B) Increased membrane-associated 5-HT$_{2A}$ receptor protein levels in PFCx of CP55940-treated rats. β-actin was used as a loading

control. Representative Western blots are shown in this figure and IOD was calculated as described in Experimental Procedures (*p<0.05, significant effect of CP55940 treatment compared to vehicle-treated animals). (C) Increased 5-HT_{2A} receptor mRNA levels and reduced CB_1 and CB_2 mRNA levels in PFCx of CP55940 treated rats compared to controls (*p <0.05, significant effect compared to vehicle-treated rats). The data represent mean ± SEM (n=4-6).

CP55940 exposure enhances 5-HT$_{2A}$ receptor signaling and expression in a neuronal cell model, CLU213 cells

CLU213 cells were used to better study the mechanisms involved in the upregulation of 5-HT$_{2A}$ receptors. Initially, we examined the effect of chronic incubation with CP55940 on the mRNA levels of 5-HT$_{2A}$ and cannabinoid receptors. CP55940 produced a significant ($p<0.05$) upregulation of 5-HT$_{2A}$ mRNA levels in CLU213 cells (Fig. 8A). Cells treated with CP55940 showed an approximate two-fold increase in 5-HT$_{2A}$ receptor mRNA levels compared to controls. CB$_1$ and CB$_2$ mRNA levels were significantly ($p<0.05$) downregulated in CP55940-treated cells (Fig. 8A). CB$_1$ and CB$_2$ mRNA levels were 80% and 65% lower in CP55940-treated cells compared to vehicle-treated cells. CP55940-treated cells also showed a significant ($p<0.01$) increase in the membrane-associated levels of 5-HT$_{2A}$ receptors compared to vehicle treated cells (approximate 80% increase, Fig. 8B). Indeed, CLU213 cells exposed to CP55940 for 72 hours showed a two-fold increase in the membrane-associated levels of 5-HT$_{2A}$ receptors compared to controls (Fig. 8B). No significant effect on the protein levels of 5-HT$_{2A}$ receptors were detected after 24 hours of exposure to CP55940 (data not shown). We also studied the effect of CP55940 on the activity of 5-HT$_2$ receptors in CLU213 cells by measuring the (-)DOI-induced phosphoinositol (PI) hydrolysis [276]. In this assay, we measured the (-)DOI-stimulation of PI hydrolysis in cells incubated with either vehicle or CP55940 (1nM) for 72 hours. We found that (-)DOI stimulated PI hydrolysis in vehicle- and CP55940-treated cells (Fig. 8C). Indeed, 10^{-6} M (-)DOI produced a 60% and 98% increase in PI hydrolysis compared to controls (vehicle- and CP55940-treated cells, respectively). Importantly, (-)DOI-induced PI hydrolysis was significantly ($p<0.05$) higher in CP55940-treated cells compared to vehicle-treated controls.

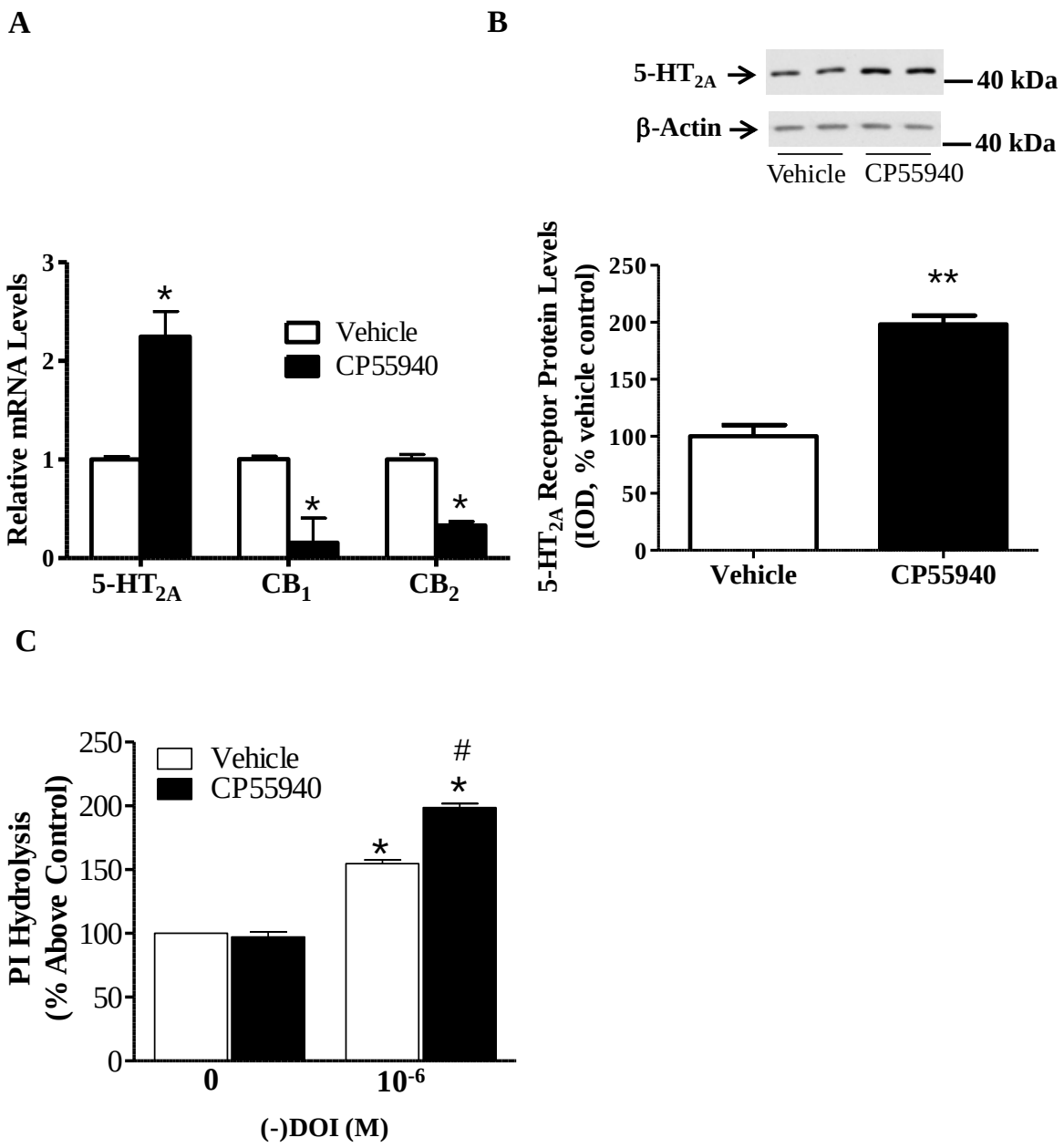


Figure 8. CP55940-induced enhanced activity and upregulation of 5-HT_{2A} receptors in CLU213 cells.

(A) CP55940-induced increased $5HT_{2A}$ receptor mRNA levels and reduced CB_1 and CB_2 receptor mRNA levels in CLU213 cells. *$p<0.05$ significant effect of CP55940 treatment compared to controls. (B) Increased membrane-associated 5-HT_{2A} receptor protein levels in CP55940 treated cells. **$p<0.01$, significant effect of CP55940 compared to vehicle-treated

controls (C) CP55940-induced increases in 5-HT_{2A} receptor-mediated phosphoinositol (PI) hydrolysis in CLU213 cells. *$p<0.05$, significant effect of (-)DOI compared to vehicle-treated controls. $^{\#}p<0.05$, significant effect of (-)DOI on CP55940-treated cells compared with (-)DOI-treated cells. The data represent mean ± SEM (n=3).

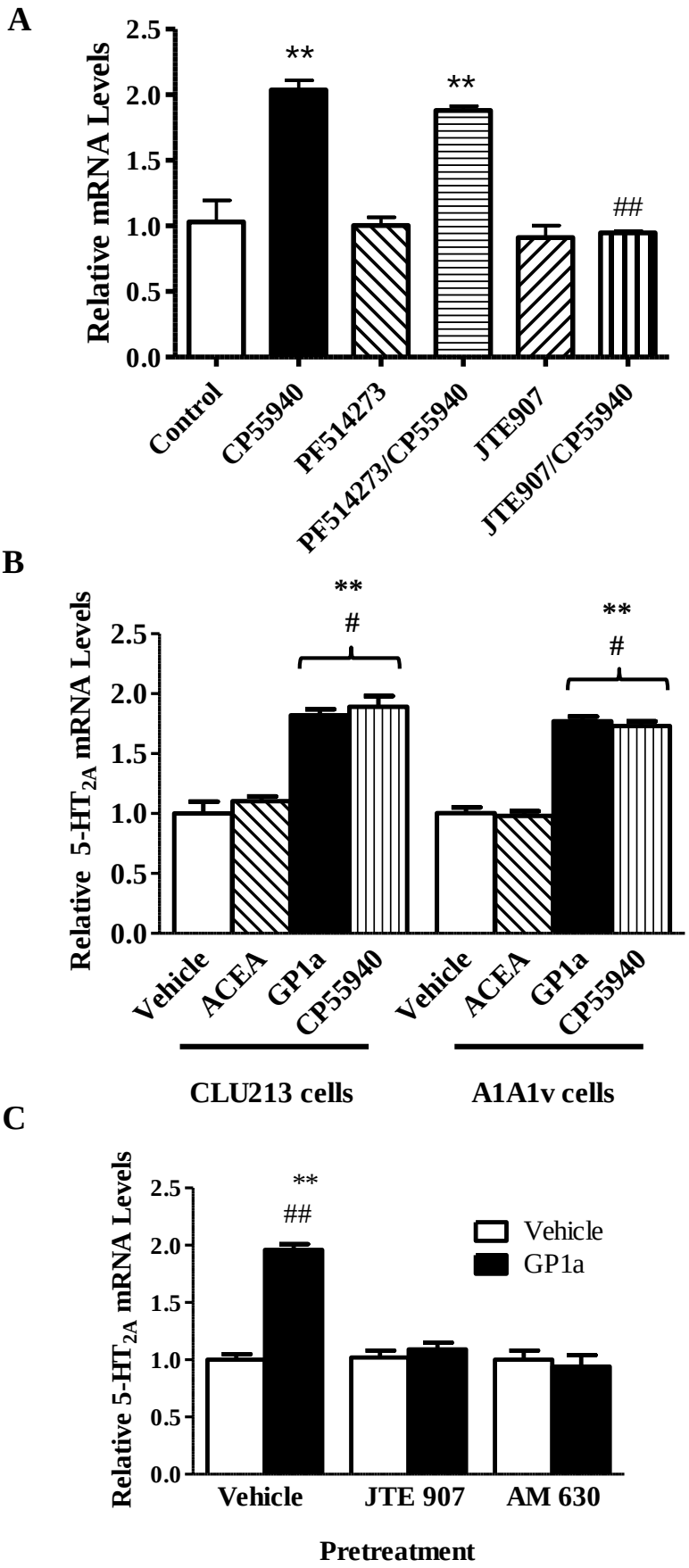
Selective CB_2 receptor agonists upregulate 5-HT_{2A} receptor mRNA in two neuronal cell models, CLU213 and A1A1v cells.

We then aimed to identify the effect of selective cannabinoid receptor antagonists, PF-514 (CB_1 antagonist) and JTE907 (CB_2 antagonist), on the CP55940-induced upregulation of 5-HT_{2A} receptor signaling in CLU213 cells as described in methods (Fig. 9A). CP55940 produced an approximate two-fold increase in 5-HT_{2A} mRNA levels (Fig. 9A). CP55940-induced 5-HT_{2A} receptor upregulation was not significantly ($p>0.05$) modified in cells pretreated with PF-514273. Moreover, CP55940-induced a 93% increase in 5-HT_{2A} mRNA compared to vehicle controls, suggesting that the CP55940-induced 5-HT_{2A} receptor upregulation would be independent of CB_1 receptors. Noteworthy, the CP55940-induced 5-HT_{2A} receptor upregulation was prevented in CLU213 cells pretreated with JTE907. Furthermore, we did not detect significant ($p>0.05$) differences in 5-HT_{2A} mRNA levels between vehicle controls and cells pretreated with JTE907 and treated with CP55940. This suggests that CP55940-induced upregulation of 5-HT_{2A} receptors might be mediated by CB_2 receptors in CLU213 cells (Fig. 9A). Neither JTE907 nor PF-514273 modified 5-HT_{2A} receptor mRNA basal levels (Fig. 9A). The two-way ANOVA did not show a significant main effect of CP55940 treatment ($F_{(1,17)}$ =2.36, $p>0.15$) but found a main effect of pretreatment with cannabinoid receptor antagonists ($F_{(2,17)}$ =20.65, $p<0.0001$). There was a significant interaction between pretreatment and treatment on 5-HT_{2A} receptor mRNA levels ($F_{(2,17)}$ =63.25, $p<0.0001$)

We also used the non-selective or selective CB_1 or CB_2 receptor agonists, CP55940, ACEA or GP1a respectively, to study their effect on 5-HT_{2A} receptor upregulation on either CLU213 or A1A1v cells [18;280-282]. This experiment was designed to study whether these cannabinoid agonists can mediate similar effects in two different and independent neuronal cell

lines that endogenously express 5-HT_{2A} receptors. CP55940 and GP1a significantly ($p<0.01$) upregulated 5-HT_{2A} mRNA in both CLU213 and A1A1v cells (Fig. 9B). Specifically, CP55940 increased 5-HT_{2A} receptor mRNA levels by 89 ± 9% or 73 ± 4% in CLU213 or A1A1v cells compared to vehicle controls, respectively. GP1a increased 5-HT_{2A} receptor mRNA levels by 82 ± 5% or 77 ± 3% in CLU213 or A1A1v cells compared to controls, respectively (Fig. 9B). ACEA was unable to upregulate 5-HT_{2A} receptor mRNA levels in either cell type compared to controls. No significant ($p>0.05$) differences in either the CP55940- or the GP1a-induced upregulation of 5-HT_{2A} receptor mRNA was detected between CLU213 and A1A1v cells. The two-way ANOVA analysis did not show a main effect on cell type used ($F_{(1,23)} =0.12$, $p>0.1645$) but found a main effect on the cannabinoid agonists used ($F_{(3,23)} =167.98$, $p<0.0001$) and a main interaction between these two factors ($F_{(3,23)} =189.12$, $p<0.0001$).

Our previous data suggest that selective CB_2 receptor agonists induce a strong regulation of 5-HT_{2A} receptors. JTE907 and AM630 are two specific CB_2 receptor antagonists used in the literature to study the specific effects of CB_2 receptors in different animal or cell culture models [61;62;248]. In these experiments we studied the effect of these two antagonists on the GP1a-induced upregulation of 5-HT_{2A} receptors (Fig. 9C). GP1a induced a significant ($p<0.01$) approximately two-fold increase in 5-HT_{2A} mRNA levels in CLU213 cells compared to vehicle controls (Fig. 9C). This effect was significantly ($p<0.01$) prevented in cells pretreated with either JTE907 or AM630 prior to the GP 1a treatment. The two-way ANOVA found a main effect of antagonist pretreatment ($F_{(2,17)} =35.76$, $p<0.0001$) and agonist treatment ($F_{(1,17)} =43.63$, $p>0.0001$) and a significant interaction between these two factors ($F_{(2,17)} =36.91$, $p<0.0001$).


A
Relative mRNA Levels
2.5
2.0
1.5
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**
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##
Control
CP55940
PF514273
PF514273/CP55940
JTE907
JTE907/CP55940
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Relative 5-HT2A mRNA Levels
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Vehicle
ACEA
GP1a
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ACEA
GP1a
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CLU213 cells
A1A1v cells
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Relative 5-HT2A mRNA Levels
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##
Vehicle
GP1a
Vehicle
JTE 907
AM 630
Pretreatment

Figure 9. Selective CB_2 receptor antagonists prevent cannabinoid-induced upregulation of 5-HT_{2A} receptors in neuronal cells.

(A) Effect of antagonists of CB_1 and CB_2 receptors on the CP55940-induced upregulation of 5-HT_{2A} receptor mRNA. JTE907 pretreatment, a CB_2 receptor antagonist, prevented the 5-HT_{2A} receptor upregulation in CP55940-treated cells. PF514273 pretreatment, a CB_1 receptor antagonist, did not modify the effect of CP55940-treatment on 5-HT_{2A} mRNA levels. **$p<0.01$, significant effect of CP55940 on 5-HT_{2A} receptor mRNA levels compared with vehicle-treated cells. $^{\#\#}p<0.01$, significant effect of JTE907 pretreatment on the CP55940-induced upregulation of 5-HT_{2A} mRNA. (B) Effect of CP55940, ACEA, and GP1a on 5-HT_{2A} receptor mRNA levels in CLU213 and A1A1v cells. **$p<0.01$, significant effect of GP1a or CP55940 treatment on 5-HT_{2A} receptor mRNA levels compared with vehicle-treated CLU213 or A1A1v cells. $^{\#}p<0.05$, significant effect of GP1a or CP55940 treatment compared to ACEA treatment on 5-HT_{2A} receptor mRNA levels in CLU213 or A1A1v cells. (C) JTE907 and AM630, selective CB_2 receptor antagonists, prevented the GP1a-induced upregulation of 5-HT_{2A} receptor mRNA. $^{**}p<0.01$, significant effect of GP1a treatment on 5-HT_{2A} receptor mRNA levels compared to vehicle treated cells. $^{\#\#}p<0.01$, significant effect of JTE907 or AM630 pretreatment on the GP1a-induced upregulation of 5-HT_{2A} receptor mRNA in CLU213 cells. The data represent mean ± SEM (n=3).

GP1a-induces upregulation of 5-HT_{2A} receptor mRNA via ERK1/2 signaling pathway in two neuronal cell models

CB_2 receptors couple through the $G_{i/o}$ class of G-proteins to the ERK signaling pathway [265]. We aimed to identify the effect of a selective ERK1/2 inhibitor on the GP1a-induced upregulation of 5-HT_{2A} receptor signaling in CLU213 cells as described in the methods (Fig. 10A). GP1a mediated a significant ($p<0.01$) upregulation of 5-HT_{2A} receptor mRNA in both cell types. Indeed, cells incubated with GP1a (1nM) exhibited a 90 ± 4% and 84 ± 4% increase in 5-HT_{2A} mRNA in CLU213 and A1A1v cells (Fig. 10A). This strong GP1a-induced 5-HT_{2A} receptor upregulation was significantly ($p<0.01$) prevented in both cell types by PD198306 pretreatment, suggesting that the GP1a-induced activation of ERK1/2 signaling mediates the 5-HT_{2A} receptor upregulation in both CLU213 and A1A1v cell lines. No significant differences in the basal levels of 5-HT_{2A} receptor mRNA was detected between cells pretreated with either vehicle or PD198306. The three-way ANOVA found a main effect of pretreatment with PD198306 ($F_{(1,23)}$ =0.4524, $p<0.0001$) and treatment with GP1a ($F_{(1,23)}$ =116.33, $p<0.0001$) but not of the cell type used ($F_{(1,23)}$ =0.4524, $p>0.8343$). No main interaction was detected between either pretreatment ($F_{(1,23)}$ =0.7159, $p<0.4099$) or treatment ($F_{(1,23)}$ =21316, $p<0.6505$) with the cell type used. A main interaction was detected between pretreatment and treatment ($F_{(1,23)}$ =1152387, $p<0.0001$). No main interaction was detected between these three factors ($F_{(1,23)}$ =2.01, $p<0.1754$).

We measured nuclear levels of pERK to verify the effect of PD 198306 on preventing the activation of ERK signaling. Fig. 10B illustrates the effect of PD 198306 on the GP1a-induced increase of nuclear-associated pERK protein levels. GP1a produced a significant ($p<0.01$) increase in the nuclear levels of pERK compared to vehicle-treated cells (97% increase over

control, Fig. 10B). While CLU213 cells pretreated with PD 198306 and treated with vehicle showed a 55% reduction ($p<0.05$) in nuclear pERK protein levels compared to controls, cells pretreated with PD198306 and treated with GP1a showed a 23% reduction ($p>0.05$) compared to vehicle controls. The two-way ANOVA showed a main effect of pretreatment ($F_{(1,17)}$ =616.80, $p<0.0001$), treatment ($F_{(1,17)}$ =563:85, $p<0.0001$), and a main interaction ($F_{(1,17)}$ =244.71, $p<0.0001$) between pretreatment and treatment on nuclear pERK levels. We then studied whether ERK1/2 inhibition with PD198306 was able to prevent the GP1a-induced increase on 5-HT_{2A} receptor protein levels (Fig. 10C). CLU213 cells were pretreated with either vehicle or PD198306 prior to a treatment with either vehicle or GP1a (1nM). This process was repeated for 3 days and cells were collected 24 h after the last incubation. We found that GP1a produced a significant ($p<0.05$) increase in membrane-associated 5-HT_{2A} receptor protein levels compared to controls (71% increase over controls, Fig. 10C). PD198306 pretreatment significantly ($p<0.05$) inhibited this GP1a-induced upregulation of 5-HT_{2A} receptors (Fig. 10C). Furthermore, pretreatment with PD198306 significantly ($p<0.05$) reduced the membrane-associated protein levels of 5-HT_{2A} receptors compared with vehicle controls. The two-way ANOVA showed a main effect of pretreatment ($F_{(1,11)}$ =451.11$p<0.0001$), treatment ($F_{(1,11)}$ =370.15, $p<0.0001$) and a main interaction ($F_{(1,11)}$ =452.63, $p<0.0001$) between pretreatment and treatment on membrane-associated 5-HT_{2A} receptor protein levels.

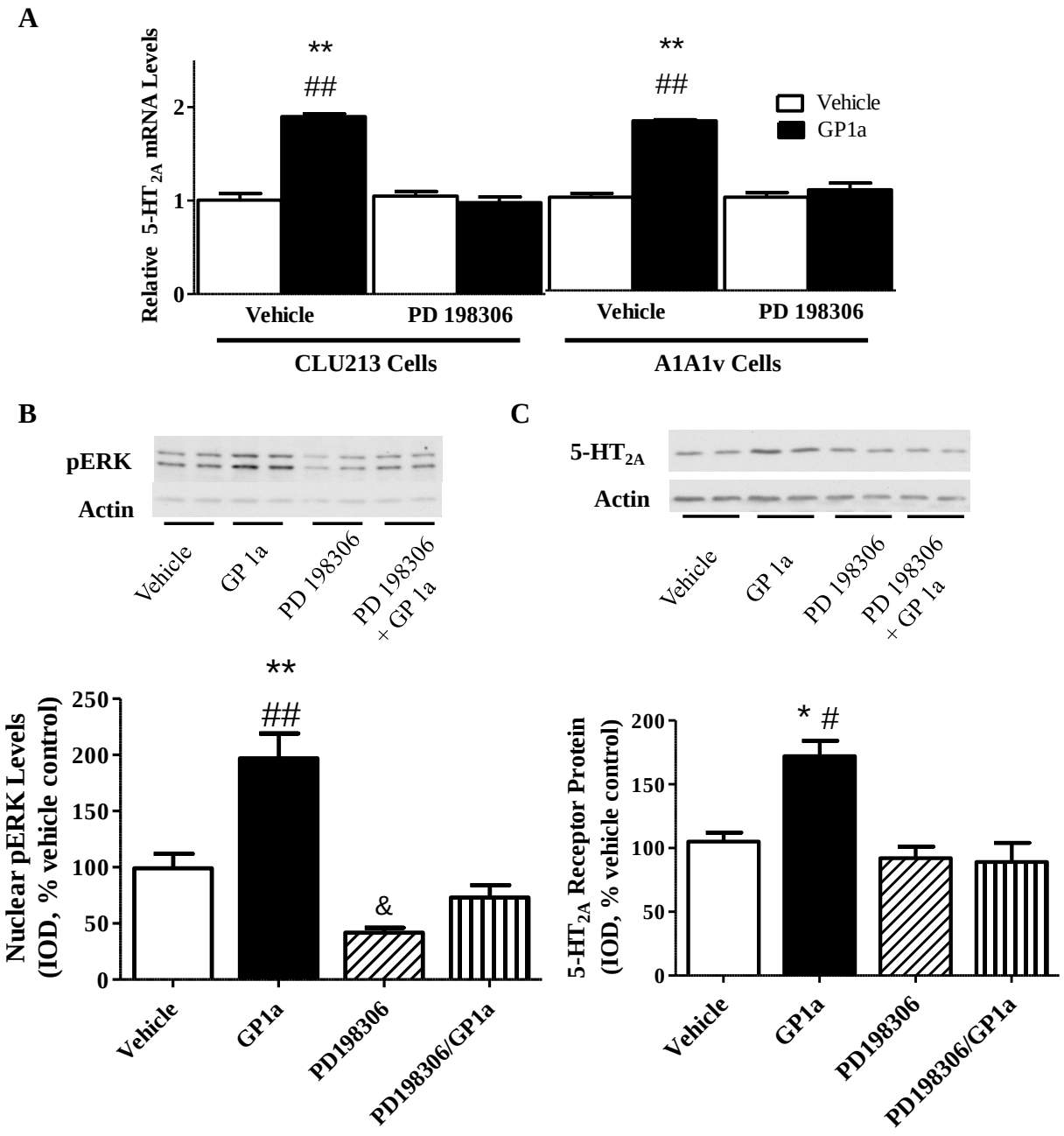


Figure 10. GP1a upregulates 5-HT_{2A} receptors via ERK1/2 signaling in CLU213 and A1A1v cells.

(A) PD198306, a selective ERK1/2 inhibitor, prevented the effect of GP1a on 5-HT_{2A} receptor mRNA levels in CLU213 and A1A1v cells. $^{**}p<0.01$, significant effect of GP1a treatment on 5-

HT_{2A} receptor mRNA levels compared to vehicle treated CLU213 or A1A1v cells. $^{\#\#}p<0.01$, significant effect of PD198306 pretreatment on the GP1a-induced upregulation of 5-HT_{2A} receptor mRNA in CLU213 cells. (B) PD198306 prevented GP1a-induced ERK1/2 activation. $^{**}p<0.01$, significant effect of GP1a treatment on nuclear pERK levels compared to vehicle control. $^{\#\#}p<0.01$, significant effect of GP1a treatment on nuclear pERK levels compared to the effect of GP1a treatment in PD198306 pretreated cells. $^{\&}p<0.05$, significant effect of PD198306 pretreatment compared to vehicle controls (C) PD198306 prevented the GP1a-induced increases in 5-HT_{2A} receptor protein levels in CLU213 cells. $^{*}p<0.05$, significant effect of GP1a treatment on 5-HT_{2A} receptor protein levels compared to vehicle treated cells. $^{\#}p<0.05$, significant effect of GP1a treatment on 5-HT_{2A} receptor protein levels compared to the effect of GP1a treatment in PD198306 pretreated cells. The data represent mean ± SEM (n=3-6).

Repeat GP1a exposure enhances ERK1/2 activation in a neuronal cell model

Finally, we studied whether the GP1a-induced activation of ERK in CLU213 cells was modified by a previous exposure to GP1a 24 hours earlier (Fig. 11). Here, cells were treated with either vehicle or GP1a (1nM) for 24h. After rinsing the cells, they were treated with either vehicle or GP1a (1nM) for 15 min and nuclear and cytosolic proteins were isolated as previously described. We found that a single treatment with GP1a induced a significant ($p<0.05$) activation of ERK1/2 in CLU213 cells and this response was significantly enhanced ($p<0.01$) in cells that were exposed to GP1a 24 hours earlier (Fig. 11A). Nuclear levels of pERK were increased by 86% or 350% after either a single or repeated exposure to GP1a. The two-way ANOVA detected a main effect of pretreatment ($F_{(1,12)} =5.23$, $p>0.0332$), treatment ($F_{(1,12)} =16.82$, $p>0.0006$) and an interaction between pretreatment and treatment ($F_{(1,12)} =5.19$, $p>0.0339$) on nuclear levels of pERK. We measured cytosolic levels of ERK1/2 protein as an index of the overall levels of this protein. We found that cytosolic ERK1/2 protein levels were not significantly ($p>0.05$) affected by repeated exposure to the CB_2 agonist GP1a (Fig. 11B). The two-way ANOVA did not find a main effect of pretreatment ($F_{(1,12)} =0.17$, $p>0.687$) or treatment ($F_{(1,12)} =0.88$, $p>0.3594$) nor an interaction between pretreatment and treatment ($F_{(1,12)} =0.27$, $p>0.6076$) on cytosolic levels of ERK1/2 protein.

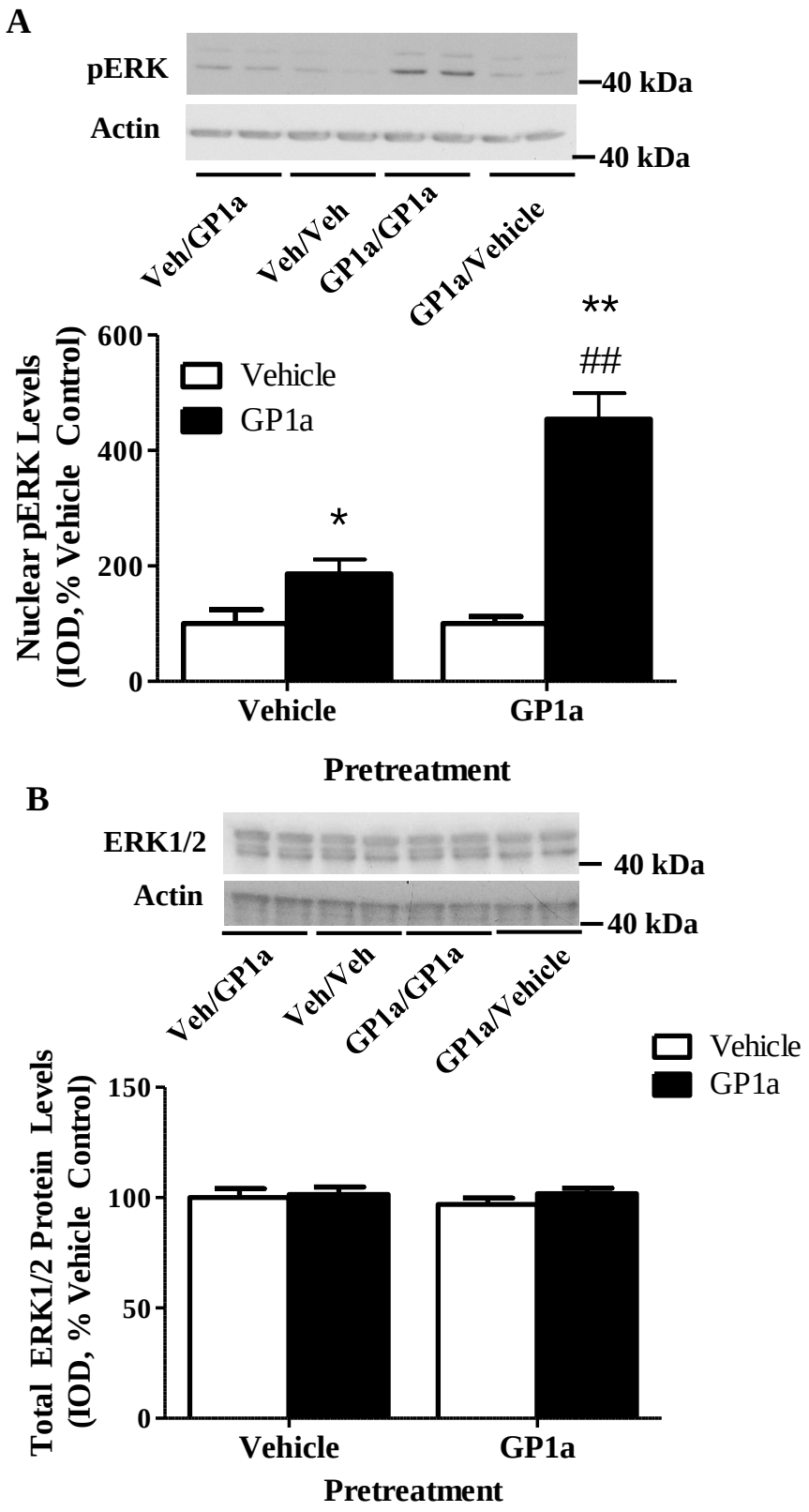


Figure 11. Multiple GP1a treatments enhance nuclear-associated pERK levels in CLU213 cells.

(A) Repeat GP1a treatment enhances nuclear pERK protein levels over a single GP1a treatment. *$p<0.05$, significant effect of one GP1a treatment on nuclear associated pERK levels compared to vehicle treated controls. **$p<0.01$, significant effect of two GP1a treatments on nuclear associated pERK levels compared to vehicle treated controls. ##$p<0.01$, significant effect of two GP1a treatments on nuclear associate pERK levels compared to one GP1a treatment in CLU213 cells. (B) GP1a treatment does not significantly ($p>0.05$) alter total cytosolic ERK1/2 protein levels. The data represent mean ± SEM (n=4).

4.5 Discussion

Several behavioral reports suggest that cannabinoids can regulate 5-HT neurotransmission, and more specifically 5-HT_{2A} receptors [32-35]. However, these studies did not identify the molecular mechanisms by which cannabinoid receptors would modify 5-HT_{2A} receptor signaling. Here we used CP55940, a synthetic CB_1/CB_2 receptor agonist, which displays high and roughly similar affinity for both cannabinoid receptors to study the cannabinoid regulation of 5-HT_{2A} receptors in rats and culture cells [58;211]. Indeed, we found that CP55940 induced a significant increase in the 5-HT_{2A} receptor-mediated PLCβ activity in rat PFCx that was associated with increased 5-HT_{2A} receptor protein and mRNA levels in this brain area (Fig. 7). 5-HT_{2A} receptor-mediated PLCβ activity measures the phosphoinositide breakdown to diacylglycerol (DAG) and inositol trisphosphate (IP_3) via stimulation of phosphoinositide-specific PLCβ [269;270]. This assay involves measuring receptor-stimulated PLCβ activity in brains via the conversion of radiolabeled phosphatidylinositol (^{3}H-PI) to inositol monophosphate (^{3}H-IP) [269;270] and makes it feasible to investigate treatment-induced changes in various components of the 5-HT_{2A} receptor effector systems in a well-controlled *in vitro* situation that is entirely mediated by activation of 5-HT_{2A} receptors in this brain region [269;270].

The 5-HT_{2A} receptor-mediated increases in PLCβ activity seem to be mediated by increased levels of membrane-associated 5-HT_{2A} receptors in PFCx. Enhanced transcription of the 5-HT_{2A} receptor gene and translation of the 5-HT_{2A} receptor mRNA could explain this increase in 5-HT_{2A} receptor protein levels as we detected an approximate two-fold increase in 5-HT_{2A} receptor mRNA in PFCx (Fig. 7). Noteworthy, this increase in mRNA levels seems to be specific for 5-HT_{2A} receptors as no upregulation of 5-HT_{1A} receptors and $G\alpha_q$ was found. 5-HT_{1A} and 5-HT_{2A} receptors exhibit overlapping distributions in various brain regions, including frontal

and prefrontal cortex [283;284] and $G\alpha_q$ is a G-protein that couples 5-HT_{2A} receptors to the PLCβ signaling pathway. The lack of changes in $G\alpha_q$ seems to indicate that the cannabinoid-induced increases in membrane-associated 5-HT_{2A} receptors are sufficient to enhance PLCβ activity in this brain area. We also noted decreased CB_1 and CB_2 receptor mRNA levels in PFCx of CP55940-treated rats. This could explain how chronic exposure to CP55940 is associated with downregulation and a significant loss of CB_1 and CB_2 receptors in cortex as measured by receptor binding experiments [285;286].

We used CLU213 cells as cellular model to investigate the molecular mechanism by which cannabinoid receptors might mediate the upregulation and enhanced activity of 5-HT_{2A} receptor signaling in a neuronal cell line. This neuronal cell line endogenously expresses 5-HT_{2A} receptors coupled to the stimulation of PI hydrolysis. Interestingly, we detected CP 55,940-induced increases in 5-HT_{2A} receptor mRNA and protein levels and enhanced 5-HT_2 receptor-mediated PI hydrolysis in these neuronal cells (Fig. 8). Although, SB242084, a selective 5-HT_{2C} receptor antagonist [277], was added to prevent the activation of 5-HT_{2C} receptors, the lack of selective 5-HT_{2A} receptor agonist hindered our ability to identify a CP55940-mediated specific increase in 5-HT_{2A} receptor activity in this cell line. The two-fold increase in both 5-HT_{2A} receptor mRNA and protein levels suggest that an increase in the activity of 5-HT_{2A} receptors might be associated with the exposure to the non-selective cannabinoid agonist, CP55940.

We used PF-514273 (CB_1 antagonist) [57] and JTE907 (CB_2 receptor antagonist) [61;62] to study the involvement of cannabinoid receptors in the CP55940-induced increases in 5-HT_{2A} receptor mRNA (Fig. 9A). We found that JTE907, but not PF-514273, produced a complete inhibition of the CP55940-induced increases in 5-HT_{2A} receptor mRNA. These results suggest that blockade of the CB_2 receptors, but not CB_1 receptors, with selective antagonists can prevent

the CP55940-induced upregulation of 5-HT_{2A} receptors in CLU213 cells. We also studied the effect of CP55940, ACEA and GP1a (non-selective and selective CB_1 and CB_2 receptor agonists, respectively) on 5-HT_{2A} receptor upregulation in both CLU213 and A1A1v cells (Fig. 9B). Noteworthy, we detected similar upregulation of 5-HT_{2A} receptors in both CLU213 and A1A1v cells. This suggests that selective CB_2 receptor agonists can upregulate 5-HT_{2A} receptors in two independent neuronal cells that endogenously express 5-HT_{2A} receptors. We also found confirmatory evidence of the role of CB_2 receptor agonists on the upregulation of 5-HT_{2A} receptors in CLU213 cells through studying the effect of two selective CB_2 receptor antagonists (JTE907 and AM630) on cells exposed to the selective CB_2 receptor agonist, GP1a (Fig. 9C). Both CB_2 receptor antagonists completely prevented the GP1a-induced upregulation of 5-HT_{2A} receptors in this neuronal cell line, supporting the role of the CB_2 receptor agonist in this phenomenon. In summary, the previous results suggest that cannabinoid agonists would upregulate 5-HT_{2A} receptor mRNA via activation of CB_2 receptors in neuronal cells that endogenously express 5-HT_{2A} and CB_2 receptors.

The CB_2 receptor is a seven transmembrane receptor that was initially identified in the periphery but not in the brain [246;247]. Indeed, brain expression of CB_2 receptors was much less well established and characterized in comparison to the expression of brain CB_1 receptors. However, more recent reports have established the expression of CB_2 receptors in neurons in PFCx, amygdala, hypothalamus, and hippocampus of the healthy brain [18;19;21;264]. Furthermore, recent studies indicate that CB_2 receptors are mainly localized in post synaptic neurons [19;21] where they might mediate their effects by activation of $G_{i/o}$ G-proteins, ERK signaling and Jun N-terminal kinase (JNK) which are signaling pathways that regulate nuclear transcription factors [88;98;265]. Interestingly**,** the ERK1/2 signaling cascade can regulate

transcription factors which have consensus sequences within the rat 5-HT_{2A} receptor promoter region [235;236].

In our experiments we tested the effect of PD 198306, a potent ERK1/2 inhibitor, on the GP1a-induced increases in 5-HT_{2A} receptor mRNA levels in CLU213 and A1A1v cells [279;287]. We found that ERK1/2 inhibitor pretreatment prevented the GP1a-induced increases in 5-HT_{2A} receptor mRNA levels in both CLU213 and A1A1v cells (Fig. 10A). This would indicate that the GP1a-induced upregulation of 5-HT_{2A} receptors might require ERK1/2 activation and suggests similarities in the mechanism involved in the upregulation of 5-HT_{2A} receptors in both cell types. Since ERK1/2 is activated (phosphorylated) in the cytoplasm and then translocates to the nucleus, we measured nuclear-associated levels of pERK as an index of CB_2 receptor-induced ERK activation [98;288]. We found that PD 198306 pretreatment also prevented GP1a-induced increases in nuclear associated pERK protein levels and membrane-associated 5-HT_{2A} receptor protein levels in CLU213 cells (Fig. 10B and 10C). This evidence might indicate that CB_2 receptor agonists would induce an increase in the nuclear levels of activated ERK (pERK) that would mediate the upregulation of 5-HT_{2A} receptor mRNA and increase synthesis of the 5-HT_{2A} receptor protein.

Agonists of CB_2 receptors might upregulate 5-HT_{2A} receptor mRNA; transcription factors that are located downstream of ERK and that target the promoter region of the 5-HT_{2A} receptor gene**,** such as cyclic AMP response element binding protein (CREB), specificity protein 1 (SP-1), and activator protein 1 (AP-1), could be involved [232;289]. Indeed, we have recently found that AP-1, but not CREB, plays a role in the cannabinoid-induced upregulation of 5-HT_{2A} receptors [263].

Interestingly, we found that repeated stimulation of CB_2 receptors produced an enhanced activation of ERK1/2 in CLU213 cells (Fig. 11). While a single administration of GP1a triggered an increase in the nuclear-associated levels of pERK, we observed a greater response in cells that were exposed to GP1a 24 hours earlier (Fig. 11A). Furthermore, GP1a treatment did not have an effect on the total ERK1/2 protein levels (Fig. 11B). Previous reports that predominantly examined the ability of cannabinoid agonists to stimulate [(^{35})S]guanylyl-5'-O-(gamma-thio)-triphosphate binding to the Gα subunits of the G-proteins indicated that short exposure to cannabinoids produces desensitization of cannabinoid receptors [265]. However, our results would suggest that repeat exposure to cannabinoid agonists might mediate enhanced responses of the CB_2 receptor-mediated regulation of ERK activation. Interestingly, studies by Lefkowitz et al. (2005) suggest that seven transmembrane receptors can regulate activation of ERK1/2 through either G-proteins or β-arrestins [257]. Indeed, G-protein coupled receptors, such as CB_2 receptors, could regulate long-term activation of ERK1/2, independent of G-proteins through β-arrestins which can form scaffolding complexes with several kinases such as ERK1/2 [257;290]. Therefore, it is possible that while repeated exposure induces the desensitization of CB_2 receptor-mediated activation of G-proteins, the CB_2 receptor-mediated activation of ERK1/2 signaling could be enhanced by a mechanism that is mediated by β-arrestins and is independent of G-protein activation. Future experiments in our lab will try to identify the mechanisms involved in ERK1/2 activation after repeated exposure to CB_2 receptor agonists.

The results presented here suggest that exposure to selective CB_2 receptor agonists can induce the upregulation of 5-HT_{2A} receptors by a mechanism that involves ERK1/2 activation. Recent reports have provided new evidence linking chronic use of cannabinoids to an earlier onset of psychiatric disorders, including psychosis, schizophrenia and anxiety [11-13;93].

Indeed, these studies proposed that repeated exposure to cannabinoids might precipitate the onset of cognitive disorders in individuals predisposed to developing them [11-13]. Interestingly, preclinical evidence links CB_2 receptors to several neuropsychiatric disorders such as schizophrenia, anxiety, and depression [93;202;248]. This evidence highlights that need to identify the mechanism by which CB_2 receptors may be inducing and/or contributing to neuropsychiatric disorders. The CB_2 receptor agonist-induced upregulation of 5-HT_{2A} receptors in PFCx could provide a molecular mechanism by which exposure to cannabinoid receptor agonists might contribute to the pathophysiology of cognitive disorders, since psychosis and schizophrenia are disorders associated with dysfunction of 5-HT_{2A} receptor signaling in PFCx [28;31;137;173].

5-HT_{2A} receptors, which have been shown to regulate the dopamine mesoaccumbens pathway, play a critical role in the regulation of stress, mood, impulse control and the behavior effects of drugs of abuse [31;137;173]. Furthermore, impaired function of cortical 5-HT_{2A} receptors has been identified in several neurological and psychiatric disorders such as schizophrenia, Alzheimer's disease, depression, anxiety and eating disorders [24]. Moreover, the therapeutic benefits of atypical antipsychotics, which are more potent 5-HT_{2A} receptor antagonists than dopamine D_2 receptor antagonists [28;179], are proposed to be mediated by antagonism and subsequent desensitization of 5-HT_{2A} receptor signaling in PFCx [29]. Actually, it has been suggested that blockade of pyramidal neurons in PFCx (particularly enriched in 5-HT_{2A} receptors) may underlie the beneficial effects of atypical antipsychotic drugs [28;29]. Additionally, a recent report suggests that crosstalk between 5-HT_{2A} and corticotrophin receptors in PFCx regulates anxiety-like behaviors and mood disorders, as well [31]. Indeed, they provide evidence that over-expression of 5-HT_{2A} receptors in the PFCx can contribute to anxiety-like

behaviors [31]. Therefore, the cannabinoid receptor-mediated upregulation of 5-HT_{2A} receptor neurotransmission in PFCx could be responsible for anxiety-like behaviors reported after exposure to cannabinoid agonists [291]. Indeed, a recent report indicates that while chronic exposure to selective CB_2 receptor agonists induced anxiogenic-like behaviors, chronic antagonism of CB_2 receptors induces anxiolytic-like behaviors in rodents [93].

Emerging evidence indicates that selective CB_2 receptor agonists might have wide therapeutic application in the treatment of stroke, neurogenerative diseases, and neuropathic pain [2;5]. However, the cannabinoid-induced upregulation of 5-HT_{2A} receptors might represent a potential negative side-effect of the long term exposure to selective CB_2 receptor agonists. Our studies might provide a molecular mechanism by which chronic use of cannabinoids might precipitate the onset of some psychiatric disorders in people predisposed to developing them and contribute to the mechanisms associated with the therapeutic use of selective cannabinoid agonists.

Chapter 5: Cannabinoid 2 Receptor- and Beta Arrestin 2-Dependent Upregulation of Serotonin 2A (5-HT_{2A}) Receptors

(Franklin J.M., Vasiljevik T., Prisinzano T.E., Carrasco G.A. (2012) Cannabinoid 2 Receptor- and Beta Arrestin 2-Dependent Upregulation of Serotonin 2A (5-HT_{2A}) Receptors. *European Journal of Neuropsychopharmacology*, **23:** 760-767)

5.1 Abstract

Recent evidence suggests that cannabinoid receptor agonists may regulate 5-HT_{2A} receptor neurotransmission in the brain, although no molecular mechanism has been identified. Here, we present experimental evidence that sustained treatment with a non-selective cannabinoid agonist (CP55940) or selective CB_2 receptor agonists (JWH133 or GP1a) upregulate 5-HT_{2A} receptors in a neuronal cell line. Furthermore, this cannabinoid receptor agonist-induced upregulation of 5-HT_{2A} receptors was prevented in cells stably transfected with either CB_2 or β-Arrestin 2 shRNA lentiviral particles. Additionally, inhibition of clathrin-mediated endocytosis also prevented the cannabinoid receptor-induced upregulation of 5-HT_{2A} receptors. Our results indicate that cannabinoid agonists might upregulate 5-HT_{2A} receptors by a mechanism that requires CB_2 receptors and β-Arrestin 2 in cells that express both CB_2 and 5-HT_{2A} receptors. 5-HT_{2A} receptors have been associated with several physiological functions and neuropsychiatric disorders such as stress response, anxiety, depression, and schizophrenia. Therefore, these results might provide a molecular mechanism by which activation of cannabinoid receptors might be relevant to some cognitive and mood disorders in humans.

5.2 Introduction

Marijuana (*Cannabis sativa* L.) is the most commonly abused illicit drug in the United States [7]. According to recent epidemiological data, marijuana and synthetic cannabinoids are the most prevalent illicit drugs used by 12^{th} graders in the United States [9]. Indeed, more than one-third (36.4%) of high school seniors reported using marijuana in 2011, including 11.4% who reported using synthetic cannabinoids [9].

Cannabinoid agonists produce their effects by acting upon two cannabinoid receptors in the brain, CB_1 and CB_2 receptors [88;286;292]. These receptors bind endocannabinoids and exogenous cannabinoids (such as Δ9-THC) with high affinity [15]. CB_1 and CB_2 receptors, which couple to Gαi/o class of G-proteins, have presynaptic or postsynaptic distribution in the brain [15;19;94;266]. Furthermore, these receptors can activate ERK1/2 signaling possibly through a β-Arrestin 2 (β-Arr2) dependent pathway [88;293].

Behavioral reports have suggested that cannabinoid receptor agonists can regulate the activity of 5-HT_{2A} receptors [32;34]. However, the molecular mechanism by which cannabinoid regulates 5-HT_{2A} receptor signaling in the brain is unknown. 5-HT_{2A} receptors, which regulate the dopamine mesoaccumbens pathway, play an important role in the regulation of stress, mood and impulse control and the behavioral effects of several drugs of abuse [137;173]. Furthermore, impaired function of cortical 5-HT_{2A} receptors has been identified in several neurological and psychiatric disorders such as schizophrenia, Alzheimer's disease, depression, anxiety, and eating disorders [24].

Here, we studied some mechanisms involved in the cannabinoid-induced upregulation of 5-HT_{2A} receptors in a neuronal cell line. Our results support the cannabinoid-induced upregulation of 5-HT_{2A} receptors through a CB_2 receptors and β-Arr2-dependent mechanism.

5.3 Materials and Methods

Cell Culture Protocol

CLU213 cells, a rat neuronal cell line, were purchased from Cedarlane Laboratories (Burlington, NC). Cells were grown on 100-mm^2 plates treated with polystyrene (Corning Incorporated, Corning, NY) and maintained in 5% CO_2 at 37°C, in Dulbecco's modified eagle medium (Mediatech Inc, Manassas,VA) containing 10% fetal bovine serum (Thermo Scientific, Logan, UT).

Quantitative Real-Time PCR

These reactions were prepared using QuantiFast SYBR Green PCR Kit (Qiagen, Valencia, CA) and the ABI 7500 fast real time PCR system (Applied Biosystems, Foster City, CA) as previously described [29]. The primers used in this manuscript were: 5-HT_{2A} (F:5'-AACGGTCCATCCACAGAG-3',R:5'-AACAGGAAGAACACGATGC-3'), CB_2 (F:'5-CCAACATGTAGCCAGCTTGACT-3',R: 5'-TGCAGGAACCAGCATATGA-3') β-Arr2 (F:5'-AGCACCGCGCAGTACAAGT-3',5'-R:CACGCTTCTCTCGGTTGTCA-3'), and GAPDH (F:5'- TGGAGTCTACTGGCGTCTTCAC-3',R:5'-GGCATGGACTGTGGTCATGA-3'). These primers have been validated in the literature [29;274;294].

In all real-time PCR experiments, measurements were made from the number of cycles required to reach the threshold fluorescence intensity [cycle threshold (Ct)]. Ct values for each reaction were subtracted from Ct values for GADPH and then subtracted from Ct values for vehicle-treated controls that served as a baseline, and the result was referred to as ΔΔCt. Fold changes in gene expression were calculated as $2^{-\Delta\Delta Ct}$ to reflect the fact that, under optimal conditions, the amount of PCR product doubles with each amplification cycle. Results were normalized to those obtained for amplifications of the same cDNA samples using primers

designed against GADPH, which acts as an internal standard, and averaged for each treatment group.

To study the effect of non-selective and selective cannabinoid receptor agonists on 5-HT_{2A} mRNA, CLU213 cells were incubated with either vehicle (ethanol 0.01% final concentration), CP55940 1 nM (non-selective CB_1/CB_2 agonist, K_i: 0.58 nM and 0.68 nM for CB_1 and CB_2 receptors, respectively) [50;293], ACEA 15 nM (selective CB_1 agonist, K_i:1.4 nM and 3.1 μM for CB_1 and CB_2 receptors, respectively) [55;72], GP1a 1 nM (highly selective CB_2 agonist, K_i: 0.037 nM and 353 nM for CB_2 and CB_1 receptors, respectively) [60], or JWH133 30 nM (selective CB_2 agonist, K_i: 3.4 nM and 677 nM for CB_2 and CB_1 receptors, respectively) [295;296] for 24 hours. In the Concanavalin A (ConA) experiment, cells were pretreated with 250 μg/ml ConA for 20 minutes [297] and then treated with different cannabinoid agonists.

In a separate experiment utilized to examine the effect of an aminoalkylindole on 5-HT_{2A} receptor mRNA levels, cells were treated with either vehicle (ethanol 0.01% final concentration), GP1a 1 nM, CP55940 1 nM, or JWH073 40 nM (mildly selective CB_1 receptor agonist, K_i: 8.9 nM and 38 nM for CB_1 and CB_2 receptors, respectively) [52;295] for 24 hours. In the Concanavalin A (ConA) experiment, cells were pretreated with 250 μg/ml ConA for 20 minutes [297] and then treated with different cannabinoid agonists.

Western Blot

Membrane-associated and cytosolic-associated fractions were isolated using the ProteoExtract™ Native Membrane Protein Extraction kit (Calbiochem, La Jolla, CA). Nuclear-associated fractions were isolated using NE-PER ® Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, IL). Expression of membrane-associated 5-HT_{2A} receptors, CB2 receptors, β-Arr2, and nuclear-associated pERK was determined by Western blot as previously

described [272]. The anti-5-HT_{2A} receptor antibody was a generous gift from Dr. Nancy A. Muma and the specificity of this antibody has been validated in the literature [194]. CB_2 receptor, β-Arr2, and pERK antibody were purchased from Santa Cruz, CA. Protein loading for each lane was verified using an anti-actin antibody (Santa Cruz Biotechnology, Inc.). Negative controls included either the omission of primary antibody or addition of preimmune rabbit immunoglobulins. Films were analyzed densitometrically as described [272]. All samples were standardized to controls and normalized to their respective actin levels.

To study the effect of GP1a, CP55940, or ACEA on membrane-associated 5-HT_{2A} receptor levels, CLU213 cells were treated with either vehicle (ethanol 0.01% final concentration), GP1a (1nM), CP55,940 (1 nM), or ACEA (15 nM) for 72 hours. Cells were washed with PBS every 24 hours and fresh vehicle, GP1a, CP55940, or ACEA was added. To study the effect of ConA on cytosolic-associated CB_2 or nuclear-associated pERK levels, cells were pretreated with ConA (250 μg/ml) and then 20 minutes later treated with either vehicle, GP1a (1nM), or CP55940 (1nM) for 15 minutes.

Lentivirus and stable transduction of shRNAs in CLU213 cells

β-Arr2 shRNA (r), CB_2 shRNA (r), control shRNA lentiviral particles, polybrene, and puromyocin were purchased from Santa Cruz, CA. Cells were infected with either control, β-Arr2, or CB_2 shRNA lentivirial particles following manufacturer instructions in complete medium containing 2 μg/ml Polybrene. 48 hours post-infection, medium was changed to a virus-free complete medium. Puromyocin selection was initiated 24 hours later. Cells were analyzed for β-Arr2 or CB_2 knockdown one week later.

Statistics

All data are expressed as the mean ± S.E.M. Each sample was measured in triplicate. Data was analyzed by an unpaired Student's t-test or ANOVA (Newman-Keuls post-hoc test).

5.4 Results

Cannabinoid-induced upregulation of 5-HT_{2A} receptors

Cells treated with either CP55940 (CB_1/CB_2 agonist), GP1a (CB_2 agonist) or JWH133 (CB_2 agonist) showed significant ($p<0.01$) increases in 5-HT_{2A} mRNA levels compared to vehicle-treated controls (Fig. 12A). However, no significant changes were detected in cells treated with ACEA, selective CB_1 agonist. Next we determined the effect of CP55940 (1 nM), ACEA (15 nM) and GP1a (1 nM) on membrane-associated 5-HT_{2A} receptor protein levels. GP1a exhibits higher selectivity for CB_2 receptors than JWH133 therefore we utilized GP1a to assess whether a highly selective CB_2 receptor agonist would increase 5-HT_{2A} receptor protein levels (approximate 9,000- and 200-fold selectivity between CB_2/CB_1 receptors, respectively) [60;296]. We found that CP55940 treatment and GP1a treatment significantly ($p<0.01$) increased membrane-associated 5-HT_{2A} receptor protein levels (61±10% and 72 ± 10% increase, respectively)(Fig. 12B and Fig. 12D). Treatment with ACEA did not significantly ($p>0.05$) modify the membrane-associated 5-HT_{2A} receptor protein levels compared to controls (Fig. 12C).

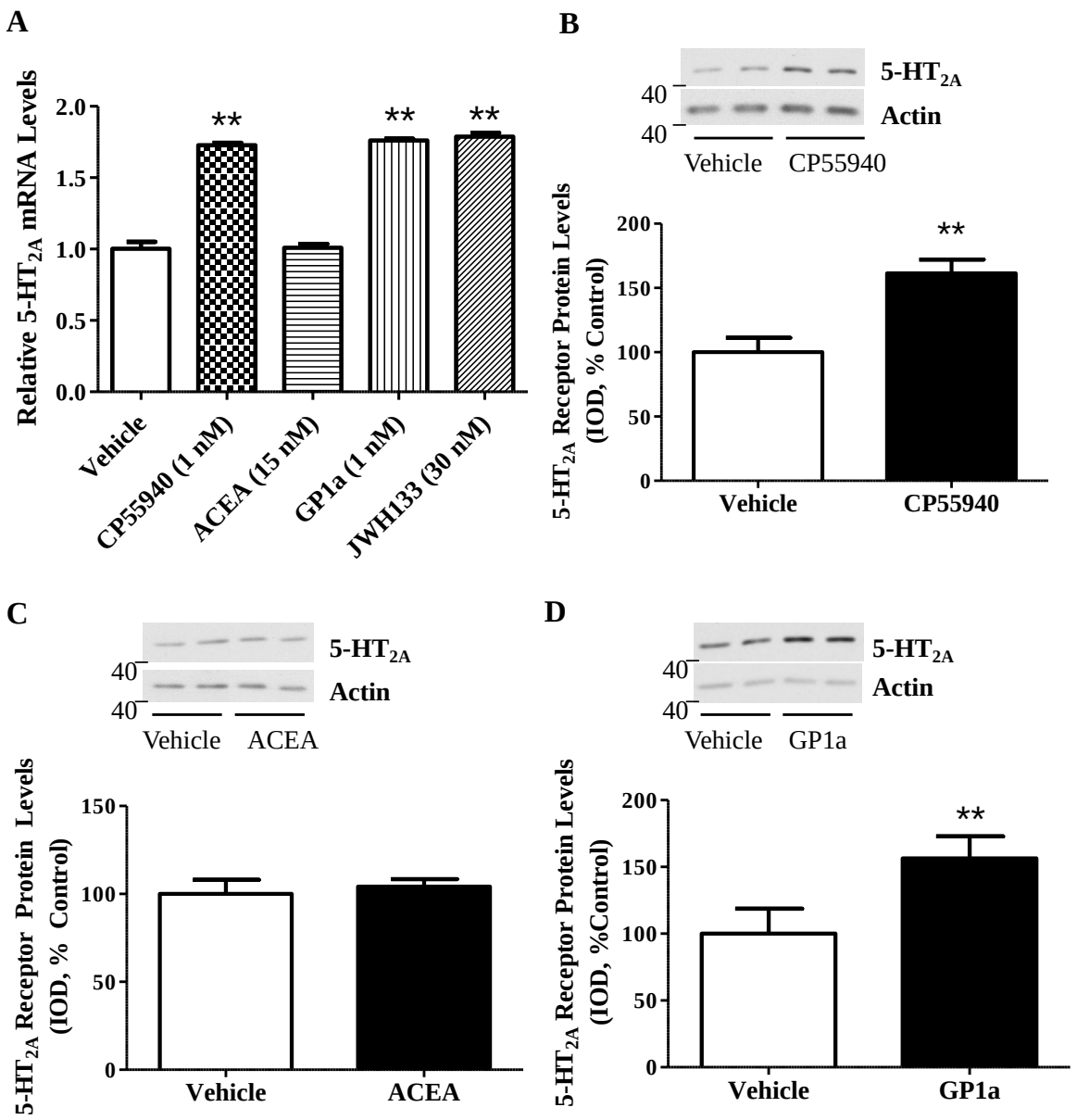


Figure 12. Cannabinoid-induced upregulation of 5-HT$_{2A}$ receptors.

(A) Increased 5-HT$_{2A}$ receptor mRNA levels in cells treated with either CP55940, JWH133, or GP1a. (**p<0.01 significant effect of CP55940, JWH133, and GP1a treatment compared to vehicle-treated controls). (B) Increased membrane-associated 5-HT$_{2A}$ receptor protein levels in cells treated with CP55940. (**p<0.01 significant effect of CP55940 treatment compared to vehicle-treated controls). (C) ACEA treatment does not alter membrane-associated 5-HT$_{2A}$

receptor protein levels ($p>0.05$). (D) Increased membrane-associated 5-HT_{2A} receptor protein levels in cells treated with GP1a. (**$p<0.01$ significant effect of GP1a treatment compared to vehicle-treated controls). The data represent mean ± SEM (n=3).

CB_2 receptors are necessary for CP55940, JWH133, and GP1a-induced upregulation of 5-HT_{2A} receptors

We used cells stably transfected with CB_2 shRNA or control shRNA lentiviral particles to study whether the effect of cannabinoid agonists is mediated by CB_2 receptors. CB_2 shRNA lentiviral particle treatment significantly ($p<0.01$) reduced both CB_2 mRNA (Fig. 13A) and protein levels (Fig. 13B) by approximately 80% compared to control-treated cells. CP55940, JWH133, and GP1a upregulated 5-HT_{2A} mRNA in control shRNA cells (85 ± 5%, 84 ± 5% and 84 ± 5% increase in 5-HT_{2A} mRNA, respectively). Noteworthy, cells stably transfected with CB_2 shRNA showed no CP55940, GP1a, or JWH133-induced increases in 5-HT_{2A} mRNA levels (Fig. 13C). The two-way ANOVA showed main effects of transfection ($F_{(1,23)} =173.47$, $p<0.0001$), CB_2 agonists ($F_{(3,23)} =15.54$,$p<0.0001$), and a main interaction between these two factors ($F_{(3,23)} =27.66$,$p<0.0001$) on 5-HT_{2A} mRNA levels.

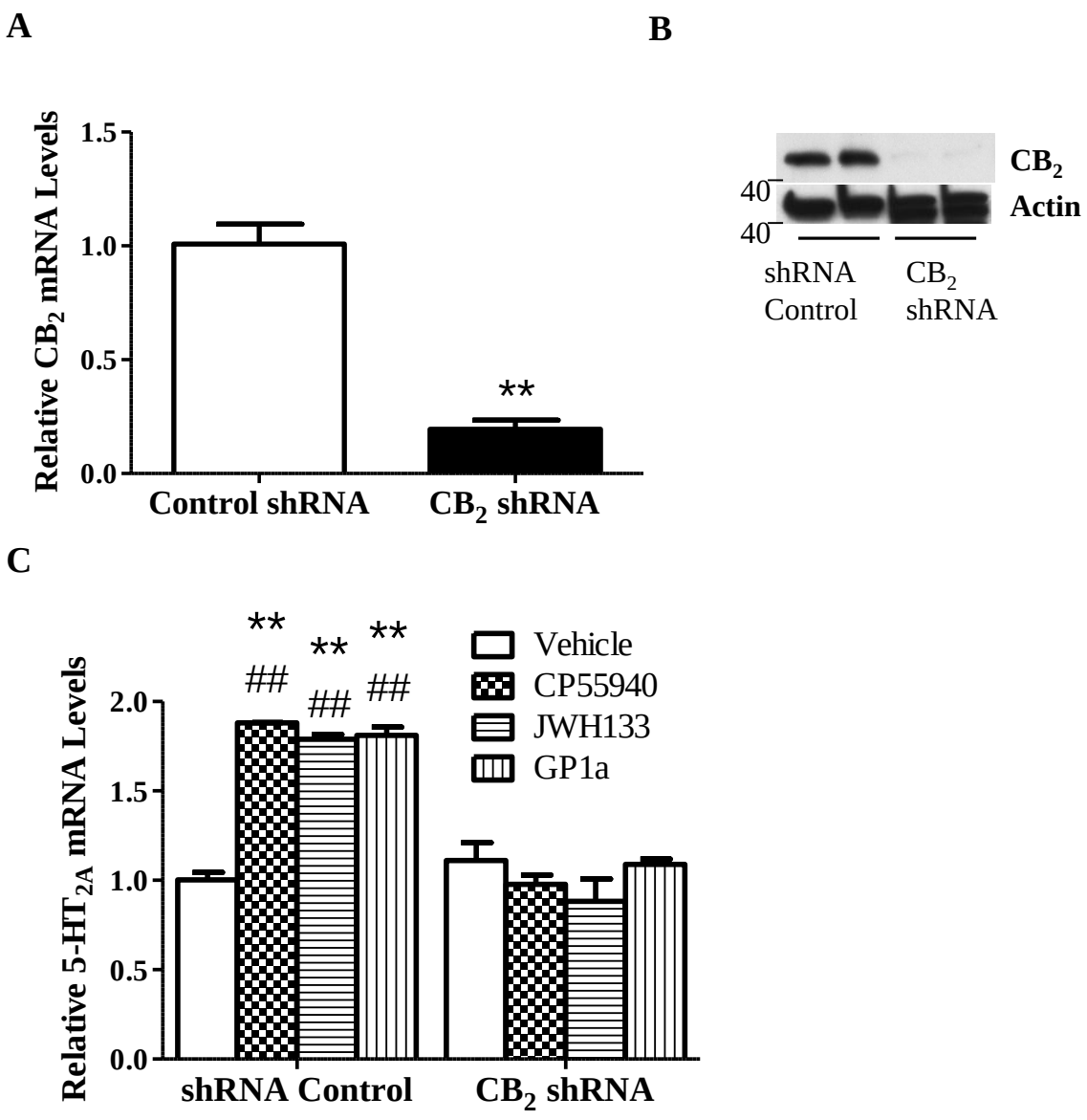


Figure 13. CB_2 receptor is necessary for CP55940, JWH133, and GP1a-induced upregulation of 5-HT_{2A} receptors.

(A) Reduced CB_2 mRNA levels in cells treated with CB_2 shRNA lentiviral particles. (**$p<0.01$ significant effect of CB_2 shRNA compared to control shRNA treated cells). (B) Reduced CB_2 protein levels in cells treated with CB_2 shRNA lentiviral particles compared to control shRNA lentiviral particle treated controls. (C) CB_2 shRNA lentivirus transfection prevents CP55940,

JWH133 and GP1a-induced increases in 5-HT_{2A} receptor mRNA. **p<0.01, significant effect of CP55940, JWH133, and GP1a treatment on 5-HT_{2A} receptor mRNA levels in control shRNA lentivirus tranfected cells compared to vehicle-treated controls. ##p<0.01, significant effect of CB_2 shRNA lentivirus transfection on the CP55940, JWH133, and GP1a-induced upregulation of 5-HT_{2A} receptors. The data represent mean ± SEM (n=3).

β-Arr2 is necessary for CP55940, JWH133, and GP1a-induced upregulation of 5-HT_{2A} receptors

We also used cells stably transfected with either β-Arr2 shRNA or control shRNA lentiviral particles to study the contribution of β-Arr2 on the cannabinoid-induced upregulation of 5-HT_{2A} receptors. Treatment with β-Arr2 shRNA lentiviral particles significantly ($p<0.01$) reduced both β-Arr2 mRNA (Fig. 14A) and protein levels (Fig. 14B) by approximately 85%. CP55940, JWH133, and GP1a upregulated 5-HT_{2A} mRNA in control shRNA treated cells by 94 ± 10%, 84 ± 10% or 95 ± 7%, respectively. The cannabinoid agonist-induced upregulation of 5-HT_{2A} mRNA levels was significantly ($p<0.05$) reduced in cells stably transfected with β-Arr2 shRNA lentiviral particles. CP55940-, JWH133-, or GP1a-mediated increases in 5-HT_{2A} mRNA levels was 19 ± 9%, 0 ± 2% or 40 ± 10%, respectively (Fig. 14C). There was no significant difference ($p>0.05$) in the response mediated by either CP55940 or JWH133. However, treatment with GP1a significantly ($p<0.05$) increased 5-HT_{2A} receptor mRNA levels in β-Arr2 shRNA lentivirus treated cells compared to vehicle treated controls. The two-way ANOVA showed main effects of transfection ($F_{(1,22)} = 40.47, p<0.0001$), CB_2 agonists ($F_{(3,22)} = 13.92$,$p<0.0001$), and a main interaction between these two factors ($F_{(3,22)} = 4.12$,$p<0.025$) on 5-HT_{2A} mRNA levels.

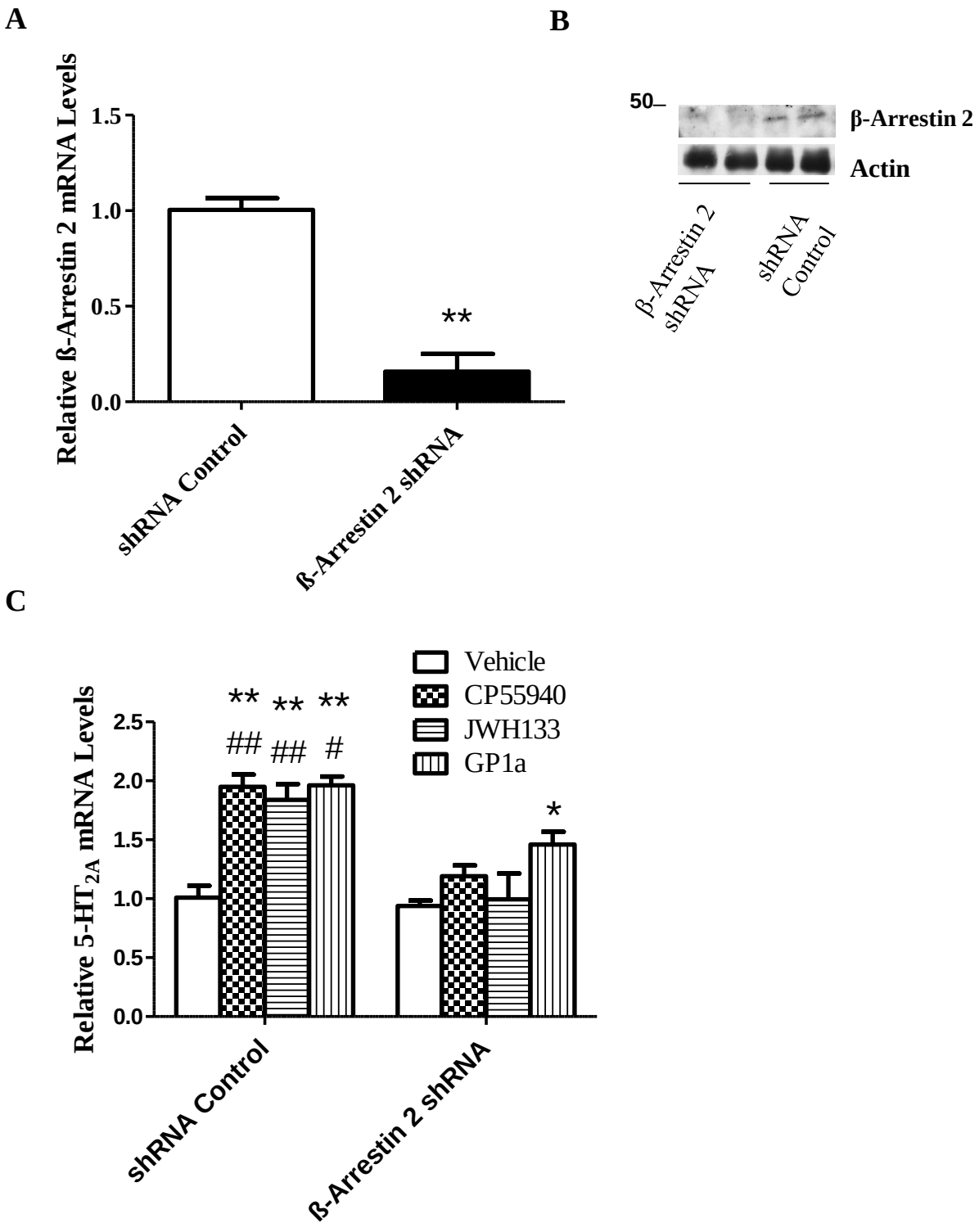


Figure 14. β-Arrestin 2 is necessary for CP55940, JWH133, and GP1a-induced upregulation of 5-HT_{2A} receptors.

(A) Reduced β-Arr2 mRNA levels in cells treated with β-Arr2 shRNA lentiviral particles. (**$p<0.01$ significant effect of β-Arr2 shRNA compared to control shRNA treated cells). (B) Reduced β-Arr2 protein levels in cells treated with β-Arr2 shRNA lentiviral particles compared to control shRNA lentiviral particle treated controls. (C) β-Arr2 shRNA lentivirus transfection prevents CP55940, JWH133 and GP1a-induced increases in 5-HT_{2A} receptor mRNA. **$p<0.01$, significant effect of CP55940, JWH133, and treatment on 5-HT_{2A} receptor mRNA levels in control shRNA tranfected cells compared to vehicle-treated controls. ##$p<0.01$ or #$p<0.05$, significant effect of β-Arr2 shRNA transfection on the CP55940 and JWH133 or GP1a-induced upregulation of 5-HT_{2A} receptors. @$p<0.05$, significant effect of GP1a treatment in β-Arr2 shRNA transfected cells compared to vehicle-treated β-Arr2 shRNA transfected cells. The data represent mean ± SEM (n=3).

CP55940 and GP1a may upregulate 5-HT_{2A} receptors via CB_2 receptor internalization

We then investigated whether CB_2 receptor internalization is involved in the cannabinoid agonist-induced upregulation of 5-HT_{2A} receptors. Different methods are commonly used to prevent clathrin-mediated internalization of receptors such as ConA treatment, use of hypertonic sucrose, or depletion of intracellular potassium [297;298]. We pretreated cells with vehicle or ConA prior to treatment with either vehicle, CP55940 or GP1a. As expected, CP55940 and GP1a significantly upregulated 5-HT_{2A} mRNA levels compared to vehicle treated cells (approximately 90% increase, Fig. 15A). ConA pretreatment significantly ($p<0.01$) blocked the effect of CP55940 (-9 ± 2% reduction) and GP1a (-5 ± 2% reduction) on 5-HT_{2A} mRNA levels compared to controls (Fig. 15A). The two-way ANOVA showed a main effect of ConA ($F_{(1,23)}=146.47$, $p<0.0001$), CB_2 agonists ($F_{(2,23)}=28.95$,$p<0.0001$), and a main between these two factors ($F_{(2,23)}=26.10$, $p<0.0001$) on 5-HT_{2A} mRNA levels.

We then evaluated whether ConA pretreatment can modify the agonist-induced cytosolic localization of CB_2 receptors in a neuronal cell line. We isolated cytosolic fractions from cells pretreated with vehicle or ConA and then treated with either vehicle, CP55940 or GP1a. Control cells treated with either CP55940 or GP1a showed significant increases in the cytosolic levels of CB_2 receptors (112 ± 30% and 90 ± 9%, respectively) compared to controls (Fig. 15B). ConA pretreatment significantly ($p<0.01$) reduced the vehicle, CP55940- and GP1a-induced increases in CB_2 cytosolic levels. Furthermore, CP55940 treatment in the ConA pretreated cells significantly increased CB_2 receptor cytosolic levels compared to ConA/Vehicle or ConA/GP1a treated cells. The cytosolic CB_2 levels were: 32 ± 7%, 7.5 ± 2%, 85 ± 13% for vehicle, GP1a and CP55940 treated cells, respectively. The two-way ANOVA showed a main effect of ConA

pretreatment ($F_{(1,21)}$ =129.66 ,p<0.0001), CB_2 agonist treatment ($F_{(2,21)}$ =19.28 ,p<0.0001), and a main interaction between these two factors ($F_{(2,21)}$ =8.91 ,p<0.025) on CB_2 cytosolic levels.

Recent evidence shows that some cannabinoid ligands are more efficacious CB_2 receptor internalizers than others [293]. While CP55940 is a classified as a good CB_2 receptor internalizer, JWH073 would classify as a poor CB_2 receptor internalizer [293]. There is no data regarding the properties of GP1a [293]. We then tested the effect of either vehicle, JWH073, GP1a or CP55940 on 5-HT_{2A} mRNA levels in cells (Fig. 15C). We found that both GP1a and CP55940 treatment produced significant (p<0.01) increases in the 5-HT_{2A} mRNA levels (110 ± 8% and 90 ± 7%, respectively) but JWH073 did not have any effect.

CB_2 internalization might also play a role in ERK1/2 activation [257]. Indeed, CB_2 receptors might upregulate 5-HT_{2A} receptors via activation of ERK1/2 [232]. We studied the effect of ConA pretreatment on ERK1/2 activation by measuring nuclear levels of pERK protein in cells treated with either vehicle or GP1a. Significant (p<0.05) increases in the nuclear levels of pERK (35 ± 3% compared to controls) were found in GP1a treated cells (Fig. 15D). ConA pretreatment significantly reduced the basal and GP1a-induced increases in nuclear-associated pERK levels (Fig. 15D). The two-way ANOVA showed a main effect of ConA pretreatment ($F_{1,829}$ =55647,p<0.0001), GP1a treatment ($F_{2,30.60}$ =2052,p<0.0001) and a main interaction between these two factors ($F_{1,6.53}$ =438,p<0.021) on nuclear pERK levels.

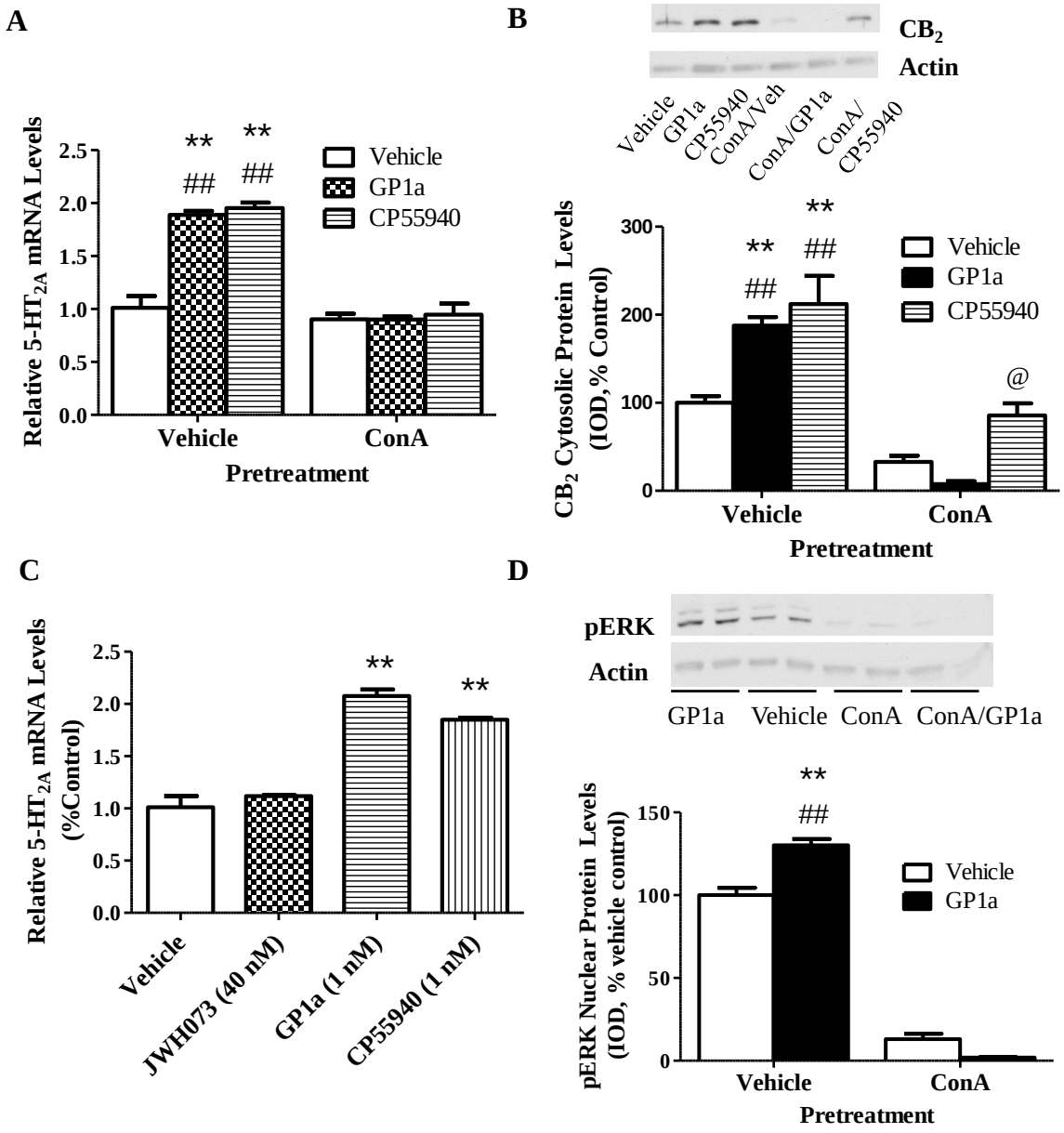


Figure 15. CP55940 and GP1a may upregulate 5-HT$_{2A}$ receptors via CB$_2$ receptor internalization.

(A) ConA pretreatment prevents CP55940 and GP1a-induced increases in 5-HT$_{2A}$ receptor mRNA. **p<0.01, significant effect of CP55940 and GP1a treatment on 5-HT$_{2A}$ receptor mRNA levels compared to vehicle-treated controls. ##p<0.01, significant effect of ConA pretreatment on

the CP55940 and GP1a-induced upregulation of 5-HT_{2A} receptors. (B) ConA pretreatment prevents CP55940 and GP1a-induced increases in cytosolic-associated CB_2 receptor protein levels. **p<0.01, significant effect of CP55940 and GP1a treatment on cytosolic CB_2 receptor protein levels compared to vehicle-treated controls. ##p<0.01, significant effect of ConA pretreatment on the CP55940 and GP1a-induced increases in cytosolic CB_2 receptor protein levels. @p<0.05, significant effect CP55940 treatment in ConA pretreated cells compared to ConA/vehicle or ConA/GP1a treated cells. (C) JWH073 does not significantly (p>0.05) alter 5-HT_{2A} receptor mRNA levels. **p<0.01, significant effect of CP55940 and GP1a treatment on 5-HT_{2A} receptor mRNA levels compared to vehicle-treated controls. (D) ConA pretreatment prevents GP1a-induced increases in nuclear pERK. **p<0.01, significant effect of GP1a treatment on nuclear pERK levels compared to vehicle-treated controls. ##p<0.01, significant effect of ConA pretreatment on GP1a-induced increases in nuclear pERK levels. The data represent mean ± SEM (n=3).

5.5 Discussion

CB_2 receptors are located not only in the periphery but also in several brain areas including PFCx, hippocampus, and amygdala [18;19;21;202]. Furthermore, recent studies reported that CB_2 receptors are mainly localized post-synaptically [19;94]. These recent findings have led to a re-evaluation of the roles of CB_2 receptors in the brain. Interestingly, here we found that a potent non-selective cannabinoid agonist (CP55940) and selective CB_2 receptor agonists (JWH133 and GP1a) induced a strong upregulation of 5-HT_{2A} receptors in neuronal cells. This cannabinoid–induced upregulation was not mimicked by a CB_1 agonist (ACEA) and was absent in cells stably transfected with CB_2 shRNA (Fig. 13) indicating that CB_2 receptors are required for this cannabinoid-mediated upregulation of 5-HT_{2A} receptors.

Accumulating evidence shows that different CB_2 receptor agonists can distinctly regulate multiple effector pathways, a phenomenon known as functional selectivity [257;286;293]. This transmission of extracellular signals to the interior of the cell is a function of plasma membrane receptors that can direct the recruitment, activation, and scaffolding of cytoplasmic signaling complexes via β-arrestins [257]. Specifically, a β-Arr2 dependent pathway could mediate the CB_2-induced activation of ERK1/2 signaling and the internalization of CB_2 receptors [293;299]. Our results support a key role of β-Arr2 in the cannabinoid-induced upregulation of 5-HT_{2A} receptors as cells stably transfected with β-Arr2 shRNA lentivirus (Fig. 14) failed to upregulate 5-HT_{2A} receptors in response to either CP55940 or JWH133. GP1a-mediated upregulation of 5-HT_{2A} mRNA levels was significantly reduced in β-Arr2 shRNA lentivirus treated cells compared to controls shRNA treated cells. Although, we do not know the mechanism mediating this effect we speculate that the remaining β-Arr2 protein levels (approximately 15-20%) could be enough to produce significant increases in 5-HT_{2A} mRNA levels. Alternatively, GP1a could be triggering

this upregulation (at least in part) through a different signaling mechanism than the one triggered by CP55940 or JWH 133.

β-Arr2 could mediate the upregulation of 5-HT_{2A} receptors by regulating internalization of CB_2 receptors [293]**.** ConA pretreatment of cultured cells is used to prevent clathrin-mediated internalization of receptors [297;298]. Interestingly, ConA pretreatment prevented the CP55940- and GP1a-induced increases in 5-HT_{2A} receptor mRNA levels in cultured cells (Fig. 15A). Our results also showed that ConA pretreatment also prevented the CP55940 or GP1a increases in CB_2 receptor cytosolic levels (Fig. 15B). Noteworthy, ConA pretreatment even reduced the basal cytosolic CB_2 protein levels (Fig. 15B). This suggests that ConA might prevent the basal trafficking of CB_2 receptors between the membrane and cytosol in neuronal cells. Similarly, Atwood et al (2012) described significant increases in membrane-associated levels of CB_2 receptors in sucrose treated cells [293] further indicating that protocols utilized to prevent clathrin-mediated endocytosis alter basal trafficking of the CB_2 receptors between the membrane and the cytosol.

The previous experiments suggest that the degree of internalization of CB_2 receptors could play an important role in the upregulation of 5-HT_{2A} receptors. Atwood et al. (2012) identified different classes of CB_2 agonists that differ substantially in their ability to promote CB_2 receptor internalization [293]. Cannabinoid agonists such as CP55940 and JWH133 were the most efficacious CB_2 receptor internalizers, while aminoalkylindoles which include cannabinoid ligands, such as JWH073 and WIN55,212-2, were the least effective or failed to promote CB_2 receptor internalization [293]. We found that CP55940, but not JWH073, induced 5-HT_{2A} upregulation (Fig. 15C), suggesting that upregulation of 5-HT_{2A} receptors is dependent on the ability of the agonists to promote internalization of CB_2 receptors. This is consistent with

the experiments previously discussed where ConA pretreatment prevented the cannabinoid-mediated increases in 5-HT_{2A} mRNA levels (Fig. 15A).

CB_2 receptor-mediated upregulation of 5-HT_{2A} receptors might involve transcription factors that are located downstream of ERK and that target the promoter region of the 5-HT_{2A} receptor gene such as CREB and AP-1 [232]. Here, we found that a selective CB_2 receptor agonist (GP1a) induced ERK1/2 activation (Fig. 15D), suggesting that the CB_2 receptor mediated-upregulation of 5-HT_{2A} receptors involve activation of the ERK1/2 signaling pathway.

In summary, our results indicate that cannabinoid agonists would upregulate 5-HT_{2A} receptors by a mechanism that requires CB_2 receptors and β-Arr2 activation in cells that express both CB_2 and 5-HT_{2A} receptors. This upregulation could be mediated by a mechanism that involves internalization of CB_2 receptors and ERK1/2 activation. Indeed, compounds that do not internalize CB_2 receptors such as JWH073 do not induce 5-HT_{2A} upregulation. It is then possible that cannabinoid agonists that upregulate 5-HT_{2A} receptors mediate their effect by recruiting β-Arr2 to the membrane, enhancing ERK1/2 activation, and also promoting CB_2 receptor internalization. 5-HT_{2A} receptors have been associated with several physiological functions and neuropsychiatric disorders such as stress response, anxiety, depression, and schizophrenia [24;137]. Cannabinoid-induced upregulation of 5-HT_{2A} receptors may precipitate the onset of mental disorders associated with dysfunction of 5-HT_{2A} receptor neurotransmission as it has been recently suggested [13].

Chapter 6: Cannabinoid Agonists Increase the Interaction between Beta Arrestin 2 and ERK1/2 and Upregulate Beta Arrestin 2 and 5-HT_{2A} Receptors

(Franklin J.M., Vasiljevik T., Prisinzano T.E., Carrasco G.A. (2012) Cannabinoid Agonists Increase the Interaction between Beta Arrestin 2 and ERK1/2 and Upregulate Beta Arrestin 2 and 5-HT_{2A} Receptors. *Pharmacological Research*, **68**: 46-58.)

6.1 Abstract

We have recently reported that selective CB_2 receptor agonists upregulate 5-HT_{2A} receptors by enhancing ERK1/2 signaling in PFCx. Increased activity of cortical 5-HT_{2A} receptors has been associated with several neuropsychiatric disorders such as anxiety and schizophrenia. Here we examine the mechanisms involved in this enhanced ERK1/2 activation in rat PFCx and in a neuronal cell model. Sprague-Dawley rats treated with a non-selective cannabinoid agonist (CP55940) showed enhanced co-immunoprecipitation of β-Arrestin 2 and ERK1/2, enhanced pERK protein levels, and enhanced expression of β-Arrestin 2 mRNA and protein levels in PFCx. In a neuronal cell line, we found that selective CB_2 receptor agonists upregulate β-Arrestin 2, an effect that was prevented by selective CB_2 receptor antagonist JTE-907 and CB_2 shRNA lentiviral particles. Additionally, inhibition of clathrin-mediated endocytosis, ERK1/2, and the AP-1 transcription factor also prevented the cannabinoid receptor-induced upregulation of β-Arrestin 2. Our results suggest that sustained activation of CB_2 receptors would enhance β-Arrestin 2 expression, possibly contributing to its increased interaction with ERK1/2 thereby driving the upregulation of 5-HT_{2A} receptors. The CB_2 receptor-mediated upregulation of β-Arrestin 2 would be mediated, at least in part, by an ERK1/2-dependent activation of AP-1. These data could provide the rationale for some of the

adverse effects associated with repeated cannabinoid exposure and shed light on some CB_2 receptor agonists that could represent an alternative therapeutic approach because of their minimal effect on serotonergic neurotransmission.

6.2 Introduction

Cannabinoid receptor agonists are being shown to have wide therapeutic applications in the treatment of conditions such as stroke, neuropathic pain, neurodegenerative diseases, and cocaine addiction [2-5]. However, recent and independent clinical studies provide strong evidence indicating that sustained use of nonselective cannabinoid agonists may precipitate the onset of mental disorders associated with dysfunction of 5-HT_{2A} receptor neurotransmission in PFCx, such as schizophrenia, psychosis and anxiety [10-12;14]. Although the precise mechanism by which repeated cannabinoid exposure may precipitate these disorders is unknown, we have recently provided evidence that cannabinoid agonists induce a strong upregulation and increase activity of 5-HT_{2A} receptors *in vivo* and *in vitro* [262;263].

Cannabinoid agonists can produce their physiological effects through the activation of two G-protein coupled cannabinoid receptors in the brain, CB_1 and CB_2 receptors [15;98]. CB_1 and CB_2 receptors bind endocannabinoids, synthetic cannabinoids, and cannabinoids found in nature (such as *Cannabis sativa* L.) with high affinity [15;98]. Although only CB_1 receptors were initially identified in the brain [246], later studies have identified CB_2 receptors in several brain areas such as PFCx, hippocampus, amygdala, substantia nigra, and cerebellum [18;21], triggering a reevaluation of the possible roles that CB_2 receptors may play in the brain. These cannabinoid receptors couple to $G_{i/o}$ class of G-proteins and can activate ERK1/2 signaling in either a G-protein or β-Arrestin dependent pathway [15;88]. While G-protein-mediated activation of ERK1/2 is transient and peaks within 2-5 minutes [171;300], β-Arrestins can form a scaffolding complex with Raf-1, MEK, and ERK1/2 which can regulate the long-term activation of ERK1/2 after β-Arrestin mediated internalization of the G-protein coupled receptor or GPCR [171;257;300].

We recently reported that 5-HT_{2A} receptors are upregulated by repeated exposures to cannabinoid agonists through a mechanism that would involve CB_2 receptor-mediated activation of ERK1/2 signaling, and that is independent of CB_1 receptor activation [262;263]. Moreover, we presented experimental evidence that sustained treatment with a non-selective cannabinoid agonist (CP55940) or selective CB_2 receptor agonists (JWH133 or GP1a) upregulate 5-HT_{2A} receptors in a neuronal cell line, an effect that was not replicated by selective CB_1 agonists [263]. The CB_2 receptor is a class A GPCR which means it would preferentially interact with β-Arrestin 2 to form a scaffolding complex with ERK1/2 [301]. Accordingly, we also reported that the cannabinoid receptor agonist-induced upregulation of 5-HT_{2A} receptors was prevented in cells stably transfected with either CB_2 or β-Arrestin 2 shRNA lentiviral particles [262].

Here we examined mechanisms which could contribute to the CB_2 receptor- and ERK1/2-mediated enhanced activation of 5-HT_{2A} receptors. We studied the involvement of selective CB_1 and CB_2 receptor agonists on the regulation of β-Arrestin 2 expression and the formation of a β-Arrestin 2 and ERK1/2 protein complex. Our results indicate that repeated exposure to cannabinoids enhance the protein interaction between β-Arrestin 2 and ERK1/2. Furthermore, cannabinoid agonists upregulated β-Arrestin 2 by a mechanism that would require internalization of CB_2 receptors, activation of ERK1/2, and activation of the transcription factor AP-1. We also detected a strong CB_2, but not CB_1, receptor activation upregulation of β-Arrestin 2 in a neuronal cell line. We hypothesize that the data presented here could provide, at least in part, a molecular mechanism by which repeated exposure to cannabinoids might be relevant to some cognitive and mood disorders by upregulating and enhancing the activity of 5-HT_{2A} receptors.

6.3 Materials and Methods

Drugs

(-)-*cis*-3-[2-Hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol (CP55940), a CB_1 and CB_2 receptor agonist; (6a*R*,10a*R*)-3-(1,1-Dimethylbutyl)-6a,7,10,10a-tetrahydro-6,6,9-trimethyl-6*H*-dibenzo[*b*,*d*]pyran (JWH-133) a selective CB_2 agonist; N-(Piperidin-1-yl)-1-(2,4-dichlorophenyl)-1,4-dihydro-6-methylindeno[1,2-c]pyrazole-3-carboxamide (GP1a) a highly selective CB_2 receptor agonist; N-(2-Chloroethyl)-5Z,8Z,11Z,14Z-eicosatetraenamide (ACEA) a highly selective CB_1 receptor agonist; 2-(2-Chlorophenyl)-3-(4-chlorophenyl)-7-(2,2-difluoropropyl)-6,7-dihydro-2*H*-pyrazolo[3,4-*f*][1,4]oxazepin-8(5*H*)-one (PF-514273), a selective CB_1 receptor antagonist; *N*-(1,3-Benzodioxol-5-ylmethyl)-1,2-dihydro-7-methoxy-2-oxo-8-(pentyloxy)-3-quinolinecarboxamide (JTE-907) a selective CB_2 receptor antagonist/inverse agonist; *N*-(Cyclopropylmethoxy)-3,4,5-trifluoro-2-[(4-iodo-2-methylphenyl)amino]-benzamide (PD198306) a potent and selective ERK1/2 inhibitor; and (*E*,*E*,*Z*,*E*)-3-Methyl-7-(4-methylphenyl)-9-(2,6,6-trimethyl-1-cyclohexen-1-yl)-2,4,6,8-nonatetraenoic acid (SR11302), an inhibitor of activating protein-1 transcription factor activity were purchased from Tocris (Ellisville, MO). Naphthol AS-E phosphate, a CREB inhibitor, was purchased from Sigma-Aldrich Inc. (St. Louis, MO). Naphthalen-1-yl-(1-butylindol-3-yl)methanone (JWH073), a CB_1/CB_2 receptor agonist, was synthesized in the laboratory of Dr. Thomas Prisinzano as described previously [49].

Animal Experimental Protocol

Male Sprague-Dawley rats (225-275 g; Harlan Laboratories, Indianapolis, IN) were housed two per cage in a temperature-, humidity-, and light-controlled room (12 hr light/dark

cycle, lights on 7:00 AM-7:00 PM). Food and water were available *ad libitum*. All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals as approved by the University of Kansas Institutional Animal Care and Use Committee (IACUC).

After arrival, the rats were allowed to acclimate to their environment for at least 4 days prior to the start of the treatment period. Eight rats were randomly assigned to each group, cage mates were assigned to the same treatment group. The body weight of each rat was recorded every other day. All solutions were made fresh before administration and rats were injected with either vehicle (Tween-80/ethanol/saline (1:1:18); 1mL/kg, i.p.) or CP55940 (0.05 mg/kg, i.p.) once a day for 7 days. Rats were sacrificed by decapitation 48 h after the last CP55940 injection. The dose and time course for CP55940 were chosen based upon the literature that reported that similar doses induced increased anxiety-like behaviors [302;303] and upregulation of 5-HT_{2A} receptors in rat PFCx [263]. In our preliminary experiments we also noticed that doses higher than 0.2 mg/kg prevent weight gain in rats after 2 days of CP55940 exposure. After sacrifice, brains were immediately removed and PFCx was dissected and frozen in dry ice.

Co-Immunoprecipitation

Co-immunoprecipitation (co-IP) was conducted with the Thermo Scientific Pierce co-IP kit following manufacturer's protocol and as previously described [263]. The β-Arrestin 2 and ERK1/2 antibody was purchased from Santa Cruz, CA. Briefly, β-Arrestin 2 antibody was incubated with AminoLink Plus coupling resin for 2 hrs. This resin was incubated with pre-cleared PFCx lysate (300 µg) from either vehicle or CP55940 treated rats overnight. A negative control included a non-reactive resin that was incubated with β-Arrestin 2 antibody for 2 hrs and then pre-cleared PFCx lysate from either CP55940 or vehicle-treated rats overnight. After the

overnight incubation, the resins were washed (3x) and the protein eluted using elution buffer**.** Samples were analyzed by Western blot using ERK1/2 antibody. The specificity of the β-Arrestin 2 or ERK1/2 antibody has been verified in the literature [262;304;305].

Western Blot

Membrane-associated fractions were isolated using the ProteoExtract^{TM} Native Membrane Protein Extraction kit (Calbiochem, La Jolla, CA) and Nuclear-associated fractions were isolated using NE-PER ® Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific, IL). Expression of β-Arrestin 2, pERK, and ERK1/2 were determined by western blot analysis as previous described [269]. pERK antibody was purchased from Santa Cruz, CA. The specificity of the antibody has been verified in the literature [306]. Protein loading for each lane was verified using an anti-actin antibody (Santa Cruz Biotechnology, Inc.). Negative controls included either the omission of primary antibody or addition of preimmune rabbit immunoglobulins. Films were analyzed densitometrically with values calculated from the integrated optical density (IOD) of each band using Scion Image software (Scion Corporation, Frederick, MD, USA), as previously described [269;272]. All samples were standardized to controls and normalized to their respective actin levels.

Ex vivo assay for CP55940-induced ERK1/2 activation in PFCx tissue

This assay was based upon a PLCβ assay that has been previously described [269]. PFCx tissue from vehicle or CP55940 treated rats was homogenized in homogenization buffer (1 mL/0.1 g of tissue) with protease inhibitor for 3 seconds. Samples were vortexed and centrifuged at 13,000 rpm at 4ºC for 10 minutes. The supernatant was removed, 500 µL of homogenate buffer was added to the pellet, samples were vortexed and centrifuged at 13,000 rpm at 4ºC for 10 minutes. This wash process was repeated three times. Protein levels were measured and

equalized. Vehicle and CP55940 samples were treated with 1 nM CP55940 and incubated for 15 minutes. Western blot analysis was utilized to measure pERK levels as previously described above.

Quantitative Real-Time PCR

These reactions were prepared using QuantiFast SYBR Green PCR Kit (Qiagen, Valencia, CA) and the ABI 7500 fast real time PCR system (Applied Biosystems, Foster City, CA) and then data were analyzed using the comparative cycle threshold (Ct) method as previously described [263]. The primers used in this manuscript were: 5-HT_{2A} (F:5'-AACGGTCCATCCACAGAG-3',R:5'-AACAGGAAGAACACGATGC-3'), β-Arrestin 2 (F:5'-AGCACCGCGCAGTACAAGT-3',5'-R:CACGCTTCTCTCGGTTGTCA-3') and GAPDH (F:5'-TGGAGTCTACTGGCGTCTTCAC-3',R:5'-GGCATGGACTGTGGTCATGA-3'). These primers have been previously validated in the literature [194;262;263;304].

Cell Culture Protocol

CLU213 cells, a rat neuronal cell line that co-expresses 5-HT_{2A}, D_2, CB_1 and CB_2 receptors, were purchased from Cedarlane Laboratories (Burlington, NC). CLU213 were grown on 100-mm^2 plates treated with polystyrene (Corning Incorporated, Corning, NY) and maintained in 5% CO_2 at 37°C, in Dulbecco's modified eagle medium (DMEM; Mediatech Inc, Manassas, VA) containing 10% fetal bovine serum (FBS; Thermo Scientific, Logan, UT).

Effect of Non-Selective and Selective CB_1 and CB_2 Receptor Agonists on β-Arrestin 2 mRNA and protein levels

CLU213 cells were incubated with either vehicle (ethanol 0.01% final concentration), CP55940 1 nM (CB_1 and CB_2 agonist, K_i: 0.58 nM and 0.68 nM for CB_1 and CB_2 receptors, respectively) [50], JWH133 30 nM (selective CB_2 agonist, K_i: 3.4 nM and 677 nM for CB_2 and

CB_1 receptors, respectively) [59], GP1a 1 nM (highly selective CB_2 agonist, K_i: 0.037 nM and 353 nM for CB_2 and CB_1 receptors, respectively) [60], ACEA 15 nM (selective CB_1 agonist, K_i: 1.4 nM and 3.1 μM for CB_1 and CB_2 receptors, respectively) [55], or or JWH073 40 nM (CB_1 and CB_2 agonist, K_i: 8.9 nM and 38 nM for CB_1 and CB_2 receptors, respectively) [49;52] for 24 hours. mRNA was isolated and qRT-PCR for β-Arrestin 2 mRNA were performed as described above.

In a separate experiment, cells were treated with vehicle (ethanol 0.01% final concentration), CP55940 (1 nM), JWH133 (30 nM) or GP1a (1 nM) for 72 hours. Cells were washed (3x) with PBS every 24 hours and fresh vehicle, CP55940, JWH133 or GP1a were added. Expression of membrane-associated β-Arrestin 2 was determined by Western blot as previously described.

Effect of selective cannabinoid receptor antagonists on the CP55940-induced upregulation of β-Arrestin 2

CLU213 cells were pretreated with either vehicle (ethanol 0.01% final concentration); PF-514273 20 nM (CB_1 antagonist, K_i: 1 nM and >10,000 nM for CB_1 and CB_2 receptor, respectively)[57] or JTE-907 10 nM (CB_2 antagonist, K_i: 0.38 and 1,050 nM for CB_2 and CB_1 receptors, respectively) [62]. Twenty minutes later cells were incubated with either vehicle (ethanol 0.01% final concentration) or CP55940 1 nM for 24 hours. mRNA was isolated and qRT-PCR for β-Arrestin 2 mRNA was performed.

Effect of selective CB_2 receptor antagonist on GP1a-induced upregulation of β-Arrestin 2

CLU213 cells were pretreated with either vehicle (ethanol 0.01% final concentration) or JTE-907 (10 nM, a CB_2 receptor antagonist) [62]. Twenty min later cells were incubated with

either vehicle (ethanol 0.01% final concentration) or GP1a (1nM) for 24 hours. mRNA was isolated and qRT-PCR for β-Arrestin 2 mRNA was determined.

Lentivirus and stable transduction of shRNAs in CLU213 cells

β-Arrestin 2 shRNA (r), CB_2 shRNA (r), CB_1 shRNA (r), copGFP control, control shRNA lentiviral particles, polybrene, and puromyocin were purchased from Santa Cruz, CA. Optimal transduction conditions were elucidated utilizing copGFP control lentiviral particles prior to transduction with β-Arrestin 2 shRNA or CB_2 shRNA lentiviral particles and then transduction was conducted as previously described [262]. Cells were analyzed for β-Arrestin 2, CB_2, or CB_1 knockdown one week after initiation of puromyocin selection.

Effect of CB_2 or CB_1 shRNA lentivirus transfection on cannabinoid-induced upregulation of β-Arrestin 2

After confirming that treatment with the CB_2 or CB_1 shRNA lentivirus reduced CB_2 or CB_1 mRNA 80% and 70%, respectively, control, CB_2, or CB_1 shRNA treated cells were treated with either vehicle (ethanol 0.01% final concentration), CP55940 1 nM, JWH133 30 nM, or GP1a 1 nM for 24 hours. mRNA was isolated from cells and qRT-PCR was performed for β-Arrestin 2 mRNA levels as previously described above.

Effect of Concanavalin A (ConA) treatment on cannabinoid-induced increases in β-Arrestin 2 mRNA

Cells were pretreated with either vehicle or 250 µg/mL ConA for 20 minutes [262;297]. Twenty minutes later cells were incubated with either vehicle (ethanol 0.01% final concentration), CP55940 (1 nM), or GP1a (1 nM). mRNA was isolated and qRT-PCR was performed for β-Arrestin 2 mRNA levels.

Effect of a selective ERK1/2 inhibitor on GP1a-Induced Increases in β-Arrestin 2 mRNA

CLU213 cells were treated with either vehicle (ethanol 0.01% final concentration) or PD198306 (200 nM) [263]. Twenty minutes later cells were incubated with either vehicle (ethanol 0.01% final concentration) or GP1a (1 nM). mRNA was isolated and qRT-PCR was used to measure β-Arrestin 2 mRNA levels.

Effect of β-Arrestin 2 shRNA lentivirus transfection on cannabinoid-induced ERK1/2 activation

After confirming that treatment with the β-Arrestin 2 lentivirus particles reduced β-Arrestin 2 mRNA and protein levels 85%, control or β-Arrestin 2 treated cells were treated with either vehicle (ethanol 0.01% final concentration), CP55940 1 nM or GP1a 1 nM for 15 minutes. Nuclear fractions were isolated and western blot analysis was utilized to measure nuclear-associated pERK levels.

Effect of Transcription Factor Inhibitors on GP1a-Induced Upregulation of β-Arrestin 2 mRNA

CLU213 cells were treated with either vehicle (ethanol 0.01%), Naphthol AS-E phosphate (10 μM) [224], or SR11302 (1 μM) [226] for 20 minutes. Cells were then treated with either vehicle (ethanol 0.01%) or GP1a (1 nM) for 24 hours. mRNA was isolated and qRT-PCR for β-Arrestin 2 mRNA was performed as previously described.

Effect of β-Arrestin 2 shRNA lentivirus treatment on GP1a-induced upregulation of 5-HT_{2A} mRNA

Control or β-Arrestin 2 shRNA lentivirus treated cells were treated with either vehicle (ethanol 0.01% final concentration), GP1a 1 nM, ACEA 15 nM or JWH073 40 nM for 24 hours. mRNA was isolated and qRT-PCR for β-Arrestin 2 mRNA was performed.

Statistics

All data are expressed as the mean ± S.E.M., where *n* indicates the number of rats or cell culture plates per group. Data was analyzed by an unpaired Student's t-test or ANOVA

(Newman-Keuls post-hoc test). GB-STAT software (Dynamic Microsystems, Inc., Silver Spring, MD, USA) was used for all statistical analyses.

6.4 Results

Chronic CP55940 treatment induces enhanced β-Arrestin 2 and ERK1/2 interaction in PFCx

Our previous work has shown that some cannabinoid agonists can enhance 5-HT_{2A} receptor expression by means of a mechanism that involves CB_2 receptor regulation of ERK1/2 activation [262;263]. Cannabinoid receptors could produce a long-term ERK1/2 activation by a mechanism that may involve a β-Arrestin-ERK1/2 scaffolding complex [171;257;300]. Specifically, CB_2 receptors that are a class A GPCR would preferentially interact with β-Arrestin 2, which may facilitate and enhance the interaction between β-Arrestin and ERK1/2 resulting in long-term ERK1/2 activation [301]. Here, we used co-immunoprecipitation protocols to study the effect of CP55940 treatment on the physical interaction between β-Arrestin 2 and ERK1/2 in rat PFCx (Fig. 16A). We used β-Arrestin 2 antibody as bait and ERK1/2 antibody as prey. Inactive columns which are unable to bind β-Arrestin 2 antibody were used as a control as described in methods. We found that ERK1/2 co-precipitates with β-Arrestin 2 when we used β-Arrestin 2 as bait (Fig. 16A, lanes 3 & 4). Interestingly, we detected a significant ($p<0.05$) two-fold increase in the interaction between β-Arrestin 2 and ERK1/2 in PFCx of CP55940-treated rats compared to vehicle treated controls (Fig. 16A, lane 3 and 4, vehicle- and CP55940-treated animals, respectively). No co-precipitation of β-Arrestin 2 and ERK1/2 was detected using the inactive columns (Fig. 16A, lanes 5 & 6).

Chronic CP55940 treatment enhances ERK1/2 activation in PFCx homogenates after an acute challenge with CP55940

The increased interaction between β-Arrestin 2 and ERK1/2 proteins could lead to an enhanced ERK1/2 signaling pathway activity. We then designed an *ex vivo* experiment to measure acute CP55940-induced ERK phosphorylation in PFCx homogenates of vehicle and

CP55940-treated rats. ERK activation (phosphorylation) was induced by a short (15min) incubation of the homogenates with 1nM CP55940. We found that this CP55940 challenge induced a significantly ($p<0.01$) greater ERK1/2 phosphorylation in PFCx homogenates of CP55940 treated rats compared to vehicle controls (78 ± 5% increase in CP55940 compared to controls, Fig. 16B). No significant differences ($p>0.05$) in total ERK1/2 protein levels were detected between both experimental groups.

Chronic CP55940 treatment upregulates β-Arrestin 2 expression but not ERK1/2 in rat PFCx

We also studied the effect of repeated exposure of CP55940 on β-Arrestin 2 and ERK1/2 protein expression in rat PFCx since changes in the levels of these proteins could explain the enhanced: (1) β-Arrestin 2 and ERK1/2 interaction (Fig. 16A); and (2) ERK1/2 signaling pathway (Fig. 16B) in PFCx of CP55940-treated rats compared to vehicle controls. Here, we examined the effect of repeated CP55940 (50μg/kg for 7 days) exposure on the expression of membrane-associated β-Arrestin 2 and total ERK1/2 protein levels in rat PFCx. CP55940 treatment produced a significant ($p<0.01$) increase in β-Arrestin 2 membrane-associated protein levels compared to vehicle treated controls (108 ± 13% increase compared to controls, Fig. 16C). Interestingly, CP55940 treatment did not significantly alter ($p>0.05$) total ERK1/2 protein levels in rat PFCx (Fig. 16D). Actin was used as a protein loading control in the Western blots as described in methods. Cannabinoid-induced upregulation of β-Arrestin 2 mRNA synthesis could contribute to the changes in membrane-associated β-Arrestin 2 protein levels, so we also determined the effect of CP55940 treatment on β-Arrestin 2 mRNA levels. β-Arrestin 2 mRNA was significantly ($p<0.05$) increased (two-fold) in PFCx of CP55940-treated rats compared to vehicle-treated controls (107 ± 15% increase compared to controls, Fig. 16E).

β-Arrestin 2 is involved in the cannabinoid-induced upregulation of 5-HT_{2A} receptors

We used neuronal cells stably transfected with either β-Arrestin 2 or control shRNA lentiviral particles to study the contribution of β-Arrestin 2 on the cannabinoid-induced upregulation of 5-HT_{2A} receptors. We have previously shown that GP1a (a selective CB_2 receptor agonist) upregulates 5-HT_{2A} receptors while treatment with ACEA (a selective CB_1 receptor agonist) does not significantly alter 5-HT_{2A} receptor mRNA levels [263]. Treatment with β-Arrestin 2 shRNA lentiviral particles significantly ($p<0.01$) reduced β-Arrestin 2 mRNA and protein levels by approximately 85% [262]. Here, cells were treated with either vehicle, GP1a (1 nM), or ACEA (15 nM) for 24 hours. We found that GP1a upregulated 5-HT_{2A} mRNA in control shRNA treated cells by 109 ± 2% (Fig. 16F). ACEA did not significantly modify 5-HT_{2A} receptor mRNA levels in control shRNA treated cells compared to vehicle treated controls. Noteworthy, the GP1a-induced upregulation of 5-HT_{2A} mRNA levels was significantly ($p<0.05$) reduced in cells stably transfected with β-Arrestin 2 shRNA lentiviral particles. GP1a-mediated increases in 5-HT_{2A} mRNA levels was prevented in β-Arrestin 2 shRNA treated cells (Fig. 16F). The two-way ANOVA showed main effects of transfection ($F_{(1,23)}$ =63.37 ,$p<0.0001$), CB_2 agonist ($F_{(3,23)}$ =71.59 ,$p<0.0001$), and a main interaction between these two factors ($F_{(3,23)}$ =61.22 ,$p<0.0001$) on 5-HT_{2A} mRNA levels. In summary, these data suggest that CP55940 treatment might heighten the β-Arrestin 2 and ERK1/2 interaction that would enhance ERK1/2 signaling in rat PFCx through upregulation of β-Arrestin 2. This latter protein is necessary for the cannabinoid-induced upregulation of 5-HT_{2A} receptors, as indicated in studies conducted in our neuronal cell model.

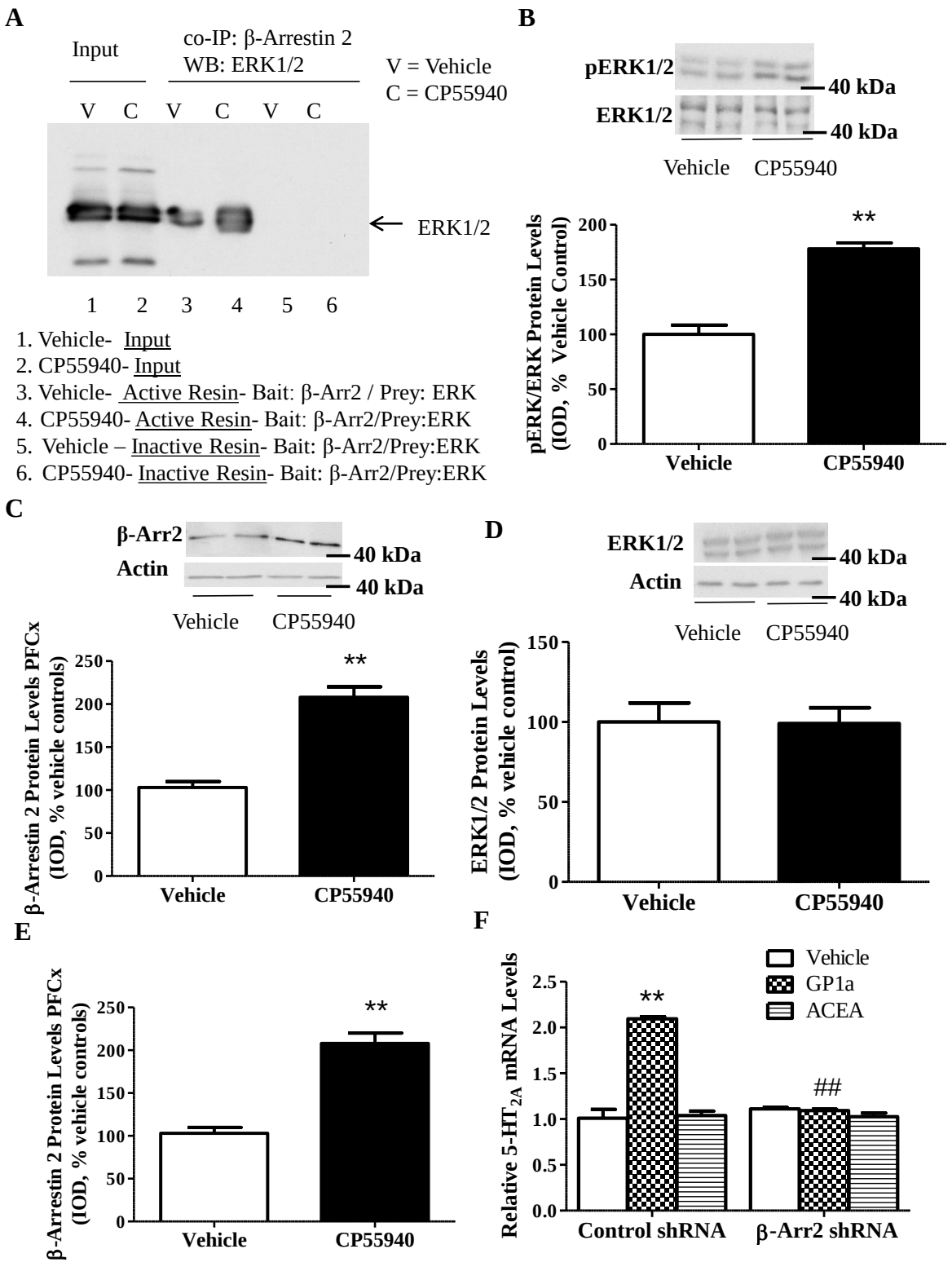


Figure 16. CP55940-induced enhanced co-immunoprecipitation of β-Arrestin 2 and ERK1/2 and increased β-Arrestin 2 protein expression in rat PFCx.

(A) Enhanced immunoprecipitation of the ERK1/2 (Lane 4) compared to vehicle-treated controls (Lane 3). Negative controls (Lanes 5 and 6) received the same concentration of β-Arrestin 2 antibody except that the coupling resin was replaced with control agarose resin that is not amine reactive. All columns were incubated with PFCx lysate (300 µg) from vehicle (Lanes 3 and 5) or CP55940 (Lanes 4 and 6) treated rats. PFCx lysate (30 µg of protein) was used as an input control (Lane 1 and 2). (B) Increased pERK protein levels in CP55940 treated rats compared to vehicle treated rats. **$p<0.01$, significant effect of CP55940 treatment compared to vehicle-treated controls. (C) Increased membrane associated β-Arrestin 2 protein levels in PFCx of CP55940 treated rats. **$p<0.01$ significant effect of CP55940 treatment compared to vehicle-treated controls. (D) CP55940 treatment does not affect total ERK1/2 expression in the PFCx. (E) Increased β-Arrestin 2 mRNA levels in PFCx of CP55940 treated rats. *$p<0.01$ significant effect of CP55940 treatment compared to vehicle treated controls. (F) β-Arr2 shRNA lentivirus transfection prevents GP1a-induced increases in 5-HT_{2A} receptor mRNA. **$p<0.01$, significant effect of GP1a treatment on 5-HT_{2A} receptor mRNA levels in control shRNA tranfected cells compared to vehicle-treated controls. ##$p<0.01$, significant effect of β-Arr2 shRNA transfection on the GP1a-induced upregulation of 5-HT_{2A} receptors. Representative Western blots are shown in this figure and IOD was calculated as described in Experimental Procedures. The data represent mean ± SEM (n=6-8).

CP55940 and selective CB_2 receptor agonists, JWH-133 and GP1a, upregulate β-Arrestin 2 in CLU213 cells

Next, we used a neuronal cell culture model to elucidate the mechanisms underlying cannabinoid-induced upregulation of β-Arrestin 2. Here, cells were incubated with either vehicle, CP55940 (CB_1/CB_2 receptor agonist, 1nM), selective CB_2 receptor agonists JWH133 (30nM) or GP1a (1nM), or a selective CB_1 agonist ACEA (15nM) for 24 hours. We found that sustained CP55940 exposure significantly ($p<0.01$) increased β-Arrestin 2 mRNA levels compared to vehicle treated cells (67 ± 5% increase, Fig. 17A). Noteworthy, sustained treatment with either JWH133 or GP1a, but not ACEA, significantly increased ($p<0.01$) β-Arrestin 2 mRNA levels (72 ± 5% and 64 ± 4% increase compared to controls, respectively)(Fig. 17A). We also determined what effect CP55940, JWH133, and GP1a treatment had on β-Arrestin 2 protein levels. We found that treatment with CP55940 (1nM), JWH133 (30nM), and GP1a (1nM) also significantly increased ($p<0.01$) β-Arrestin 2 protein levels compared to vehicle treated controls (105 ± 26%, 120 ± 12%, and 143 ± 21% increase compared to vehicle controls, respectively)(Fig. 17B).

A selective CB_2 receptor antagonist, JTE-907, prevents CP55940- and the GP1a-induced increases in β-Arrestin 2 mRNA levels

The evidence presented above suggested that the cannabinoid-induced upregulation of β-Arrestin 2 might be mediated by the CB_2 receptors. In order to examine the relative roles of CB_1 and CB_2 receptors in the upregulation of β-Arrestin 2, cells were pretreated with either vehicle, a selective CB_1 receptor antagonist PF-514273 (20nM), or a selective CB_2 receptor antagonist JTE-907 (10nM) and then treated with either vehicle or CP55940 (1nM) for 24 hours. CP55940 treatment significantly ($p<0.01$) increased β-Arrestin 2 mRNA levels compared to vehicle treated

controls (75 ± 2% increase compared to controls, Fig. 17C). We found that PF-514273 pretreatment did not inhibit or significantly ($p>0.05$) modify the CP55940-induced increases in β-Arrestin 2 mRNA levels. Noteworthy, pretreatment with a selective CB_2 receptor antagonist, JTE-907, prevented the CP55940-induced increases in β-Arrestin 2 mRNA levels ($p<0.01$, Fig. 17C). No significant ($p>0.05$) effect of PF-514273 or JTE-907 pretreatment was found on basal β-Arrestin 2 mRNA levels. The two-way ANOVA showed main effects of cannabinoid antagonists ($F_{(2,17)} =37.27$,$p<0.0001$), cannabinoid agonists ($F_{(1,17)} =143.29$,$p<0.0001$), and a main interaction between these two factors ($F_{(2,17)} =36.94$,$p<0.0001$) on β-Arrestin 2 mRNA levels.

In order to more thoroughly investigate the role of CB_2 receptors in the cannabinoid-induced upregulation of β-Arrestin 2 mRNA levels, we pretreated cells with either the vehicle or selective CB_2 antagonist JTE-907 and then treated cells with vehicle or a highly selective CB_2 receptor agonist GP1a. As expected, GP1a treatment significantly ($p<0.01$) increased β-Arrestin 2 mRNA levels (74 ± 5% increase compared to controls, Fig. 17D). This GP1a-induced upregulation of β-Arrestin 2 was prevented ($p<0.01$) in cells pretreated with JTE-907 (Fig. 17D). The two-way ANOVA showed main effects of JTE-907 pretreatment ($F_{(1,11)} =35.37$, $p<0.0003$), GP1a treatment ($F_{(1,11)} = 68.32$, $p<0.0001$), and a main interaction between these two factors ($F_{(1,11)} =37.12$, $p<0.0003$) on β-Arrestin 2 mRNA levels.

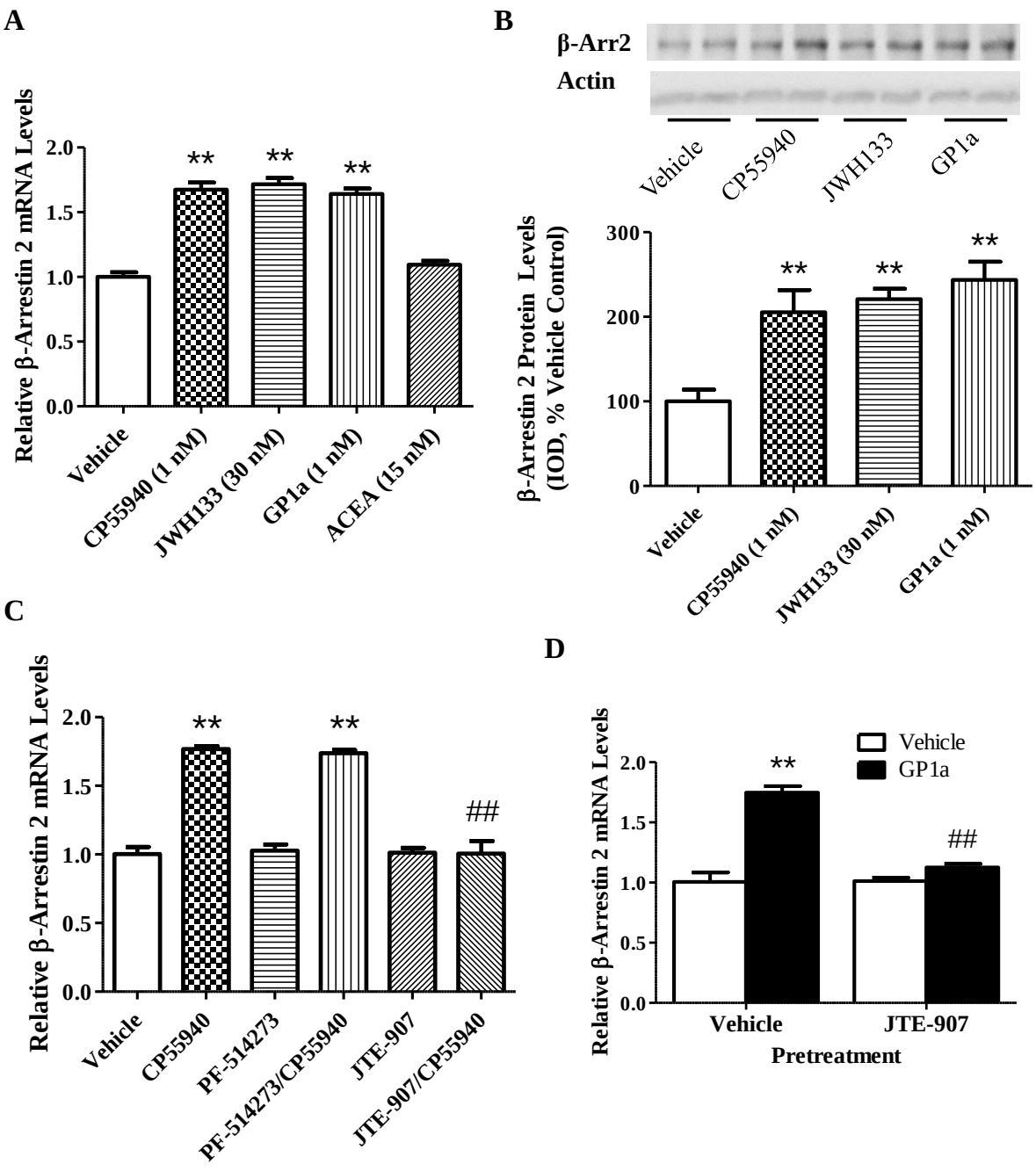


Figure 17. Selective CB_2 receptor agonists upregulate β-Arrestin 2 and selective CB_2 receptor antagonist prevents cannabinoid-induced upregulation of 5-HT_{2A} receptors.

(A) Increased β-Arrestin 2 mRNA levels in CP55940, JWH-133, and GP1a treated cells. ACEA treatment did not significantly alter β-Arrestin 2 mRNA levels. **p<0.01 significant effect of CP55940, JWH-133, or GP1a treatment compared to vehicle-treated controls. (B) Increased β-

Arrestin 2 protein levels in CP55940, JWH-133, and GP1a treated cells. **p<0.01 significant effect of CP55940, JWH-133, or GP1a compared to vehicle-treated controls (C) Selective CB_2 receptor antagonists pretreatment JTE-907 prevents CP55940-induced increases in β-Arrestin 2 mRNA. **p<0.01, significant effect of CP55940 treatment on β-Arrestin 2 mRNA levels compared to vehicle-treated controls. ##p<0.01, significant effect of JTE-907 pretreatment on the CP55940-induced upregulation of β-Arrestin 2 mRNA levels (D) JTE-907 prevents GP1a-induced increases in β-Arrestin 2 mRNA levels. **p<0.01, significant effect of GP1a treatment on β-Arrestin 2 mRNA levels compared to vehicle treated controls. ##p<0.01, significant effect of JTE-907 pretreatment on GP1a-induced upregulation of β-Arrestin 2 mRNA levels. Representative Western blots are shown in this figure and IOD was calculated as described in Experimental Procedures. The data represent mean ± SEM (n=3).

CB_2, but not CB_1, shRNA lentiviral particle treatment prevents cannabinoid-induced increases in β-Arrestin 2 mRNA level

We also used cells stably transfected with control, CB_2, or CB_1 shRNA lentiviral particles to study whether the effect of cannabinoid agonists on β-Arrestin 2 mRNA levels. CB_1 and CB_2 mRNA levels were significantly reduced by approximately 75-80% in neuronal cells treated with either CB_1 or CB_2 shRNA lentiviral particles, respectively (Fig. 18A). We have previously reported similar reductions (75-80%) in protein levels of these receptors after transfection with these shRNA lentiviral particles [262].

Control or CB_2 shRNA lentiviral transfected cells were treated with either vehicle, CP55940 (1nM), JWH-133 (30nM), or GP1a (1nM). We found that treatment with CP55940, JWH-133, or GP1a significantly ($p<0.01$) increased β-Arrestin 2 mRNA levels in control shRNA treated cells compared to vehicle-treated controls (76 ± 3%, 65 ± 2%, and 72 ± 5% increase, respectively)(Fig 18B). Noteworthy, CB_2 shRNA treatment prevented ($p<0.01$) the CP55940, JWH-133, and GP1a induced increases in β-Arrestin 2 mRNA levels. CB_2 shRNA lentivirus treatment did not significantly ($p>0.01$) alter basal β-Arrestin 2 mRNA levels. The two-way ANOVA for β-Arrestin 2 mRNA showed significant main effects of transfection ($F_{(3,23)}=97.86$,$p<0.001$) and cannabinoid agonist treatment ($F_{(1,23)}=33.23$,$p<0.0001$). There was a significant interaction between transfection and cannabinoid agonist treatment ($F_{(3,23)}=23.12$, $p<0.0001$).

We also determined what effect CP55940, JWH-133 and GP1a treatment had on β-Arrestin 2 upregulation in control or CB_1 shRNA lentivirus transfected cells. In control shRNA treated cells, CP55940, JWH-133, and GP1a treatment significantly ($p<0.01$) increased β-Arrestin 2 mRNA levels compared to vehicle treated controls (68 ± 3%, 74 ± 5%, and 71 ± 5% increase, respectively) (Fig. 18C). Noteworthy, treatment with CB_1 shRNA lentiviral particles

did not prevent or significantly modify the CP55940, JWH-133, or GP1a-induced increases in β-Arrestin 2 mRNA levels compared to control shRNA treated cells (Fig. 18C). The two-way ANOVA for β-Arrestin 2 mRNA showed significant main effects of transfection ($F_{(1,23)}$ =7.05 , p<0.0173) and cannabinoid agonist treatment ($F_{(3,23)}$ =180.67 , p<0.0001). There was no significant interaction between transfection and cannabinoid agonist treatment ($F_{(3,23)}$ =0.11, p>0.9526). This evidence indicates that CB_2, but not CB_1, receptors, would mediate the cannabinoid-induced upregulation of β-Arrestin 2.

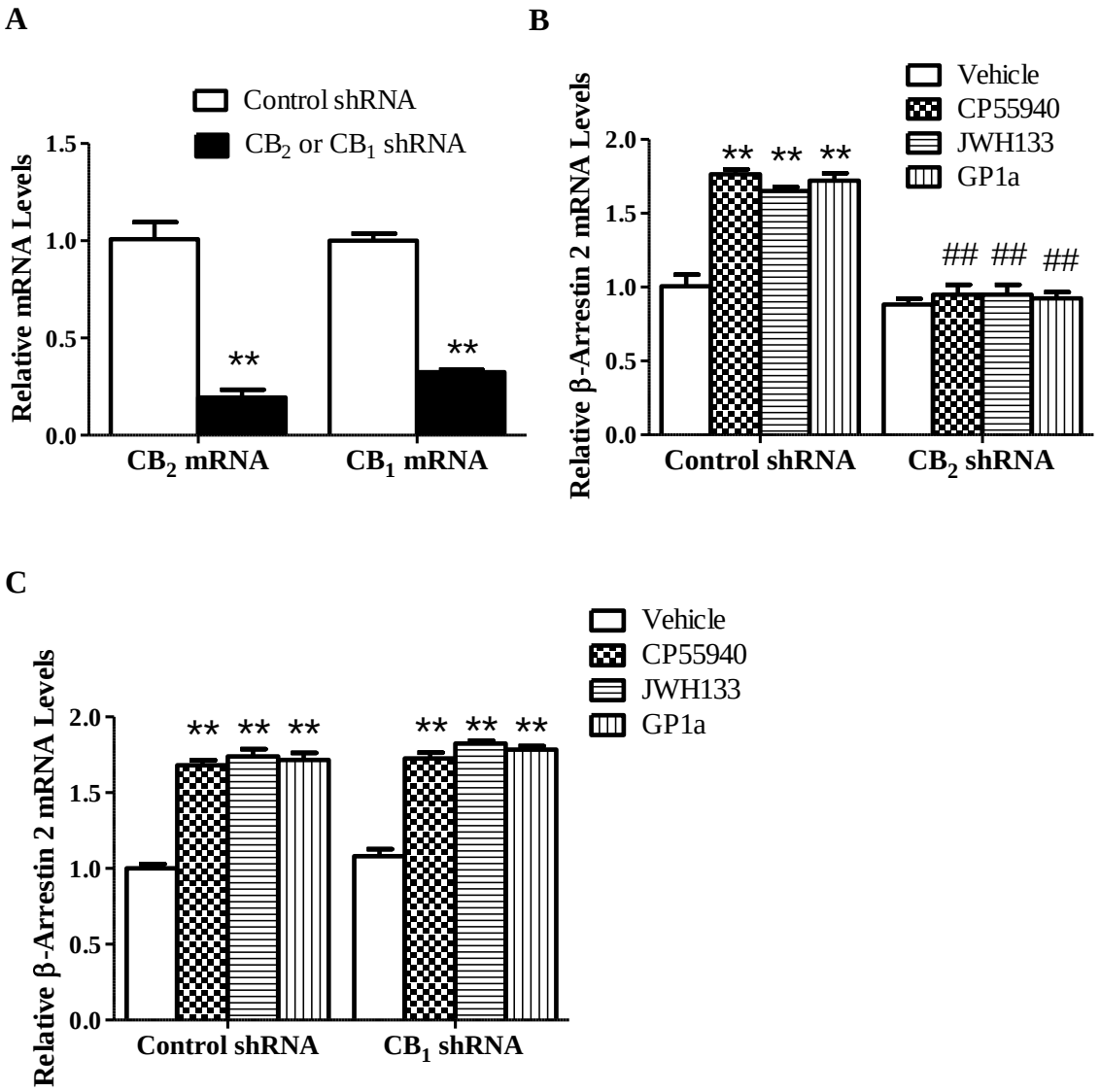


Figure 18. CB_2 receptors are necessary for CP55940, JWH133, and GP1a-induced upregulation of β-Arrestin 2 in CLU213 cells.

(A) Reduced CB_2 or CB_1 mRNA levels in cells treated with CB_2 or CB_1 shRNA lentiviral particles, respectively. **$p<0.01$ significant effect of CB_2 or CB_1 shRNA compared to control shRNA treated cells. (B) CB_2 shRNA lentivirus transfection prevents CP55940, JWH-133 and GP1a-induced increases in β-Arrestin 2 mRNA. **$p<0.01$, significant effect of CP55940, JWH-

133, and GP1a treatment on β-Arrestin 2 mRNA levels in control shRNA lentivirus transfected cells compared to vehicle-treated controls. ##p<0.01, significant effect of CB_2 shRNA lentivirus transfection on the CP55940, JWH-133, and GP1a-induced upregulation of β-Arrestin 2. (C) CB_1 shRNA lentivirus transfection does not prevent CP55940, JWH-133 or GP1a-induced increases β-Arrestin 2. **p<0.01, significant effect of CP55940, JWH-133 or GP1a treatment on β-Arrestin 2 mRNA levels in control or CB_1 shRNA lentvirus treated cells compared to vehicle-treated controls. The data represent mean ± SEM (n=3).

Effect of cannabinoid receptor internalization on the CB_2-mediated upregulation of β-Arrestin 2 mRNA

Next, we investigated whether CB_2 receptor internalization may be involved in the cannabinoid-induced upregulation of β-Arrestin 2. Internalization of membrane-associated receptors could be a very important step in the signaling of CB_2 receptors [293]. Specifically, CB_2-induced ERK1/2 activation may require the internalization of membrane-associated CB_2 receptors [293]. Here, we used two different approaches to study the role of internalization of CB_2 receptors on β-Arrestin 2 upregulation: (1) ConA treatment to prevent CB_2 receptor internalization [262]; and (2) different cannabinoid agonists that are either good or poor internalizers of CB_2 receptors [293].

Currently, there are several protocols that are commonly used to prevent clathrin-mediated internalization of GPCR which include: ConA, hypertonic sucrose, or depletion of intracellular potassium [297]. Here we pretreated cells with vehicle or ConA and then treated cells with either vehicle, CP55940 (1nM) or GP1a (1nM) for 24h. Our previous studies suggested that this ConA pretreatment decreased the cannabinoid-induced translocation of CB_2 receptors from the membrane to the cytosol and prevented the cannabinoid-induced ERK1/2 activation [262]. CP55940 and GP1a significantly ($p<0.01$) increased β-Arrestin 2 mRNA levels compared to vehicle treated cells (76 ± 3%, and 77 ± 3% increase, respectively) (Fig. 19A). ConA pretreatment significantly ($p<0.01$) blocked the effect of CP55940 and GP1a on β-Arrestin 2 mRNA levels compared to controls (Fig. 19A). The two-way ANOVA showed a main effect of ConA ($F_{(1,17)} =107.73$, $p<0.0001$), cannabinoid agonists ($F_{(2,17)} =35.88$, $p<0.0001$), and a main interaction between these two factors ($F_{(2,17)} =32.07$, $p<0.0001$) on β-Arrestin 2 mRNA levels.

Interestingly, a recent study shows that some cannabinoid agonists are more efficacious CB_2 receptor internalizers than other cannabinoid agonists [293]. Indeed, CP55940 and GP1a would be efficacious CB_2 receptor internalizers while aminoalkyindoles, such as JWH-073, were classified as poor CB_2 receptor internalizers [293]. We then tested the effect of either vehicle, JWH-073, GP1a, or CP55940 on β-Arrestin 2 mRNA levels in neuronal cells. We found that either GP1a or CP55940 treatment produced significant ($p<0.01$) increases in the β-Arrestin 2 mRNA levels while JWH-073 treatment did not significantly alter β-Arrestin 2 mRNA levels (Fig 19B). These results obtained seem to indicate that CB_2 internalization of CB_2 receptors is a critical step in the upregulation of β-Arrestin 2 mRNA.

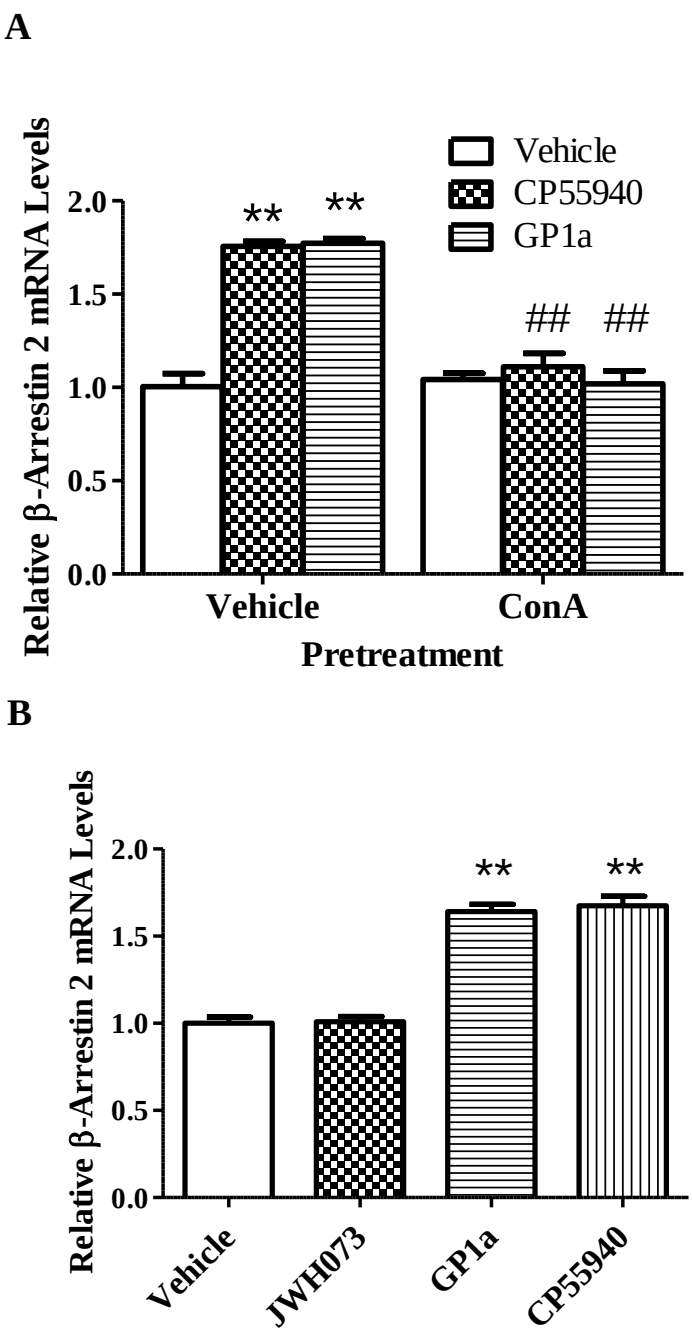


Figure 19. Selective CB_2 receptor agonists upregulate β-Arrestin 2 and selective CB_2 receptor antagonist prevents cannabinoid-induced upregulation of 5-HT_{2A} receptors.

(A) Increased β-Arrestin 2 mRNA levels in cells treated with either CP55940 or GP1a. **p<0.01 significant effect of CP55940 and GP1a treatment compared to vehicle-treated controls. (B) ConA pretreatment prevents CP55940 and GP1a-induced increases β-Arrestin 2 mRNA.

**p<0.01, significant effect of CP55940 and GP1a treatment on β-Arrestin 2 mRNA levels compared to vehicle-treated controls. ##p<0.01, significant effect of ConA pretreatment on the CP55940 and GP1a-induced upregulation of β-Arrestin 2. The data represent mean ± SEM (n=3).

Agonists of CB_2 receptors would mediate the upregulation of β-Arrestin 2 via ERK1/2 in CLU213 cells

We also aimed to investigate the mechanism by which CB_2 receptor induces the upregulation of β-Arrestin 2. CB_2 receptors are positively coupled to the ERK1/2 signaling pathway [88]. We used PD198306, a selective ERK1/2 inhibitor [288], to study the effect of GP1a-induced ERK1/2 activation on β-Arrestin 2 upregulation. GP1a exhibits higher selectivity for CB_2 receptors than JWH-133; therefore we utilized GP1a in these experiments (approximate 9,000- and 200-fold selectivity between CB_2/CB_1 receptors for GP1a and JWH-133, respectively) [59;60]. Here, cells were pretreated with either vehicle or PD198306 (200nM) for 20 min and then treated with either vehicle or GP1a (1nM) for 24h. GP1a treatment significantly ($p<0.01$) increased β-Arrestin 2 mRNA levels compared to vehicle treated controls (104 ± 10% increase, Fig. 20A). This GP1a-induced upregulation of β-Arrestin 2 was prevented ($p<0.01$) in cells pretreated with PD198306 (Fig. 20A). The two-way ANOVA for β-Arrestin 2 mRNA showed significant main effects of PD198306 pretreatment ($F_{(1,11)} =30.21$, $p<0.0006$) and GP1a treatment ($F_{(1,11)} =48.81$, $p<0.0001$). There was a significant interaction between PD198306 and GP1a treatment ($F_{(1,11)} =41.61$, $p<0.0002$).

We then used cells stably transfected with either β-Arrestin 2 or control shRNA lentiviral particles to study the contribution of β-Arrestin 2 to the cannabinoid-induced increases in nuclear-associated pERK. ERK1/2 is activated through phosphorylation and once phosphorylated this protein can translocate from the cytoplasm to the nucleus [232]. Cells transfected with either control or β-Arrestin 2 shRNA lentiviral particles were treated with vehicle or CP55940 (1nM) for 15 min. We found that treatment with CP55940 significantly ($p<0.01$) increased nuclear-associated pERK levels in control shRNA treated cells compared to

vehicle treated controls (Fig. 20B). The CP55940 induced increases in nuclear-associated pERK levels were significantly ($p<0.01$) reduced in cells stably transfected with β-Arrestin 2 shRNA lentiviral particles (Fig. 20B). Additionally, CP55940 treatment significantly ($p<0.01$) increased nuclear-associated pERK levels in β-Arrestin 2 shRNA lentiviral treated cells compared to vehicle treated controls. The two-way ANOVA for nuclear-associated pERK showed significant main effects of transfection ($F_{(1,12)} =39.41$, $p<0.0001$) and CP55940 treatment ($F_{(1,12)} = 40.79$, $p<0.0001$). There was a significant interaction between transfection and CP55940 treatment ($F_{(1,12)} =175.73$, $p<0.0001$).

Cells transfected with either control or β-Arrestin 2 shRNA lentiviral particles were also treated with either vehicle or GP1a for 15 minutes. We found that treatment with GP1a significantly ($p<0.01$) increased nuclear-associated pERK levels in control shRNA treated cells compared to vehicle treated controls (Fig. 20C). β-Arrestin 2 shRNA lentivirus transfection significantly ($p<0.01$) reduced GP1a-induced increases in pERK levels (Fig. 20C). Treatment with GP1a significantly ($p<0.01$) increased nuclear-associated pERK levels in β-Arrestin 2 shRNA treated cells compared to vehicle treated controls. The two-way ANOVA for nuclear-associated pERK showed significant main effects of transfection ($F_{(1,12)} =18.29$, $p<0.0001$) and GP1a treatment ($F_{(1,12)} = 19.76$, $p<0.0008$). There was a significant interaction between transfection and GP1a treatment ($F_{(1,12)} =350.49$, $p<0.0001$).

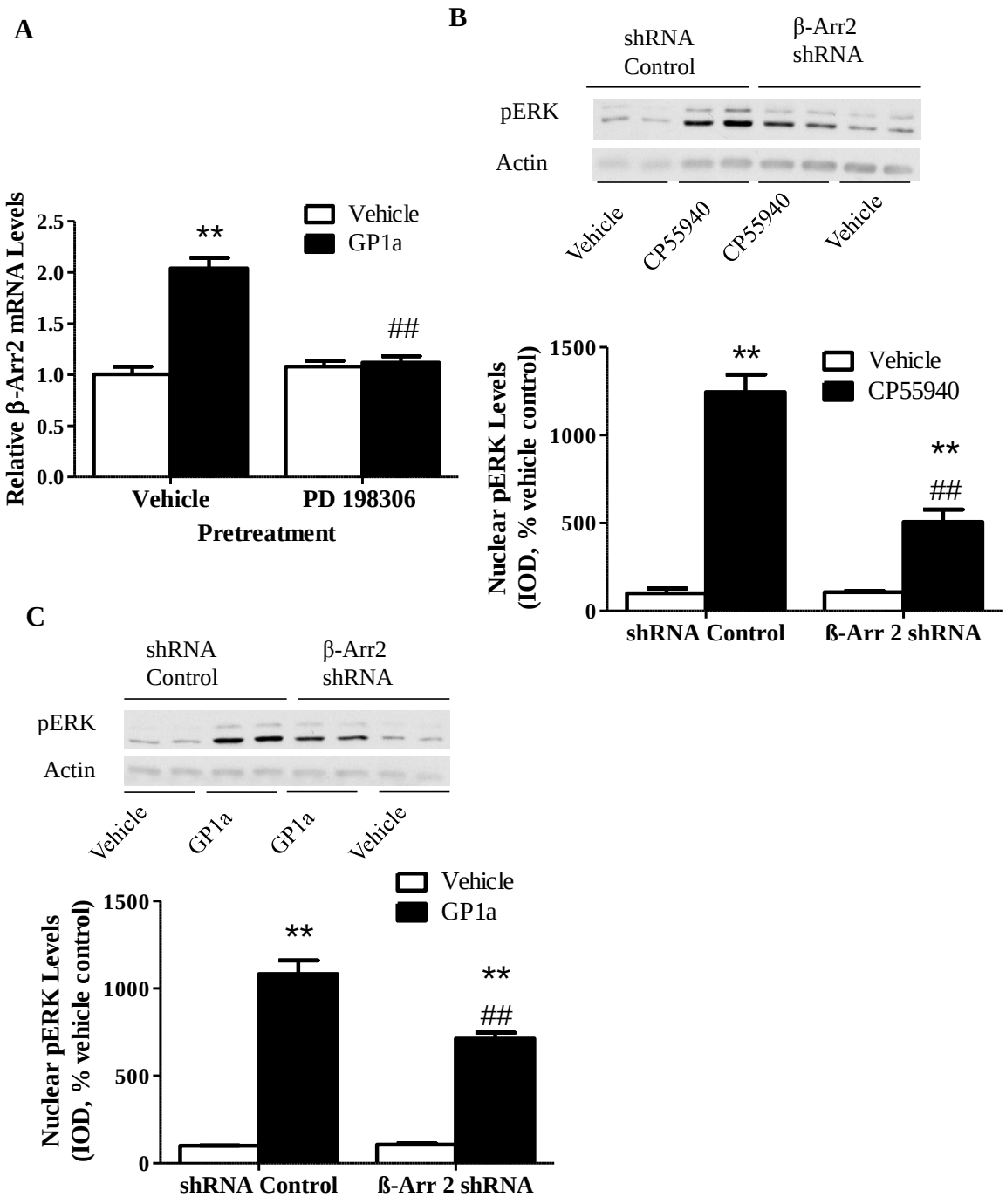


Figure 20. GP1a upregulates β-Arrestin 2 via ERK1/2 signaling in CLU213 cells.

(A) PD198306, potent ERK1/2 inhibitor, pretreatment prevents GP1a-induced increases β-Arrestin 2 mRNA levels. **p<0.01, significant effect of GP1a treatment compared to vehicle-

treated controls. ##p<0.01, significant effect of PD198306 pretreatment on GP1a-induced increases β-Arrestin 2 mRNA levels compared to vehicle-treated controls (B) β-Arrestin 2 shRNA lentivirus treatment significantly reduced CP55940-induced increased in nuclear-associated pERK levels. **p<0.01, significant effect of CP55940 treatment on nuclear-associated pERK levels in control or β-Arrestin 2 shRNA treated cells compared to vehicle-treated. ##p<0.01, significant effect of β-Arrestin 2 shRNA treatment in CP55940 treated cells compared to control shRNA transfect/CP55940 treated cells. (C) β-Arrestin shRNA lentivirus treatment significantly reduced GP1a-induced increases in nuclear-associated pERK levels. **p<0.01, significant effect of GP1a treatment on nuclear-associated pERK levels in control or β-Arrestin 2 shRNA treated cells compared to vehicle-treated controls. ##p<0.01, significant effect of β-Arrestin 2 shRNA treatment in CP55940 treated cells compared to control shRNA transfected/CP55940 treated cells. The data represent mean ± SEM (n=3).

Transcription factor AP-1, but not CREB, would be involved in GP1a induced upregulation of β-Arrestin 2 in CLU213 cells

Here we wanted to identify possible transcription factor(s) that would contribute to GP1a-induced increases in β-Arrestin 2 mRNA. The results presented above seem to indicate that ERK1/2 activation is a mechanism involved in the CB_2 upregulation of β-Arrestin 2 mRNA. In the nucleus, phosphorylated ERK (pERK) can activate several transcription factors including CREB, c-Fos, SP-1, and EGR-1 [232]. The transcription factors CREB and AP-1 have consensus sequences within the promoter region of the rat β-Arrestin 2 gene [307]. Therefore, we decided to test the effects of inhibitors of these transcription factors on the GP1a-induced upregulation of β-Arrestin 2 mRNA.

CREB is a transcription factor which binds the cAMP response element (CRE) to regulate the transcription of genes [237]. c-Fos belongs to the immediate early gene family of transcription factors and this family can dimerize with c-Jun to form the AP-1 transcription factors to upregulate transcription of various genes [239]. In our first experiment, we studied the effect of CREB inhibitor pretreatment on the GP1a-induced upregulation of β-Arrestin 2. Here, CLU213 cells were treated with either vehicle or Naphthol AS-E phosphate (10μM) for 20 min and then treated with vehicle or GP1a (1nM). Naphthol AS-E phosphate blocks cAMP-induction of CREB-dependent gene transcription (K_i=10μM) [224]. We found that Naphthol AS-E phosphate did not inhibit or significantly decrease GP1a-induced increases in β-Arrestin 2 mRNA (Fig. 21A). No significant ($p>0.05$) effect of Naphthol AS-E phosphate was found on basal β-Arrestin 2 mRNA levels either. The two-way ANOVA for β-Arrestin 2 mRNA showed no significant main effect of Naphthol AS-E phosphate pretreatment ($F_{(1,11)}=0.04$, $p>0.0557$) and a significant main effect of GP1a treatment ($F_{(1,11)}=116.6$, $p<0.0001$). There was no significant

interaction between Naphthol AS-E pretreatment and GP1a treatment ($F_{(1,11)}$ =0.68, p>0.683) on β-Arrestin 2 mRNA levels.

We also studied the effect of AP-1 inhibition on GP1a-induced increases in β-Arrestin 2 mRNA. Here neuronal cells were treated with either vehicle or SR11302 (1μM) for 20 min then vehicle or GP1a (1nM) was added to the incubation media. SR11302 is a retinoid which transrepresses AP-1 without transactivating the retinoic acid response element (E_{max}= 1 μM) [225]. As expected, GP1a induced a significant (p<0.01) increase in β-Arrestin 2 mRNA levels (Fig. 21B). SR11302 pretreatment significantly reduced (approximately 49% decrease, p<0.01) the GP1a-induced upregulation of the β-Arrestin 2 mRNA (Fig. 21B). No significant (p>0.05) effect of SR11302 was found in basal β-Arrestin 2 mRNA levels. The two-way ANOVA for β-Arrestin 2 mRNA did not show a significant main effect of SR11302 pretreatment ($F_{(1,11)}$ =4.46, p>0.0676) but did show a significant effect of GP1a treatment ($F_{(1,11)}$ p<0.0005). There was a significant interaction between SR11302 pretreatment and GP1a treatment ($F_{(1,11)}$ =8.71, p<0.0184). These results suggest that AP-1, but not CREB, would mediate, at least in part, the GP1a-induced upregulation of β-Arrestin 2.

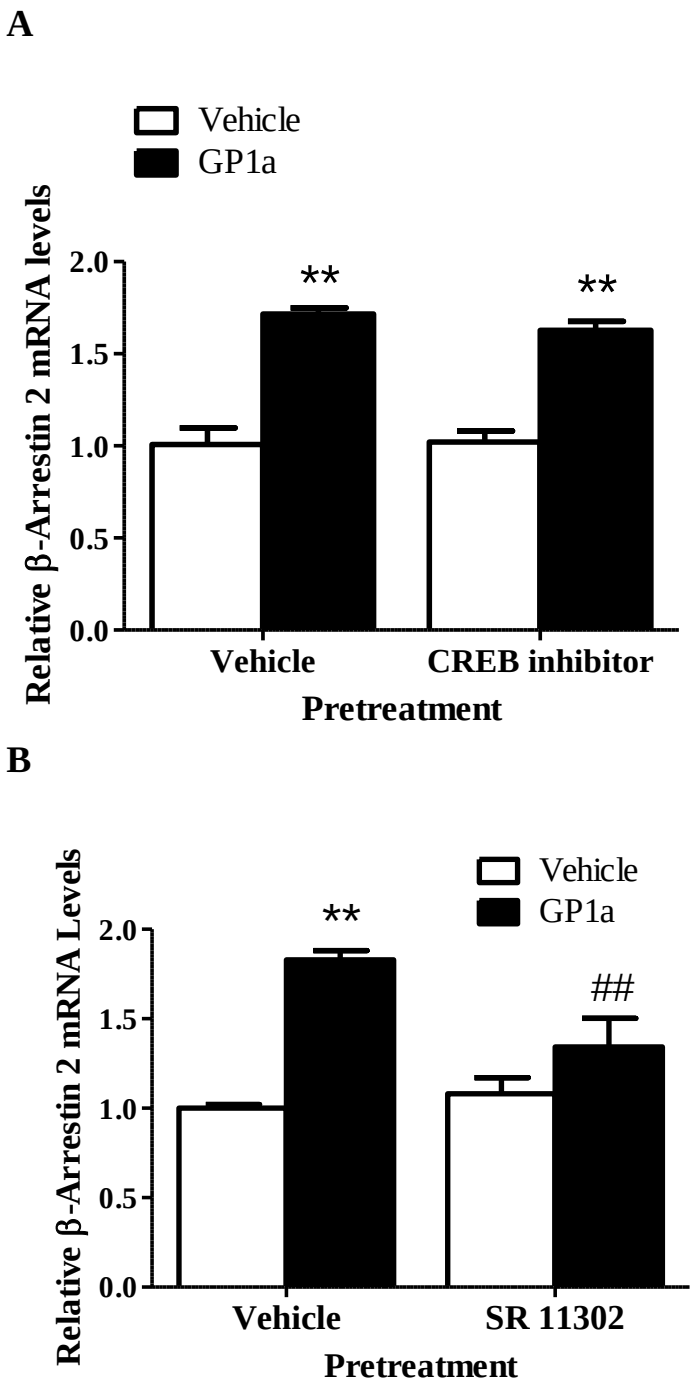


Figure 21. CB_2 receptor-induced upregulation of β-Arrestin 2 involves AP-1, but not CREB, activation.

(A) Inhibition of CREB activation did not prevent or significantly reduce GP1a-induced increases in β-Arrestin 2 mRNA. **p<0.01, significant effect of GP1a and CREB/GP1a

treatment on β-Arrestin 2 mRNA levels compared to vehicle-treated controls. (B) CB_2 receptor-mediated upregulation of β-Arrestin 2 involves AP-1 transcription factor activity. **$p<0.01$, significant effect of GP1a treatment compared to vehicle-treated controls. ##$p<0.01$, significant effect of AP-1 transcription factor inhibitor pretreatment on GP1a-induced increases in β-Arrestin 2 mRNA. The data represent mean ± SEM (n=3).

6.5 Discussion

β-Arrestin 1 and 2 were initially found to hinder G protein coupling of agonist-activated GPCRs resulting in GPCR desensitization; however, recent evidence shows that β-Arrestins can also function to activate signaling cascades independent of G protein activation and can function in receptor internalization [171;257;300]. The classical paradigm of agonist-induced GPCR mediated signal transduction involves agonist-induced dissociation of the G proteins from the GPCR and subsequent G protein regulation of secondary messengers [96;97]. It was found that GRKs could terminate this agonist-induced signaling response through phosphorylation of the receptor [171]. GRK-mediated phosphorylation of the receptor triggers the binding of β-Arrestins to the receptor preventing further G-protein mediated activation of the secondary messengers [171]. However, recent evidence has identified that β-Arrestins can recruit proteins such as ERK1/2 to the GPCR to form scaffolding complexes that can regulate the activation of signaling cascades [96;97].

Interestingly, previous reports showed that chronic exposure to THC (tetrahydrocannabinol, CB_1/CB_2 receptor agonist) upregulates β-Arrestin 1 in striatum and β-Arrestin 2 in cerebellum and hippocampus [308] but did not upregulate total ERK1/2 levels in hippocampus [309]. Currently, the consequences of this upregulation of β-Arrestins on scaffolding mediated regulation of signaling cascades are unknown. Other research has identified that β-Arrestins can bind proteins involved in receptor internalization and can bring activated receptors along with scaffolding proteins to clathrin-coated pits for endocytosis [96;97]. After internalization of the GPCR, evidence indicates that the scaffolding complex can continue to regulate signaling cascades [257].

Our results indicate that exposure to CP55940, a CB_1/CB_2 receptor agonist, increases the interaction between β-Arrestin 2 and ERK1/2 in rat PFCx (Fig. 16A). Co-immunoprecipitation has been successfully used by some groups to demonstrate an interaction between β-Arrestin 2 and its scaffolding proteins [310-312]. Interestingly, this enhanced β-Arrestin 2 and ERK1/2 interaction in the PFCx of CP55940 treated rats was associated with enhanced CP55940-induced ERK1/2 activation (Fig.16B). In previous work we have found cannabinoid-induced ERK1/2 activation is needed for the upregulation of 5-HT_{2A} receptors hence cannabinoid regulation of the β-Arrestin and ERK1/2 interaction could play an essential role in the upregulation of 5-HT_{2A} receptors through regulation of ERK1/2 signaling. Changes in β-Arrestin 2 and/or ERK1/2 expression could contribute to this cannabinoid agonist-induced enhanced β-Arrestin 2 and ERK1/2 interaction. Interestingly, we found that CP55940 treatment increased β-Arrestin 2 protein levels while ERK1/2 protein levels were not significantly altered in the PFCx of rats (Fig. 16C and 16D). Therefore changes in β-Arrestin 2 protein expression but not ERK1/2 protein expression may be contributing to this enhanced β-Arrestin 2 and ERK1/2 interaction in the PFCx. Moreover, we found increased β-Arrestin 2 mRNA in PFCx of CP55940 treated rats compared to controls (Fig.16E) suggesting that increases in β-Arrestin 2 expression most likely occur through cannabinoid-mediated enhanced transcription of the β-Arrestin 2 gene. Finally, treatment with β-Arrestin 2 lentiviral particles prevented the cannabinoid-induced upregulation of 5-HT_{2A} receptors (Fig.16F). This evidence indicates that β-Arrestin 2 is necessary for the cannabinoid-induced upregulation of 5-HT_{2A} receptors and suggests that regulation of 5-HT_{2A} receptor expression may be mediated by changes in β-Arrestin 2 expression and formation of the scaffolding complex.

We used a neuronal cell line, selective CB_1 and CB_2 receptor agonists or antagonists, and CB_1 and CB_2 shRNA lentiviral particles to determine the contribution of CB_1 and CB_2 receptors to the regulation of β-Arrestin 2 mRNA levels. In our neuronal cell line, which expresses CB_1 and CB_2 receptors, we found that treatment with the non-selective CB_1/CB_2 agonist CP55940 and treatment with the selective CB_2 receptor agonists, JWH-133 and GP1a, increased β-Arrestin 2 mRNA and protein levels (Fig. 16A and 16B). This cannabinoid–induced upregulation of β-Arrestin 2 was not mimicked by a selective CB_1 agonist (ACEA) (Fig. 17A) suggesting that CB_2 receptors may be mediating this response. Additionally, we found that a selective CB_2 receptor antagonist JTE-907, but not a selective CB_1 receptor antagonist PF-514273, prevented the CP55940-induced increases in β-Arrestin 2 mRNA levels (Fig. 17C). Moreover, treatment with CB_2 shRNA lentiviral particles, but not CB_1 shRNA lentiviral particles, prevented the CP55940, JWH-133, and GP1a-induced increases β-Arrestin 2 mRNA levels (Fig. 18B and 18C). This evidence indicates that CB_2 receptors are required for the cannabinoid-induced upregulation of β-Arrestin 2.

Recent evidence indicates that CB_2 receptor ligands can distinctly regulate the signal transduction mechanisms associated with the CB_2 receptors, a phenomenon known as functional selectivity [257;286;293]. Atwood et al. have identified different classes of cannabinoid receptor agonists which differ in their ability to induce internalization of CB_2 receptors [293]. They generalize that bicyclic cannabinoids such as CP55940 would be efficacious CB_2 receptor internalizers while aminoalkylindoles would be poor CB_2 receptor internalizers. We found that CP55940, but not JWH-073, induced β-Arrestin 2 upregulation (Fig. 19B), suggesting that upregulation of β-Arrestin 2 is dependent on the ability of the agonists to promote internalization of CB_2 receptors. To further explore this possibility, we pretreated cells with ConA, which is

commonly used to prevent clathrin mediated endocytosis of GPCRs [297;298] and then treated cells with CP55940 or GP1a. Interestingly, ConA pretreatment prevented the CP55940- and GP1a-induced increases in β-Arrestin 2 mRNA levels in cultured cells (Fig. 19A). This evidence further suggests that internalization of the CB_2 receptor is needed for the cannabinoid-induced upregulation of β-Arrestin 2.

We tested the effect of PD198306, a potent ERK1/2 inhibitor, on the CB_2-induced upregulation of β-Arrestin 2 [15;288]. Previous studies have identified that 200 nM of PD198306 can prevent ERK1/2 activation in breast cancer cells [288]. We found that the PD198306 pretreatment prevented the selective CB_2 receptor agonist induced increases in β-Arrestin 2 mRNA levels (Fig. 20A). This evidence suggests that CB_2 receptors can induce the upregulation of β-Arrestin 2 through a mechanism that involves ERK activation. Currently, the mechanism by which CB_2 receptors mediate the activation of ERK1/2 has not been well defined. It is known that β-Arrestins can form scaffolding complexes with ERK1/2, which can mediate its activation [257]. We found that β-Arrestin 2 shRNA lentiviral particle treatment significantly reduced the CP55940 and GP1a-induced increases in nuclear-associated pERK compared to vehicle treated controls (Fig. 20B and 20C). Yet treatment with CP55940 and GP1a significantly increased nuclear pERK levels in β-Arrestin 2 shRNA lentivirus treated cells compared to vehicle treated controls. This evidence suggests that CB_2 receptors can mediate ERK1/2 activation, at least in part, through β-Arrestin 2. Here the CP55940 and GP1a induced increase in pERK levels in β-Arrestin 2 shRNA lentivirus treated cells could be attributed to: (1) residual β-Arrestin 2 left over after β-Arrestin 2 shRNA lentivirus treatment (approximately 15%) and/or (2) β-Arrestin 1 mediated activation of ERK1/2 signaling. It has been found that β-Arrestins can functionally

substitute for the other isoform to some degree [97]. However, internalization of GPCRs is typically mediated primarily by one isoform of β-Arrestin [97].

Our results suggest that inhibition of AP-1, but not CREB, significantly decreased the GP1a-induced upregulation of 5-HT_{2A} receptors (Fig. 21A and 21B). The partial inhibition of the GP1a-induced increases in β-Arrestin 2 mRNA levels by SR11302 suggest that other transcription factors yet to be identified could also contribute to this upregulation.

Chronic cannabinoid receptor agonist exposure has been associated with several neuropsychiatric disorders such as schizophrenia, anxiety, and depression [11-14]. Interestingly, dysregulation of 5-HT_{2A} and D_2 receptor signaling has been associated with schizophrenia, stress response, anxiety, and depression [143;144;179;313]. While a causal link between chronic cannabinoid agonist exposure and these neuropsychiatric disorders has not been found, it has been suggested that long-term cannabinoid agonist exposure may precipitate these neuropsychiatric disorders [11-14]. Interestingly, repeated exposure to a cannabinoid agonist, CP55940, leads to increased anxiety and long-term memory impairments, that irrespective of the age at which drug exposure occurs [108]. More importantly, recent evidence indicates that repeated exposure to a selective CB_2 receptor agonist, JWH-133, induces anxiety-like behaviors in rodents that are blocked by a selective CB_2 receptor antagonist [93]. This evidence highlights the need to identify the mechanisms by which sustained cannabinoid exposure induces and/or contributes to neuropsychiatric disorders. Noteworthy, in our previous reports we found that CB_2 receptor–mediated upregulation of 5-HT_{2A} receptors [262] could contribute to the cannabinoid-induced enhanced interaction between 5-HT_{2A} and D_2 receptors in PFCx [263]. Here, we aimed to identify the molecular mechanisms contributing to this upregulation of 5-HT_{2A} receptors and specifically we focused on the role of β-Arrestin 2 in this phenomenon. In summary, our results

suggest that sustained activation of CB_2 receptors would enhance β-Arrestin 2 expression possibly contributing to its increased interaction with ERK1/2 and thereby driving the upregulation of 5-HT_{2A} receptors. The CB_2 receptor-mediated upregulation of β-Arrestin 2 would be mediated, at least in part, by an ERK1/2-dependent activation of AP-1.

In conclusion, emerging studies are identifying that selective CB_2 receptor agonists such as JWH-133 have wide therapeutic application in the treatment of conditions such as stroke, neurogenerative diseases, and neuropathic pain [2-5]. However, the mechanism that we are currently defining could represent a potential adverse effect of the long-term use of selective CB_2 receptor agonists such as GP1a and JWH-133. Repeated exposure to these agonists, that are efficacious CB_2 receptor internalizers, could be associated with potential adverse effects as mentioned above. However, our evidence suggest that CB_2 receptor agonists such as JWH-073, that are categorized as poor CB_2 receptor internalizers, could represent an alternative therapeutic approach that may have minimal effect on serotonergic neurotransmission in brain thereby reducing the adverse effects that may be associated with enhanced 5-HT_{2A} receptor function.

Chapter 7: G-Protein Receptor Kinase 5 Regulates the Cannabinoid Receptor 2-Induced Upregulation of Serotonin 2A Receptors

(Franklin J.M. and Carrasco G.A. (2013) G-Protein Receptor Kinase 5 Regulates the Cannabinoid Receptor 2-Induced Upregulation of Serotonin 2A Receptor. *Journal of Biological Chemistry*, **288:** 15712-15724.)

7.1 Abstract

We have recently reported that cannabinoid agonists can upregulate and enhance the activity of 5-HT_{2A} receptors in PFCx. Increased expression and activity of cortical 5-HT_{2A} receptors has been associated with neuropsychiatric disorders such as anxiety and schizophrenia. Here we report that repeated CP55940 exposure selectively upregulates GRK5 proteins in rat PFCx and in a neuronal cell culture model. We sought to examine the mechanism underlying the regulation of GRK5 and to identify the role of GRK5 in the cannabinoid agonist induced upregulation and enhanced activity of 5-HT_{2A} receptors. Interestingly, we found that cannabinoid agonist- induced upregulation of GRK5 involves CB_2 receptors, β-Arrestin 2 and ERK1/2 signaling as treatment with CB_2 shRNA lentiviral particles, β-Arrestin 2 shRNA lentiviral particles, or ERK1/2 inhibitor prevented the cannabinoid agonist induced upregulation of GRK5. Most importantly, we found that GRK5 shRNA lentiviral particle treatment prevented the cannabinoid agonist- induced upregulation and enhanced 5-HT_{2A} receptor mediated calcium release. Repeated cannabinoid exposure was also associated with enhanced phosphorylation of CB_2 receptors and increased interaction between β-Arrestin 2 and ERK1/2. These latter phenomena were also significantly inhibited by GRK5 shRNA lentiviral treatment. Our results suggest that sustained activation of CB_2 receptors, which upregulates 5-HT_{2A} receptor signaling,

enhances GRK5 expression; the phosphorylation of CB_2 receptors; and the β-Arrestin 2/ERK interactions. These data could provide a rationale for some of the adverse effects associated with repeated cannabinoid agonist exposure.

7.2 Introduction

We have recently reported that repeated exposure to cannabinoid agonists induces a strong upregulation and increases the activity of 5-HT_{2A} receptors in rat PFCx and in two neuronal cell models [100;262;263;314]. This cannabinoid-mediated upregulation of 5-HT_{2A} receptors was: (1) induced by nonselective CB_1/CB_2 and selective CB_2 receptor agonists [263;314]; (2) inhibited by selective CB_2, but not CB_1, shRNA lentiviral particles suggesting that CB_2 receptors mediate this phenomenon [262]. Moreover this upregulation of 5-HT_{2A} receptors was β-Arrestin 2- and ERK1/2- dependent as it was inhibited in cells stably transfected with β-Arrestin 2 shRNA lentiviral particles [262] and by ERK1/2 inhibitors [263;314].

The clinical manifestations of this CB_2 receptor-induced upregulation of 5-HT_{2A} receptors are currently under discussion. Noteworthy, recent and independent clinical studies provide evidence indicating that sustained use of nonselective cannabinoid agonists may precipitate the onset of mental disorders associated with dysfunction of 5-HT_{2A} receptor neurotransmission in PFCx, such as anxiety, schizophrenia, and psychosis [11-13;24;137]. Accordingly, recent preclinical studies indicated that chronic, but not acute, exposure to non-selective [108;315] or selective CB_2 receptor agonists induced anxiety-like behaviors in rodents [93].

CB_2 receptors have been identified in postsynaptic neurons in several brain areas of the limbic brain including brain areas such as PFCx, hippocampus, and amygdala [18;21;94;248]. The CB_2 receptor is a prototypical GPCR that couples to $G_{i/o}$ class of G-proteins and can activate $ERK_{1/2}$ signaling in an either G-protein or β-Arrestin dependent pathway [15;88]. The different signaling and trafficking profiles of this receptor would depend on the nature of post-translational modifications such as phosphorylation by GRKs that modify the interaction

between this receptor and associated signaling proteins (such as β-arrestins and G-proteins) [88] and desensitization of this receptor [316].

Here we study the role of GRKs in the cannabinoid-induced upregulation of 5-HT_{2A} receptors. GRKs such as GRK2 exert important roles in the desensitization and inhibition of β-Arrestin 2 (βArr2) signaling of GPCRs [290;317]. Of note, recent results demonstrate that some GRKs, such as GRK5 and/or GRK6, would also regulate βArr2 signaling-mediated ERK1/2 activation [317]. Here we report that agonists of cannabinoid receptors differentially regulate the expression of GRK proteins which would contribute to regulation of 5-HT_{2A} receptors in neuronal cells. We hypothesize that the data presented here could provide, at least in part, a molecular mechanism by which repeated exposure to cannabinoids might be relevant to the pathophysiology of some cognitive and mood disorders by upregulating and enhancing the activity of 5-HT_{2A} receptors.

7.3 Materials and Methods

Drugs

CP55940, GP1a, ACEA, PD198306, MDL 11,939, and SB 242084 were purchased from Tocris (Ellisville, MO). Serotonin creatine sulfate complex was purchased from Sigma-Aldrich Inc. (St. Louis, MO).

Animal Experimental Protocol

Male Sprague-Dawley rats (225-275 g; Harlan Laboratories, Indianapolis, IN) were housed two per cage in a temperature-, humidity-, and light-controlled room (12 hrs light/dark cycle, lights on 7:00 AM-19:00 PM). Food and water were available *ad libitum*. All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals as approved by the University of Kansas Institutional Animal Care and Use Committee (IACUC).

After arrival, the rats were allowed to acclimate to their environment for at least 4 days prior to the start of the treatment period. Eight rats were randomly assigned to each group; cage mates were assigned to the same treatment group. The body weight of each rat was recorded every other day. All solutions were made fresh before administration and rats were injected with either vehicle (Tween-80/ethanol/saline (1:1:18); 1ml/kg, i.p.) or CP55940 (0.05 mg/kg, i.p.) once a day for 7 days. Rats were sacrificed by decapitation 48 hrs after the last CP55940 injection. The brains were immediately removed and the PFCx was dissected and frozen in dry ice.

PhosphoProtein Purification

Phosphorylated proteins were separated by an affinity chromatography procedure using a phosphoprotein purification kit from Qiagen (Valencia, CA) as previously described in detail

[318]. Immunodetection by phospho-specific antibodies has shown that the kit yields a complete separation of non-phosphorylated (flow-through) and phosphorylated proteins (elution fraction) [319]. Briefly, tissue or cells were homogenized in 200 µl of phosphoprotein lysis buffer containing 0.25% (w/v) CHAPS solution, protease inhibitor cocktail, and Benzonase. These homogenates were incubated for 30 minutes at 4°C and then centrifuged for 30 minutes at 10,000 X g and 4 °C. Thermo Scientific Pierce BCA Assay Reagents (Rockford, IL) were utilized to determine the protein concentrations of the supernatants and then 3.5 mg of total protein, adjusted to 0.1 mg/ml with phosphoprotein Lysis buffer containing 0.25% CHAPS, was run through the phosphoprotein purification columns. The non-phosphorylated proteins were washed out of the columns with 35 mL phosphoprotein lysis buffer and the bound phosphorylated proteins were eluted with phosphoprotein elution buffer. Eluted fractions containing phosphorylated proteins were collected. The isolated phosphoprotein fractions were concentrated using Nanostep ultrafiltration columns with a molecular cutoff of 10 kDa. Thermo Scientific Pierce BCA Assay Reagents (Rockford, IL) were utilized to determine the protein concentrations and samples were analyzed by Western blot.

Western Blot

Membrane or cytosolic-associated proteins were isolated using the ProteoExtract™ Native Membrane Protein Extraction kit (Calbiochem, La Jolla, CA). Expression of GRK5, GRK2, GRK6, or CB_2 was determined by Western blot as previously described [262;263]. GRK5, GRK2, GRK6, and CB_2 antibody was purchased from Santa Cruz, CA. Antibodies were used at the following dilutions: GRK5 (1:1,000), GRK2 (1:1,000), GRK6 (1:1,000), and CB_2 (1:1,000). The specificity of these antibodies has been verified in the literature [304;320;321].

Films were analyzed densitometrically as described [262;263]. All samples were standardized to controls and normalized to their respective actin levels.

Quantitative Real-Time PCR

These reactions were prepared using QuantiFast SYBR Green PCR Kit (Qiagen, Valencia, CA), the ABI 7500 fast real time PCR system (Applied Biosystems, Foster City, CA), and then data were analyzed using the comparative cycle threshold (Ct) method as previously described [262;263]. The primers used in this manuscript were: 5-HT_{2A} (F:5'-AACGGTCCATCCACAGAG-3',R:5'-AACAGGAAGAACACGATGC-3'), GRK5 (F:5'-GAACCACCAAAGAAAGGGCTG-3',R:5'-CTAGCTGCTTCCAGTGGAG-3'), GRK2 (F:5'GATGAGGAGACACAAAAGGAATC3',R:5'TCAGAGGCCGTTGGCACTGCCACGCTG-3'),GRK6(F:5'-TTTGGGCTGGATGGGTCTGTTC-3',R:5'CGCTGCAGTTCCCACAGCAATC-3'), and GAPDH (F:5' TGGAGTCTACTGGCGTCTTCAC-3',R:5'-GGCATGGACTGTGGTCATGA-3'). These primers have been validated in the literature [29;322].

Cell Culture Protocol

CLU213 cells, a neuronal cell line that co-expresses 5-HT_{2A}, D_2, CB_1 and CB_2 receptors, were purchased from Cedarlane Laboratories (Burlington, NC). CLU213 cells were grown on 100-mm^2 plates treated by vacuum gas plasma (Corning Incorporated, Corning, NY) and maintained in 5% CO_2 at 37°C, in Dulbecco's modified eagle medium (DMEM; Mediatech Inc, Manassas, VA) containing 10% fetal bovine serum (FBS; Thermo Scientific, Logan, UT).

Effect of Non-Selective CB_1/CB_2 Receptor Agonist on GRK5 and GRK2 expression

CLU213 cells were incubated with either vehicle (ethanol 0.01% final concentration) or CP55940 (CB_1/CB_2 agonist, 1nM) [211] for 72 hrs. Cells were washed (3x) with PBS every 24

hrs and fresh vehicle or CP55940 1nM was added. Expression of GRK5, GRK6, or GRK2 was determined by Western blot or qRT-PCR.

Effect of Selective CB_1 or CB_2 Receptor Agonists on GRK5 mRNA

CLU213 cells were incubated with either vehicle (ethanol 0.01% final concentration), GP1a 1nM (selective CB_2 agonist, K_i: 0.037nM and 353nM for CB_2 and CB_1 receptors, respectively) [60], or ACEA 15nM (selective CB_1 agonist, K_i: 1.4nM and 3.1μM for CB_1 and CB_2 receptors, respectively) [55] for 72 hrs. Cells were washed (3x) with PBS every 24 hrs and fresh vehicle, GP1a, or ACEA was added. qRT-PCR for GRK5 was performed.

Lentivirus and stable transduction of shRNAs in CLU213 cells

GRK5 shRNA (r), β-Arrestin 2 shRNA (r), CB_1 shRNA (r), CB_2 shRNA (r), control shRNA lentiviral particles, polybrene, and puromyocin were purchased from Santa Cruz, CA. Optimal transduction conditions were determined and transfection of cells with lentiviral particles was conducted as previously described [262]. Cells were analyzed for GRK5, β-Arrestin 2, CB_1, or CB_2 knockdown one week after initiation of puromyocin selection.

Effect of β-Arrestin 2, CB_2, or CB_1 shRNA lentivirus transfection on cannabinoid-induced upregulation of GRK5 mRNA.

After confirming that treatment with the β-Arrestin 2, CB_2, or CB_1 shRNA lentivirus significantly reduced the respective protein levels, β-Arrestin 2, CB_2, or CB_1 shRNA treated cells were treated with either vehicle (ethanol 0.01% final concentration) or GP1a 1nM for 72 hrs. Cells were washed (3x) with PBS every 24 hrs and fresh vehicle or GP1a 1nM was added.

Effect of a selective ERK1/2 inhibitor on GP1a-induced increases in GRK5 mRNA

CLU213 cells were pretreated with either vehicle (ethanol 0.01% final concentration) or PD 198306 (200nM) [217;218]. Twenty minutes later cells were incubated with either vehicle

(ethanol 0.01% final concentration) or GP1a (1nM) for 72 hrs. Cells were washed (3x) with PBS every 24 hrs and pretreatment and treatment were repeated.

Effect of GRK5 shRNA lentivirus transfection on cannabinoid-induced upregulation of 5-HT_{2A} receptors

After confirming that treatment with the GRK5 shRNA lentivirus reduced GRK5 mRNA and protein levels approximately 70%, control or GRK5 shRNA treated cells were treated with either vehicle (ethanol 0.01% final concentration), CP55940 1nM, or GP1a 1nM for 72 hrs.

Calcium Assay to measure 5-HT_{2A} receptor activity

Optimal conditions were elucidated using different buffers, plating densities, agonists, time courses, and through reference of previously published protocols [323;324]. Cells were plated at 30,000 cells per well in complete medium and grown to 90% confluence on black-sided 96 well plates. 24 hrs prior to measuring calcium release, medium was changed to serum free medium. After 24 hrs incubation in serum free medium, cells were washed (2x) with Kreb's medium (135 mM NaCl, 5.9 mM KCl, 1.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 11.6 mM Hepes, 11.5 mM D-glucose, pH 7.3) and incubated with 4 μM Fluo 3-AM in 200 μL Kreb's medium for 60 minutes at 37^{O}C in the dark. After loading, cells were washed (2x) with Kreb's medium and incubated in 200 μL of Kreb's medium for 30 minutes to allow for de-esterfication of intracellular AM esters. Finally, cells were stimulated with a single injection of 5-HT and the response was recorded for 1 minute in 6 second intervals. Fluo 3-AM fluorescence using 485 excitation and 528 emission were measured with a BioTek fluorescence plate reader [323;324].

Initial experiments were performed after loading cells with Fluo 3-AM and included serotonin (5-HT) dose response (0.1 nM to 10 μM of 5-HT) experiments with MDL11,939 or SB242084 (5-HT_{2A} and 5-HT_{2C} receptor antagonists, respectively) added to the Kreb's medium

[323;324]. MDL11,939's K_d for either 5-HT_{2A} or 5-HT_{2C} receptors is 0.54 nM and 86 nM, respectively [323;324]. SB242084's K_d for either 5-HT_{2C} or 5-HT_{2A} receptors is 0.48 nM and 144 nM, respectively [323;324]. Cannabinoid experiments were conducted using naïve cells or transfected cells (control-, GRK5-, CB_2-shRNA lentiviral particles). These cells were pretreated with either vehicle, CP55940 1 nM, GP1a 1 nM, or ACEA 15 nM over 72 hrs. Cells were washed (3x) with PBS every 24 hrs and fresh vehicle or cannabinoid agonist were added. After loading cells with Fluo 3-AM, cells were treated with 10 nM SB242084, to inhibit 5-HT_{2C} receptor mediated Ca^{2+} release, for 20 minutes during the 30 minute incubation with Kreb's medium and then stimulated with either 0.1 nM, 1 nM, 0.4 nM, 10 nM, 100 nM, 1 μM, or 10 μM of 5-HT [323;324].

Effect of GRK5 shRNA lentivirus transfection on cannabinoid-induced CB_2 receptor phosphorylation or β-Arrestin 2/ERK interaction.

Cells stably transfected with control or GRK5 shRNA lentivirus particles were treated with vehicle (ethanol 0.01% final concentration) or GP1a 1nM for 72 hrs. Cells were washed (3x) with Kreb's buffer every 24 hrs and fresh vehicle or GP1a was added. Phosphorylated proteins were isolated from cells and Western blot was used to determine the expression of phosphorylated CB_2 receptors as described above. Co-immunoprecipitation of β-Arrestin 2/ERK was examined following the protocol listed below. Expression of phosphorylated CB_2 receptors were determined by Western blot as described above.

Co-immunoprecipitation (co-IP)

These experiments were conducted with the Thermo Scientific Pierce co-IP kit following manufacturer's protocol and as previously described in detail [100]. The β-Arrestin 2 and ERK1/2 antibody was purchased from Santa Cruz, CA. Samples were analyzed by Western blot

using ERK1/2 antibody. The specificity of the β-Arrestin 2 or ERK1/2 antibody has been verified [100].

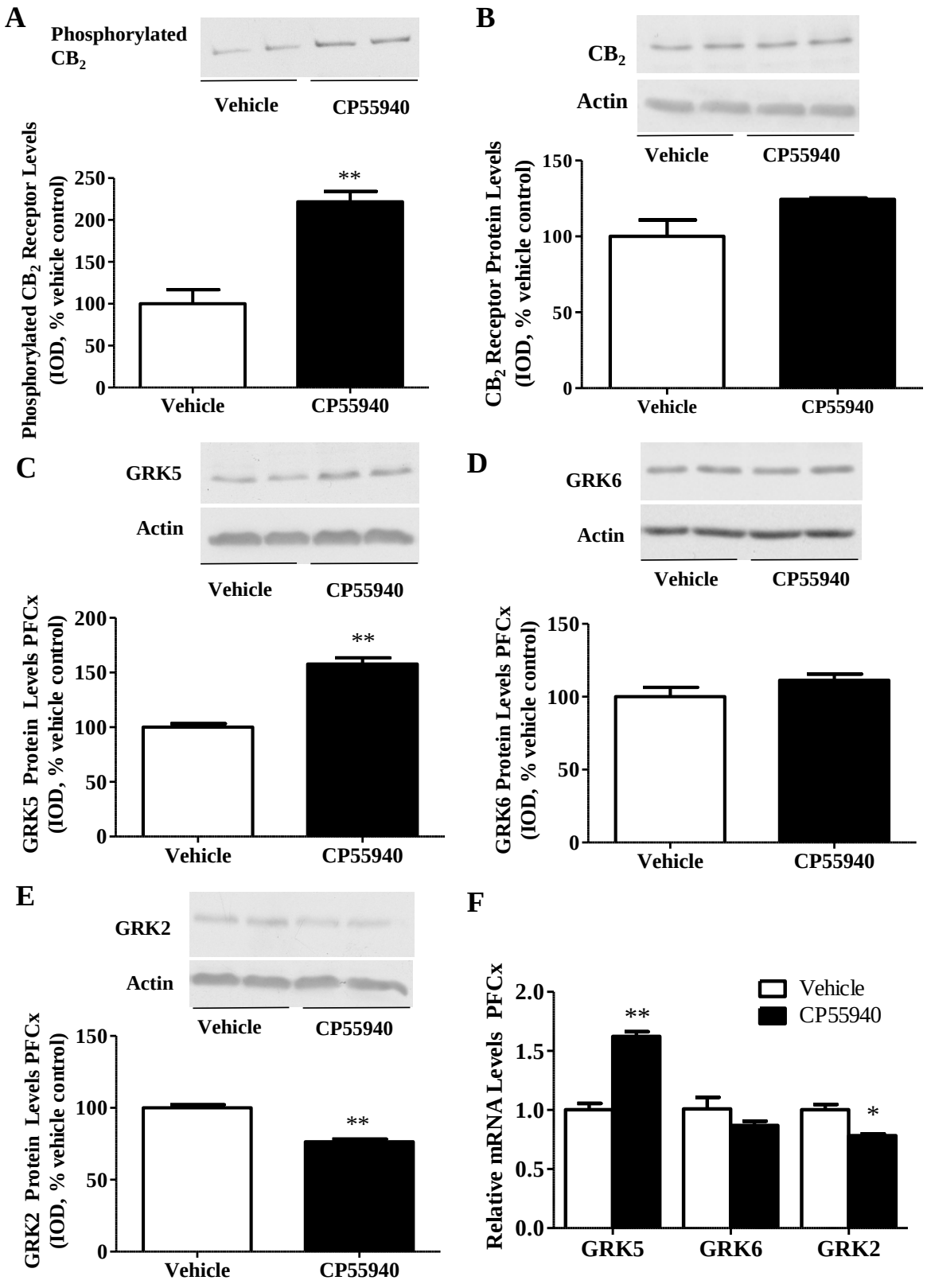
Statistics

All data are expressed as the mean ± S.E.M., where *n* indicates the number of rats or cell culture plates per group. Data was analyzed by an unpaired Student's t-test or ANOVA.

7.4 Results

Chronic exposure to cannabinoid receptor agonists could mediate the cannabinoid-induced upregulation of 5-HT_{2A} receptors, at least in part, through changes in the phosphorylation status of CB_2 receptors by GRK proteins. We initially examined the effect of repeated exposure to CP55940 (CB_1/CB_2 receptor agonist) on the phosphorylation status of the CB_2 receptors in rat PFCx (Fig. 22A). Phosphorylated proteins were separated from the PFCx of vehicle and CP55940 treated rats and Western blot was conducted as previously explained. We found a significant ($p<0.01$) increase in the phosphorylation of CB_2 receptors in CP55940-treated rats compared to vehicle-treated animals (121 ± 12% increase, Fig. 22A). Of note, CP55940 treatment did not significantly ($p>0.05$) modify the total CB_2 receptor protein expression in PFCx homogenate compared to vehicle treated controls (Fig. 22B).

Since CP55940-enhanced expression of selective GRKs could underlie the increased phosphorylation of CB_2 receptor, we studied the effect of CP55940 exposure on the protein levels of GRK5, GRK6 and GRK2 proteins in rat PFCx. Chronic administration of CP55940 produced significant ($p<0.01$) increases in GRK5 protein levels (58 ± 6% increase compared to controls, Fig. 22C). However, we did not detect any significant ($p>0.05$) changes in GRK6 protein levels in PFCx (Fig. 22D). GRK2 protein levels were significantly ($p<0.01$) reduced after repeated CP55940 exposure in rat PFCx (24 ± 2% decrease, Fig. 22E). We also examined what effect CP55940 treatment had on GRK5, GRK6 and GRK2 mRNA levels (Fig. 22F). GRK5 mRNA levels were significantly ($p<0.01$) increased in PFCx of CP55940-treated rats (62 ± 0.4% increase compared to controls). GRK6 mRNA levels did not significantly ($p>0.05$) change, while GRK2 mRNA levels were significantly ($p<0.05$) reduced in PFCx of CP55940 treated rats (22 ± 0.1% reduction compared to vehicle-treated controls, Fig. 22F).


A
Phosphorylated CB2
Vehicle
CP55940
Phosphorylated CB2 Receptor Levels (IOD, % vehicle control)
250
200
150
100
50
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Vehicle
CP55940
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CB2 Receptor Protein Levels (IOD, % vehicle control)
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Vehicle
CP55940
F
Relative mRNA Levels PFCx
2.0
1.5
1.0
0.5
0.0
**
*
Vehicle
CP55940
GRK5
GRK6
GRK2

Figure 22. CP55940-induced enhanced phosphorylation of CB_2 receptors, increased GRK5 expression, and reduced GRK2 expression in rat PFCx.

Rats were injected with CP55940 (0.05 mg/kg, i.p.) once a day for 7 days. After decapitation the brains were collected and PFCx was dissected. (A) Phosphorylated proteins were separated and detected as described under "Experimental Procedures." Thirty μg of isolated phosphorylated protein was used in Western blot detection. (B-E) CB_2 receptor and GRK protein levels were evaluated by Western blot. Proteins (8 μg) were resolved by SDS-PAGE and antibodies for (A-B) CB_2 receptor; (C) GRK5; (D) GRK6; (E) GRK2 were used to detect the proteins of interest. Representative Western blots are shown in this figure and IOD was calculated as described under "Experimental Procedures." β-Actin was used as a loading control. (F) GRK5, GRK6, and GRK2 mRNA levels were evaluated by qRT-PCR as described under "Experimental Procedures." **$p<0.01$ or * $p<0.05$, significant effect of CP55940 treatment compared to vehicle-treated controls. The data represent mean ± SEM (n=6-8).

We then used a neuronal cell line, CLU213 cells, to determine if CP55940 treatment had similar effects on GRK expression to the ones found in rat PFCx and to better examine the mechanisms involved in the cannabinoid-induced upregulation of GRK5 proteins. Here we treated cells with CP55940 for 72 hrs in order to assess the effect of repeated cannabinoid agonist exposure on the expression of GRKs since our previous findings show that repeated, but not single, exposure to cannabinoid agonists upregulate 5-HT_{2A} receptor protein expression [100;262;263;314]. CP55940 treatment in these cells significantly ($p<0.01$) increased GRK5 protein levels (67 ± 3% increase compared to vehicle-treated controls) without significant ($p>0.05$) changes in the protein levels of GRK6 (Fig. 23A and 23B, respectively). On the other hand, GRK2 protein levels were significantly ($p<0.01$) reduced compared to controls (24 ± 2% decrease, Fig 23C). CP55940 treatment also significantly ($p<0.05$) increased GRK5 mRNA levels and significantly ($p<0.05$) reduced GRK2 mRNA levels compared to vehicle-treated cells (69 ± 0.2% increase and 24 ± 0.05% decrease, respectively). There was no significant ($p>0.05$) change in GRK6 mRNA levels in CP55940 treated cells compared to vehicle treated controls (Fig. 23D).

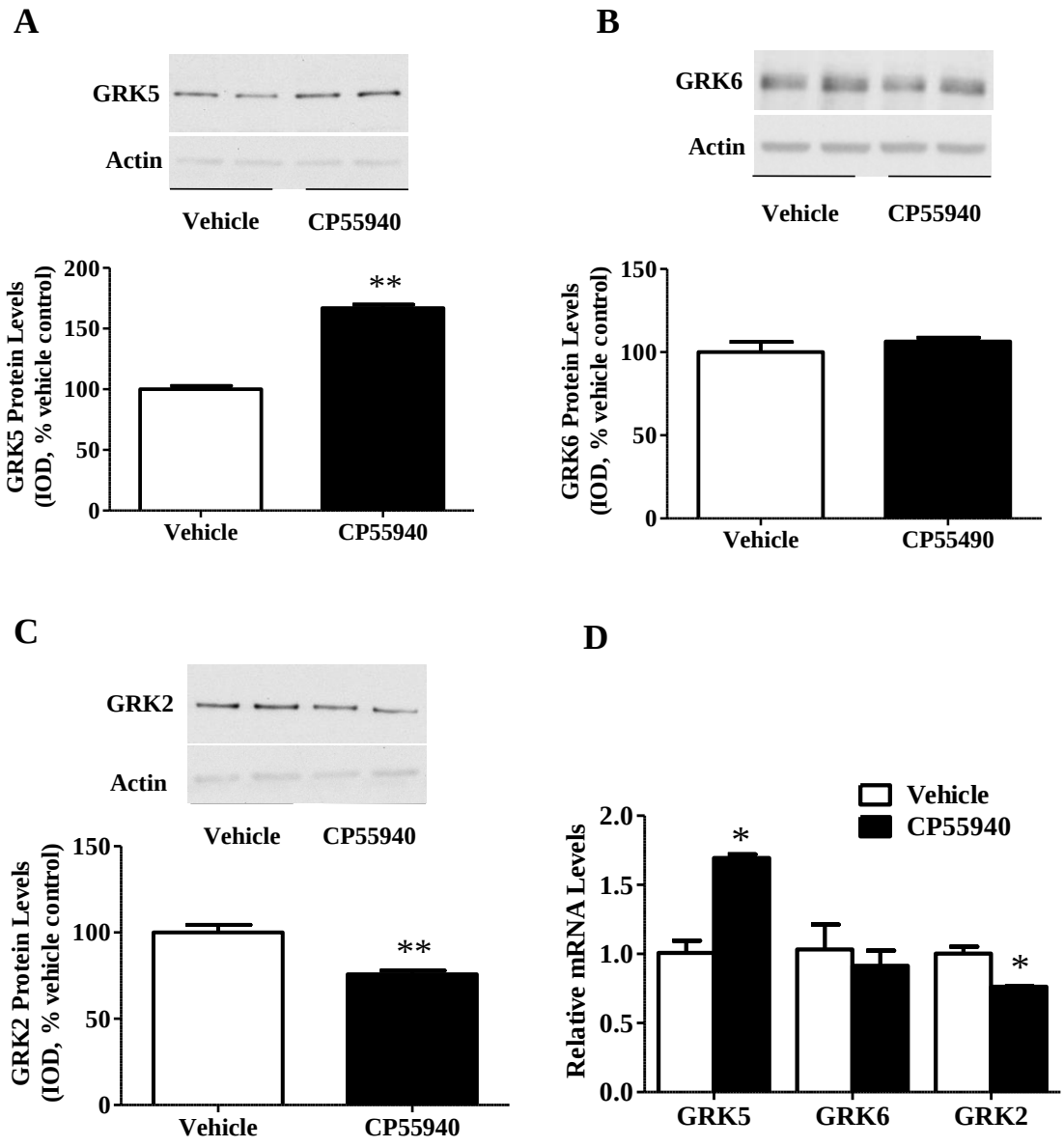


Figure 23. CP55940-induced increased GRK5 and reduce GRK2 expressions levels in CLU213 cells.

Cells were incubated with either vehicle (ethanol 0.01% final concentration) or CP55940 1 nM for 72 hrs. Cells were washed (3x) with PBS every 24 hrs and fresh vehicle or CP55940 1 nM were added. (A-B) Western blots to show alteration or no alteration in (A) GRK5, (B) GRK6, or (C) GRK2 protein levels after repeated CP55940 treatment. Proteins (8 µg) were resolved by

SDS-PAGE as described under "Experimental Procedures." (D) qRT –PCR to assess GRK5, GRK6, and GRK2 mRNA levels. **$p<0.01$ or * $p<0.05$, significant effect of CP55940 treatment compared to vehicle-treated controls. The data represent mean ± SEM (n=3).

We then aimed to identify the cannabinoid receptor involved in the upregulation of GRK5 since this could mediate the enhanced phosphorylation of CB_2 receptors detected in rat PFCx. Cells were treated with either vehicle, GP1a 1nM (selective CB_2 agonist), or ACEA 15nM (selective CB_1 agonist) over 72 hrs. We found that GP1a treatment significantly ($p<0.01$) increased (69 ± 0.05% increase), while ACEA did not significantly ($p>0.5$) modify GRK5 mRNA levels compared to controls (Fig. 24A). This evidence suggested that CB_2 receptors could mediate the upregulation of GRK5. We then tested the effect of GP1a on GRK5 mRNA levels in either control, CB_1 shRNA, or CB_2 shRNA stably transfected cells over 72 hrs. We have previously shown that treatment with the CB_1 or CB_2 shRNA lentiviral particles significantly reduces CB_1 or CB_2 receptor expression, respectively [262]. We found that treatment with GP1a significantly ($p<0.01$) increased GRK5 mRNA levels in control and CB_1 shRNA treated cells (73 ± 0.03% and 73 ± 0.04% increase compared to controls, respectively). Noteworthy, CB_2 shRNA treatment prevented ($p<0.01$) the GP1a-induced increases in GRK5 mRNA levels (Fig. 24B). Neither control, CB_1 nor CB_2 shRNA lentivirus treatment, significantly ($p>0.01$) modified basal GRK5 mRNA levels. The two-way ANOVA for GRK5 mRNA showed significant main effects of transfection ($F_{(2,17)} = 20.4$, $p<0.0001$) and cannabinoid agonist treatment ($F_{(1,17)} =187$, $p<0.0001$). There was a significant interaction between transfection and cannabinoid agonist treatment ($F_{(2,17)} =22.8$, $p<0.0001$) on GRK5 mRNA levels.

Next, we investigated whether ERK1/2 signaling pathway may be involved in the cannabinoid-induced upregulation of GRK5. CB_2 receptors are positively coupled to the ERK1/2 signaling pathway and cannabinoid agonists, such as Δ^9-THC, can regulate the expression of some GRKs through the ERK1/2 signaling pathway [308]. We used PD198306, a selective ERK1/2 inhibitor [288], to study the effect of GP1a-induced ERK1/2 activation on GRK5

upregulation. GP1a treatment significantly ($p<0.01$) increased GRK5 mRNA levels compared to vehicle treated cells (77 ± 2% increase, Fig 24D). This upregulation of GRK5 was prevented ($p<0.01$) by pretreatment with PD198306. This ERK1/2 inhibitor pretreatment did not significantly ($p>0.05$) modify basal levels of GRK5 mRNA. The two-way ANOVA for GRK5 mRNA showed significant main effects of PD198306 pretreatment ($F_{(1,11)} =22.4$, $p<0.0015$) and GP1a treatment ($F_{(1,11)} =43.3$, $p<0.0002$). There was a significant interaction between PD198306 and GP1a treatment ($F_{(1,11)} =35.2$, $p<0.0003$).

We also used cells stably transfected with either β-Arrestin 2 or control shRNA lentiviral particles to study the contribution of β-Arrestin 2 in the cannabinoid-induced upregulation of GRK5. We have previously shown that treatment with β-Arrestin 2 shRNA lentiviral particles significantly reduces β-Arrestin 2 protein expression by approximately 80% compared to control shRNA treated cells [100;262]. Here, β-Arrestin 2 or control shRNA treated cells were incubated with vehicle or GP1a for 72 hrs. GP1a significantly ($p<0.01$) upregulated GRK5 mRNA in control shRNA treated cells by 73 ± 6%. The cannabinoid agonist-induced upregulation of GRK5 mRNA levels was significantly ($p<0.01$) reduced in cells stably transfected with β-Arrestin 2 shRNA lentiviral particles (29 ± 5% increase compared to controls, Fig. 24E). The two-way ANOVA showed main effects of transfection ($F_{(1,11)} =8.72, p<0.0183$), CB_2 agonist ($F_{(1,11)} =52.3, p<0.0001$), and a main interaction between these two factors ($F_{(1,11)} =14.3, p<0.0054$) on GRK5 mRNA levels.

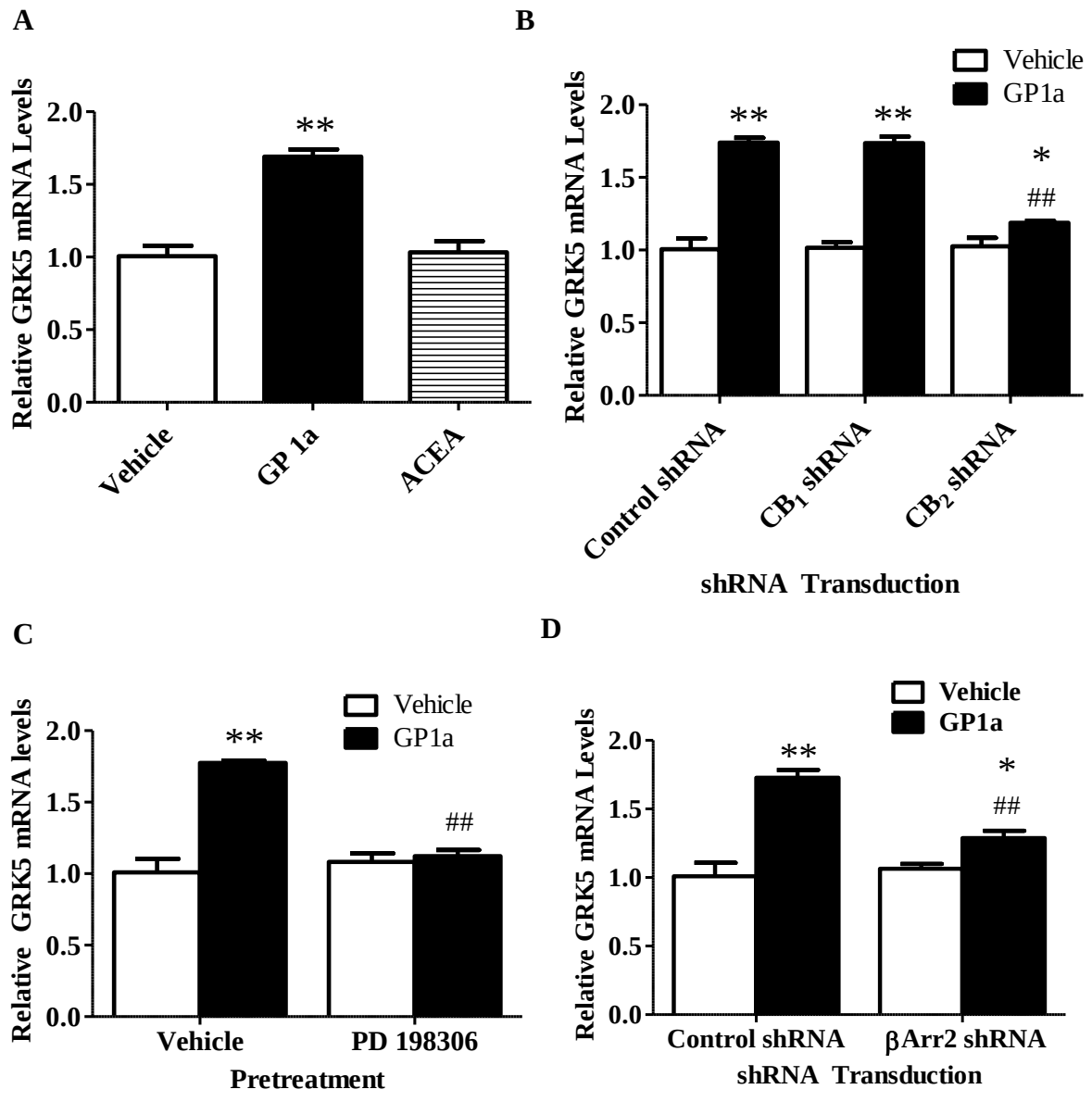


Figure 24. GP1a upregulated GRK5 via CB_2 receptors, β-Arrestin 2, and ERK1/2 signaling in CLU213 cells.

(A) Cells were incubated with either vehicle (ethanol 0.01% final concentration), 1 nM GP1a , or 15 nM ACEA for 72 hrs. qRT-PCR was used to show effect of selective CB_1 or CB_2 receptor agonist treatment on GRK5 mRNA levels. **$p<0.01$ significant effect of GP1a treatment

compared to vehicle-treated controls. (B) Cells stably transfected with control, CB_1, or CB_2 shRNA lentiviral particles were treated with vehicle or 1 nM GP1a for 72 hrs. qRT-PCR was used to examine effect of CB_1 or CB_2 receptor knockdown on GP1a-induced increases in GRK5 mRNA levels. **p<0.01, significant effect of GP1a treatment on GRK5 mRNA levels in control or CB_1 shRNA lentivirus treated cells compared to vehicle-treated controls. ##p<0.01, significant effect of CB_2 shRNA lentivirus transfection on the GP1a-induced upregulation of GRK5. *p<0.05, significant effect of GP1a treatment in CB_2 shRNA transfected cells compared to vehicle-treated /CB_2 shRNA transfected cells. (C) Cells were pretreated with vehicle or 200 nM PD198306, potent ERK1/2 inhibitor, and then treated with vehicle or 1 nM GP1a for 72 hrs. qRT-PCR was used to show effect of PD198306 pretreatment on GP1a-induced increases in GRK5 mRNA levels. **p<0.01, significant effect of GP1a treatment compared to vehicle-treated controls. ##p<0.01, significant effect of PD198306 pretreatment on GP1a-induced increases GRK5 mRNA levels compared to vehicle-treated controls. (D) Cells were stably transfected with control or β-Arrestin 2 shRNA lentivirus particles and then treated with vehicle or 1 nM GP1a for 72 hrs. qRT-PCR was used to examine the effect of β-Arrestin 2 knock down on GP1a-induced increases in GRK5 mRNA levels. **p<0.01, significant effect of GP1a treatment GRK5 mRNA levels in control shRNA lentivirus treated cells compared to vehicle-treated controls. ##p<0.01, significant effect of β-Arrestin 2 shRNA lentivirus transfection on the GP1a-induced upregulation of GRK5. *p<0.05, significant effect of GP 1a treatment in β-Arrestin 2 shRNA transfected cells compared to vehicle-treated β-Arrestin 2 shRNA transfected cells. The data represent mean ± SEM (n=3).

In order to determine whether GRK5 is involved in the cannabinoid-induced upregulation of 5-HT_{2A} receptors, we used cells stably transfected with GRK5 or control shRNA lentiviral particles. GRK5 shRNA lentiviral particle treatment significantly ($p<0.01$) reduced both GRK5 mRNA (Fig. 25A) and protein levels (Fig. 25B) by approximately 80% compared to control-treated cells. As expected, CP55940 upregulated 5-HT_{2A} mRNA in control shRNA treated cells (93 ± 2% increase, Fig. 25C). Of note, CP55940 induced increases in 5-HT_{2A} mRNA were significantly ($p<0.01$) reduced in cells stably transfected with GRK5 shRNA (Fig. 25C). In contrast, CP55940 treatment in GRK5 shRNA treated cells significantly ($p<0.01$) increased 5-HT_{2A} mRNA levels by only 29 ± 0.03%. The two-way ANOVA showed main effects of transfection ($F_{(1,11)}$ =65.3,$p<0.0001$), CP55940 ($F_{(1,11)}$ =231,$p<0.0001$), and a main interaction between these two factors ($F_{(1,11)}$ =58.1,$p<0.0001$) on 5-HT_{2A} mRNA levels. Similarly, GP1a upregulated 5-HT_{2A} mRNA in control shRNA cells (97 ± 0.02% increase, Fig. 25D) and GP1a-induced increases in 5-HT_{2A} mRNA were significantly ($p<0.01$) reduced in cells stably transfected with GRK5 shRNA (70% reduction compared to GP1a effect on control cells, Fig 25D). The two-way ANOVA showed main effects of transfection ($F_{(1,11)}$ =40.7,$p<0.0002$), GP1a ($F_{(1,11)}$ =124,$p<0.0001$), and a main interaction between these two factors ($F_{(1,11)}$=42.2,$p<0.0002$) on 5-HT_{2A} mRNA levels.

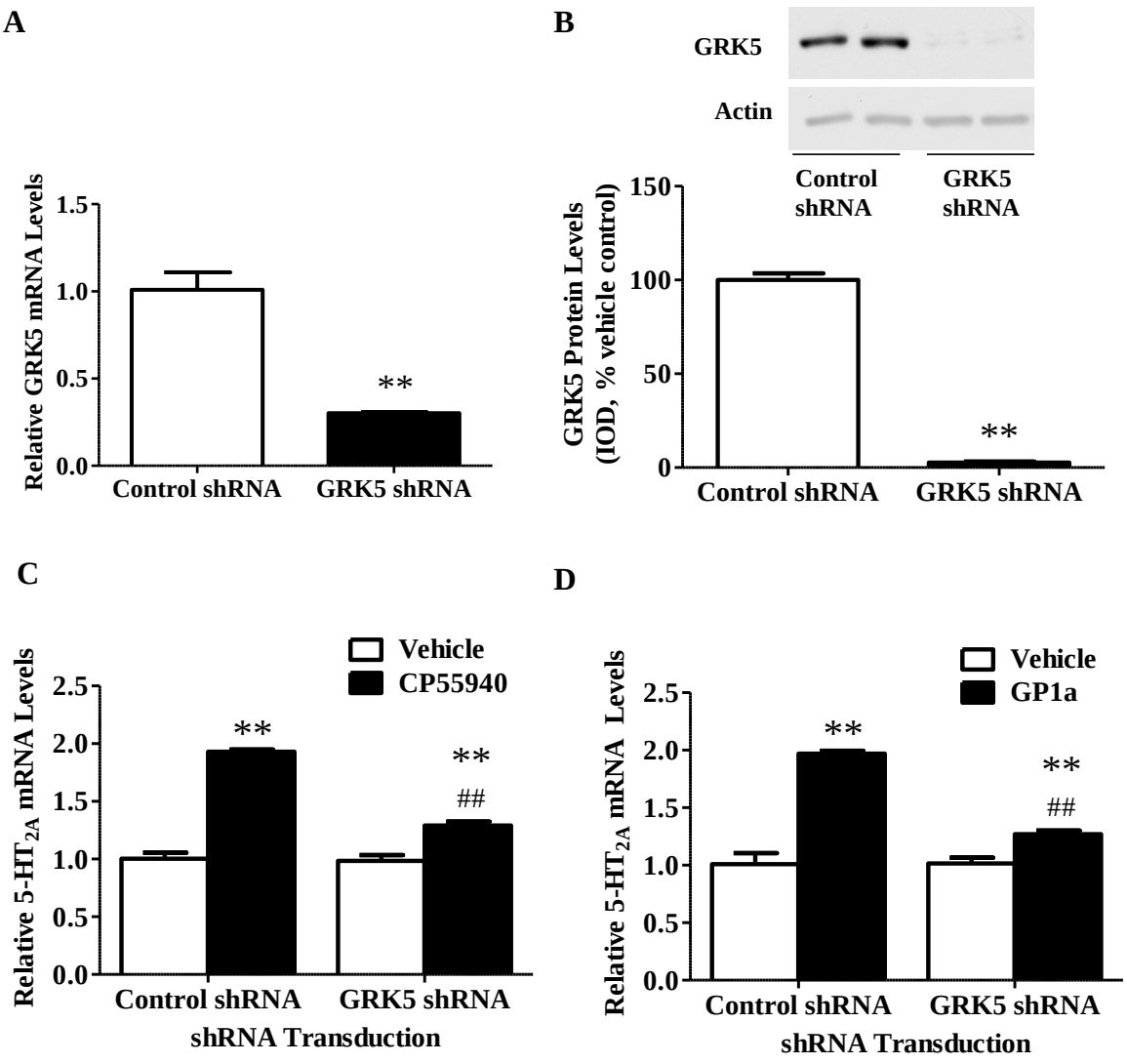


Figure 25. GRK5 is necessary for the CP55940 and GP1a-induced upregulation of 5-HT_{2A} receptors in CLU213 cells.

Cells were transfected with control or GRK5 shRNA lentiviral particles as described under "Experimental Procedures." (A) qRT-PCR to show reduced GRK5 mRNA levels in cells treated with GRK5 shRNA lentiviral particles. (B) Western blot to show reduced GRK5 protein levels in cells treated with GRK5 shRNA lentiviral particles. (A-B) **$p<0.01$ significant effect of GRK5 shRNA compared to control shRNA treated cells. (C-D) Cells stably transfected with control or GRK5 shRNA lentiviral particles were treated with either vehicle, 1 nM CP55940, or 1 nM

GP1a for 72 hrs. (C) qRT-PCR to show effect of GRK5 knockdown on CP55940-induced increases on 5-HT_{2A} receptor mRNA levels **p<0.01, significant effect of CP55940 treatment on 5-HT_{2A} receptor mRNA levels in control shRNA lentivirus transfected cells compared to vehicle-treated controls. ##p<0.01, significant effect of GRK5 shRNA lentivirus transfection on the CP55940-induced upregulation of 5-HT_{2A} receptors. **p<0.01, significant effect of CP55940 treatment in GRK5 shRNA transfected cells compared to vehicle-treated GRK5 shRNA transfected cells. (D) qRT-PCR to show effect of GRK5 knockdown on GP1a-induced increases in 5-HT_{2A} receptor mRNA levels. **p<0.01, significant effect of GP1a treatment on 5-HT_{2A} receptor mRNA levels in control shRNA lentivirus transfected cells compared to vehicle-treated controls. ##p<0.01, significant effect of GRK5 shRNA lentivirus transfection on the GP1a-induced upregulation of 5-HT2A receptors. **p<0.01, significant effect of GP1a treatment in GRK5 shRNA transfected cells compared to vehicle-treated GRK5 shRNA transfected cells. The data represent mean ± SEM (n=3).

In the next studies we examined the role of GRK5 in the cannabinoid induced increases in 5-HT_{2A} receptor activity. We have previously reported that repeated CP55940 treatment in this neuronal cell culture model significantly enhances 5-HT_{2A} receptor mediated phosphoinositol hydrolysis [314]. Here, we studied the effects of GRK5 shRNA treatment on the 5-HT_{2A} receptor mediated calcium (Ca^{2+}) release. We began conducting dose response experiments with 5-HT as described in the experimental procedures section. We used 5-HT in these experiments because previous studies have shown that the maximal response to (-)-1-2,5-dmiethoxy-4-iodoamphetamine HCL (DOI, a $5HT_{2A/2C}$ receptor agonist) is lower than the maximal response to 5-HT in two different cells lines [324]. A dose response experiment in CLU213 cells showed that 5-HT stimulated Ca^{2+} release in a dose-dependent way (Fig. 26A) with an EC_{50} of 0.11 ± 0.02 nM. To confirm that this response was the result of stimulation of 5-HT_{2A}, but not 5-HT_{2C} receptors, we measured the effect of MDL11,939 or SB242084 (5-HT_{2A} and 5-HT_{2C} receptor antagonists, respectively) [323;324] in the 5-HT-induced Ca^{2+} release in a neuronal cell model. MDL11,939 and SB242084 inhibited the 5-HT -mediated Ca^{2+} release with different affinities. While MDL11,939's IC_{50} was approximately 1nM, SB242084's IC_{50} was 0.1 µM, suggesting that the 5-HT-mediated Ca^{2+} release in CLU213 cells is mainly mediated by 5-HT_{2A} receptors at the concentration of 5-HT (0.1 nM) used in this assay (Fig. 26B). 10 nM SB242084 was added to the preincubation media in the proceeding assays to prevent the activation of 5-HT_{2C} receptors. Based on the K_d provided in experimental procedures, the fractional occupancy of 5-HT_{2C} and 5-HT_{2A} receptors at this dose of SB242094 is 95 % and 7 %, respectively.

We then studied the effect of repeated exposure (72 hrs) to either CP55940 or GP1a on the 5-HT-mediated Ca^{2+} release in neuronal cells (Fig. 26C and 26D). While in vehicle treated cells 0.1 and 0.4 nM 5-HT produced significant ($p<0.01$) increases (100 and 122%, respectively)

in Ca^{2+} release in neuronal cells, these increases were significantly ($p<0.01$) higher in cells treated with CP55940 (220% and 253% at 5-HT 0.1 nM and 0.4 nM, respectively). The two-way ANOVA showed main effects of CP55940 treatment ($F_{(1,17)}$ =70421, $p<0.0001$), 5-HT treatment ($F_{(2,17)}$ = 123689, $p<0.0001$), and a main interaction between them ($F_{(2,17)}$ =17910, $p<0.0001$). Repeated GP1a exposure (72 hrs) also enhanced the 5-HT-mediated Ca^{2+} release in CLU213 cells (Fig. 26D). Indeed, we observed a two-fold increase ($p<0.01$) in the maximal 5-HT-mediated responses in GP1a treated cells compared to vehicle controls. However, no changes ($p>0.05$) were detected in the EC_{50} values between control and GP1a treated cells (0.098 ± 0.004 and 0.095 ± 0.009 nM, respectively).

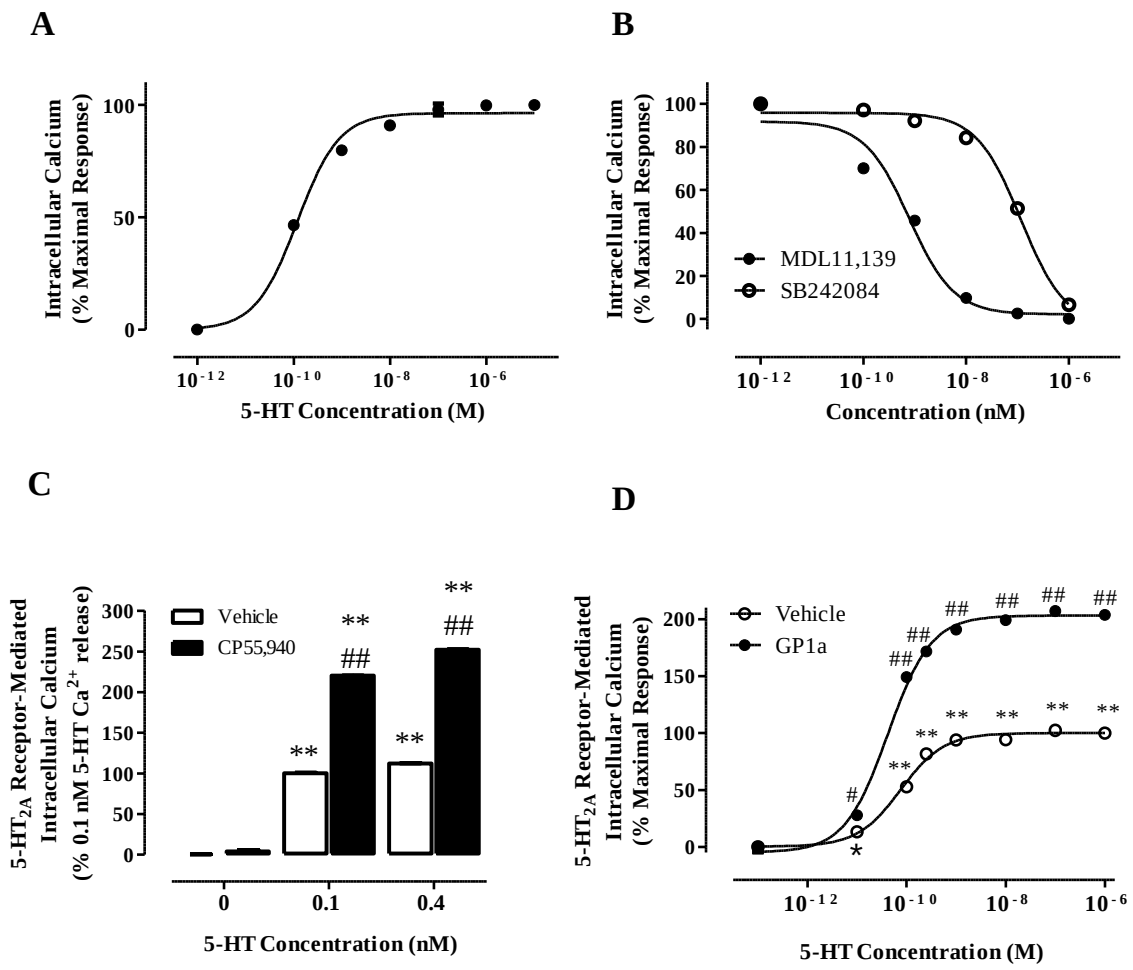


Figure 26. Repeated CP55940 and GP1a pretreatment enhanced 5-HT$_{2A}$ receptor mediated calicium release in CLU213 cells.

Calcium assays were optimized and conducted as described under "Experimental Procedures." (A) Cells were stimulated with increasing doses of 5-HT. (B) Cells were pre-incubated with increasing doses of MDL11,939 (selective 5-HT$_{2A}$ receptor antagonist, IC$_{50}$ = 1 nM) or SB242084 (selective 5-HT$_{2C}$ receptor antagonist, IC$_{50}$ = 0.1 μM) for 20 min and then stimulated with 1 μM 5-HT. (C) Cells were pretreated with vehicle or 1 nM CP55940 for 72 hrs. Cells were then pre-incubated with 10 nM SB242084 for 20 min, to inhibit 5-HT$_{2C}$ receptor mediated Ca^{2+} release, and then stimulated with either 0.1 or 0.4 nM 5-HT. **p<0.01, significant effect of

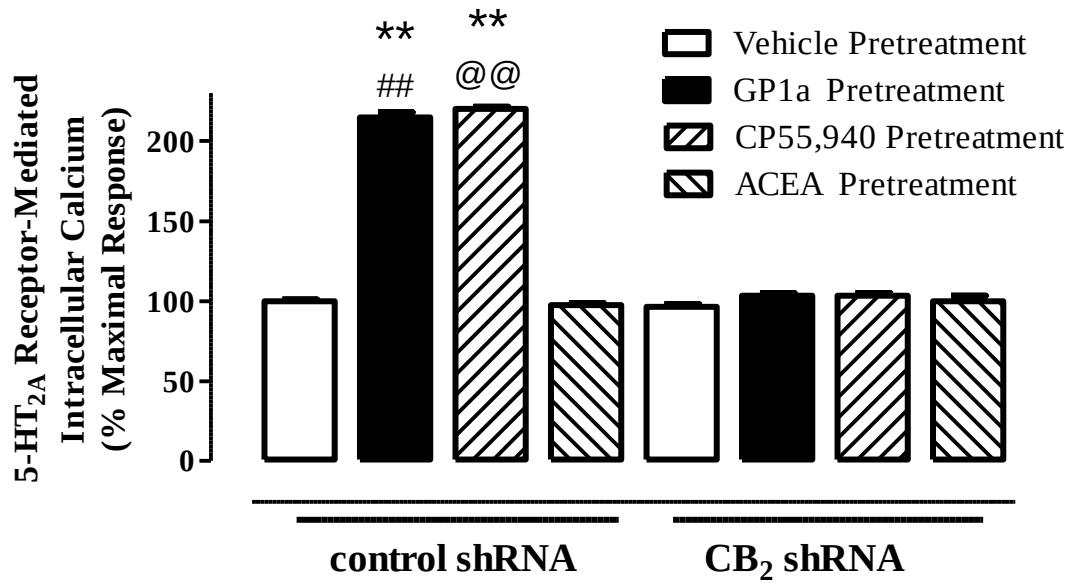
CP55940 pretreatment/5-HT stimulation or 5-HT stimulation on Ca^{2+} release in cells. ##p<0.01, significant effect of CP55940 pretreatment/5-HT stimulation compared to 5-HT stimulation in cells. (D) Cells were treated with vehicle or 1 nM GP1a for 72 hrs. Cells were then pre-incubated with 10 nM SB242084 for 20 min, to inhibit $5\text{-}HT_{2C}$ receptor mediated Ca^{2+} release, and then stimulated with either 0.1 nM, 1 nM, 0.4 nM, 10 nM, 100 nM, 1 μM, or 10 μM of 5-HT. **p<0.01, significant effect of 0.1 nM to 10 μM of 5-HT stimulation compared to vehicle treated controls. ##p<0.01, significant effect of GP1a pretreatment/5-HT stimulation compared to 5-HT stimulated cells. The data represent mean ± SEM (n=3).

Our previous results suggest that chronic exposure to agonists of CB_2 receptors enhance the 5-HT_{2A} receptor-mediated Ca^{2+} release receptors in our neuronal cell model. Our next experiment aimed to confirm the role of CB_2 receptors in this phenomenon (Fig. 27A). Here, control or stably transfected cells with CB_2 shRNA were incubated with either vehicle, CP55940 (1nM), ACEA (20 nM) or GP1a (1nM) for 72 hrs. Both CP55940 and GP1a induced a significant ($p<0.01$) and approximate two-fold increase in the 5-HT mediated Ca^{2+} release in control cells (220.6 ± 1.6% and 215.4 ± 3.0% increase over maximal response for CP55940 and GP1a treated cells, respectively). ACEA, a selective CB_1 receptor agonist, did not significantly ($p>0.05$) modify the 5-HT mediated Ca^{2+} responses in control cells. On the other hand, neither CP55940 (1nM), GP1a (1nM) nor ACEA (15nM) significantly ($p>0.05$) modified the 5-HT mediated Ca^{2+} responses in cells stably transfected with CB_2 shRNA. The two-way ANOVA showed a significant main effect of transfection ($F_{(1,23)} =2235.8$, $p<0.0001$), cannabinoid treatment ($F_{(3,23)} =3279.8$, $p<0.0001$), and a main interaction between them ($F_{(3,23)} =766.5$, $p<0.0001$).

Our next aim was to determine whether GRK5 plays a significant role in the 5-HT mediated Ca^{2+} release in a neuronal cell model. Control cells or cells stably transfected with GRK5 shRNA were incubated with either vehicle, CP55940 (1nM) or GP1a (1nM) for 72 hrs. In control cells, 5-HT (0.1 nM) mediated Ca^{2+} release was significantly increased by both CP55940 and GP1a treatment (205 ± 3% and 201 ± 4% increase over control for CP55940 or GP1a, respectively). Noteworthy, in cells stably transfected with GRK5 shRNA, neither CP55940 nor GP1a induced significant increases in the 5-HT mediated Ca^{2+} release (Fig. 27B). The two-way ANOVA showed a significant main effect of transfection ($F_{(1,17)} =215.2$, $p<0.0001$), cannabinoid treatment ($F_{(2,17)} =55.97$, $p<0.0001$), and a main interaction between them ($F_{(2,17)} =46.58$,

$p<0.0001$). In summary, these results suggest that GRK5 plays a pivotal role in the CB_2 receptor-induced upregulation of 5-HT_{2A} receptors in our neuronal cell model.

A



B

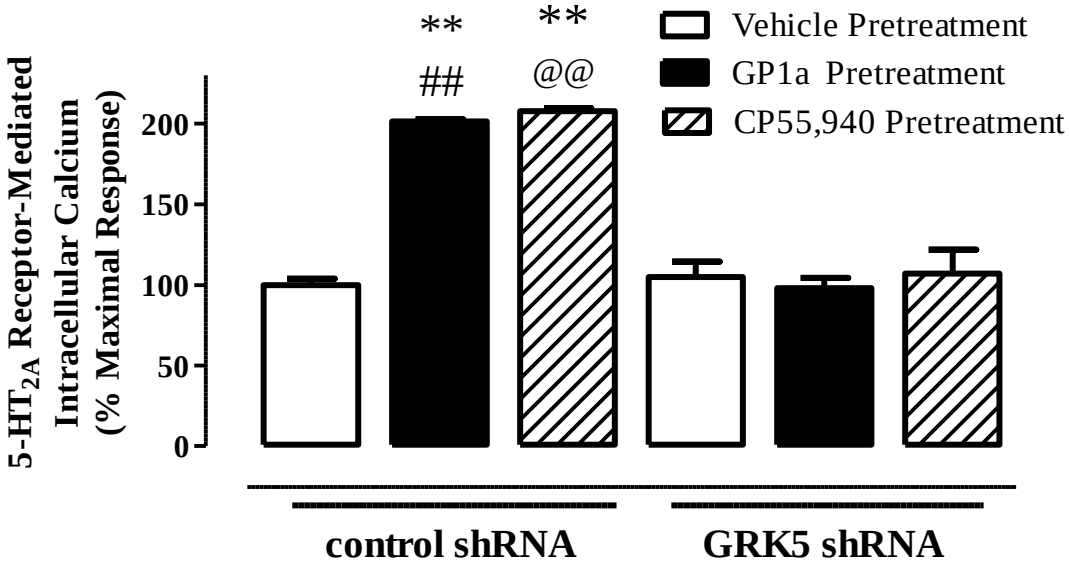


Figure 27. CB_2 and GRK5 are necessary for the CP55940 and GP1a induced increase in 5-HT_{2A} receptor mediated calcium release in CLU213 cells.

Cells were stably transfected with either control, CB_2, or GRK5 shRNA lentiviral particles as described under "Experimental Procedures." (A) Cells stably transfected with control or CB_2 shRNA lentiviral particles were pretreated with either vehicle, 1 nM CP55940, 1 nM GP1a, or 15 nM ACEA for 72 hrs. Cells were then pre-incubated with 10 nM SB242084 for 20 min and then stimulated with 0.4 nM 5-HT. (B) Cells stably transfected with control or GRK5 shRNA lentiviral particles were pretreated with either vehicle, 1 nM CP55940, or 15 nM GP1a. Cells were then pre-incubated with 10 nM SB242084 for 20 min and then stimulated with 0.4 nM 5-HT. **$p<0.01$, significant effect of CP55940 pretreatment/5-HT stimulation or GP1a pretreatment/5-HT stimulation in control shRNA transfected cells compared to vehicle treated controls. ##$p<0.01$, significant effect of GP1a pretreatment/5-HT stimulation in control shRNA transfected cells compared to GP1a pretreatment/5-HT stimulation in CB_2 or GRK5 shRNA transfected cells. @@ $p<0.01$, significant effect of CP55940 pretreatment/5-HT stimulation in control shRNA transfected cells compared to CP55940 pretreatment/5-HT stimulation in CB_2 or GRK5 shRNA transfected cells. The data represent mean ± SEM (n=3).

We then examined the role of GRK5 in the cannabinoid agonist-induced phosphorylation of CB_2 receptors. Cells stably transfected with either GRK5 or control shRNA lentiviral particles were treated with vehicle or GP1a 1nM for 72 hrs and phosphorylated proteins were isolated as described in the experimental procedures section. We found that GP1a treatment significantly ($p<0.01$) enhanced phosphorylation of CB_2 receptors in control shRNA treated cells by 36 ± 7% (Fig. 28A). Of note, this GP1a-induced enhanced phosphorylation of CB_2 receptors was prevented ($p<0.01$) in cells stably transfected with GRK5 shRNA lentiviral particles. No significant differences in the CB_2 receptor phosphorylation levels were detected between vehicle and GP1a in cells stably transfected with GRK5 shRNA lentiviral particles. The two-way ANOVA for phosphorylated CB_2 showed main effects of transfection ($F_{(1,43)}$ =21,$p<0.0001$) and GP1a ($F_{(1,43)}$ =4.8,$p<0.0333$). There was a significant interaction between transfection and GP1a treatment ($F_{(1,43)}$=8.8,$p<0.005$) on phosphorylated CB_2 receptors. We then examined the effects of GRK5 shRNA lentivirus treatment and GP1a treatment on CB_2 receptor protein levels in whole cell lysates. Repeated GP1a treatment did not significantly ($p>0.05$) modify CB_2 receptor protein levels in whole cell lysates (Fig. 28B). Furthermore, GRK5 shRNA lentivirus particle transfection did not significantly ($p>0.05$) alter the basal levels of CB_2 receptors (Fig. 28B).

We recently reported chronic CP55940 treatment can enhance β-Arrestin 2 and ERK1/2 interaction in rat PFCx [100]. This cannabinoid agonist induced enhanced interaction could be mediated by GRK5. Here, we used co-immunoprecipitation protocols to study the effect of GP1a treatment on the physical interaction between β-Arrestin 2 and ERK1/2 in control and GRK5 shRNA lentivirus transfected cells (Fig. 28C). We used β-Arrestin 2 antibody as bait and ERK1/2 antibody as prey. Inactive columns, which are unable to bind β-Arrestin 2 antibody, were used as a control as described in the methods section. We found that ERK1/2 co-

precipitates with β-Arrestin 2 when we used β-Arrestin 2 as bait (Fig. 28C, lanes 5-8). Interestingly, we detected a significant four-fold increase in the interaction between β-Arrestin 2 and ERK1/2 in GP1a/control shRNA treated cells compared to vehicle/control shRNA treated cells (Fig. 28C, lane 5 and 6, vehicle- and GP1a-treated, respectively). In GRK5 shRNA treated cells, this GP1a-induced enhanced interaction between β-Arrestin 2 and ERK1/2 was significantly reduced (approximate 1.5 fold) compared to GP1a/control shRNA treated cells (lane 6 and 8, GP1a/control shRNA and GP1a/GRK5 shRNA, respectively). No co-precipitation of β-Arrestin 2 and ERK1/2 was detected using the inactive columns (Fig. 28C, lanes 9-12). The two-way ANOVA for β-Arrestin 2/ERK co-immunoprecipitation showed main effects of transfection ($F_{(1,15)}$=718.4,$p<0.0001$) and GP1a ($F_{(1,15)}$=1252,$p<0.0001$). There was a significant interaction between transfection and GP1a treatment ($F_{(1,15)}$=649.6,$p<0.0001$) on interaction of β-Arrestin 2/ERK.

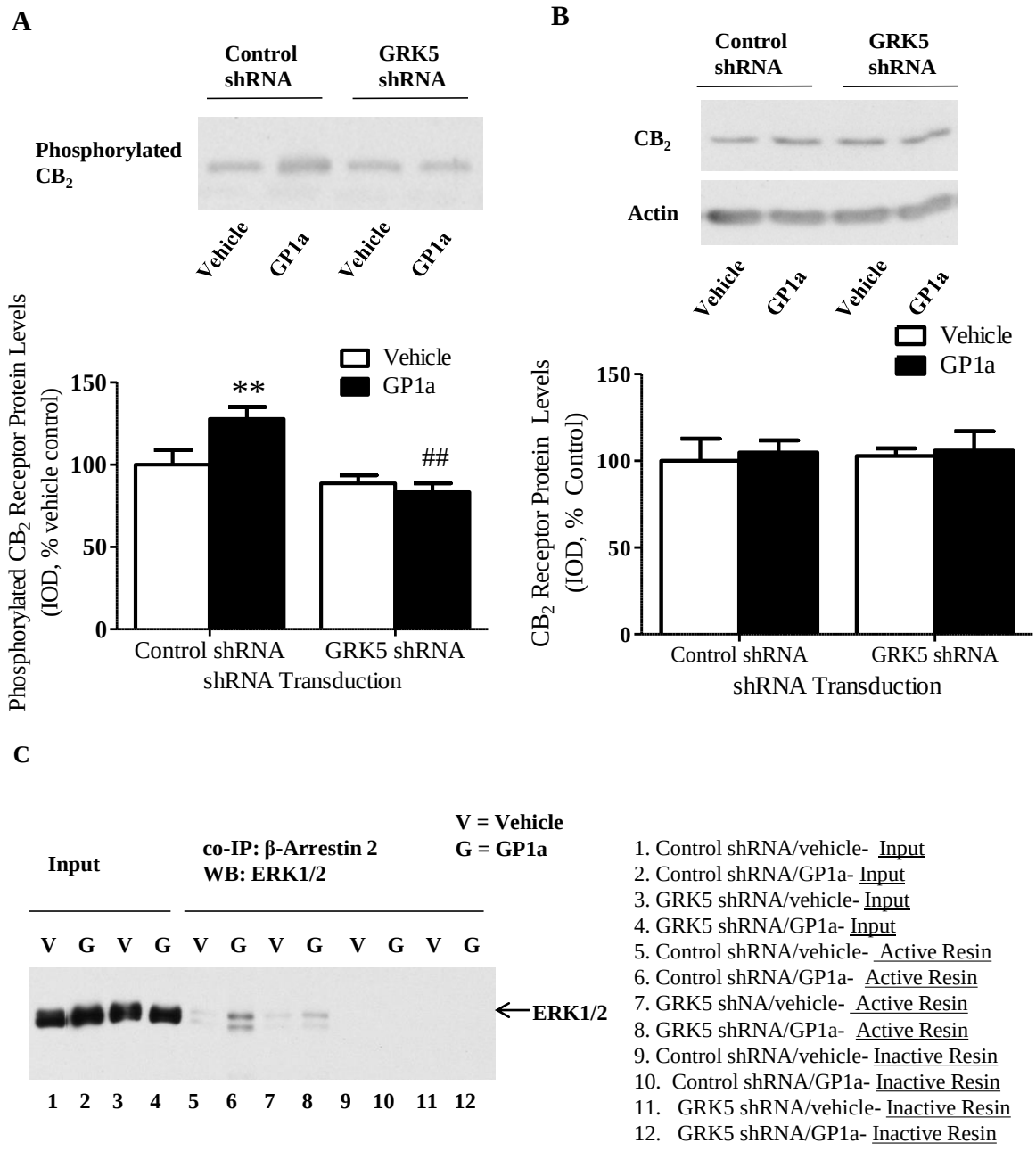


Figure 28. GRK5 mediates GP1a-induced increases in CB_2 phosphorylation and enhanced β-Arrestin 2/ERK interaction in CLU213 cells.

To examine the role of GRK5 in the GP1a-induced increases in CB_2 receptor phosphorylation and β-Arrestin 2/ERK interaction, cells stably transfected with control of GRK5 shRNA

lentiviral particles were treated with either vehicle or 1 nM GP1a for 72 hrs. (A) Phosphorylated proteins were separated and detected as described under "Experimental Procedures." Thirty µg of isolated phosphorylated protein was used in Western blot detection. **$p<0.01$, significant effect of GP1a treatment on CB_2 phosphorylation levels in control shRNA lentivirus transfected cells compared to vehicle-treated controls. ##$p<0.01$, significant effect of GRK5 shRNA lentivirus transfection on the GP1a-induced increases in CB_2 phosphorylation. The data represent mean ± SEM (n=3). (B) CB_2 receptor protein levels in whole cell lysate were evaluated by Western blot as described under "Experimental Procedures." Representative Western blots are shown in this figure and β-Actin was used as a loading control. The data represent mean ± SEM (n=3). (C) Co-Immunoprecipation was used to examine the role of GRK5 in GP1a-induced increases in β-Arrestin 2/ERK interaction. Co-Immunoprecipation was conducted as described under "Experimental Procedures." Negative controls (Lanes 9-12) received the same concentration of β-Arrestin 2 antibody except that the coupling resin was replaced with control agarose resin that is not amine reactive. All columns were incubated with whole cell lysate (300 µg) from vehicle (Lanes 1 and 3) or GP1a (Lanes 4 and 6) treated cells. Cell lysate (15 µg of protein) was used as an input control (Lane 1-4). The data represent mean ± SEM (n=4).

7.5 Discussion

Cannabinoid agonists produce their physiological effects through the activation of two G-protein coupled cannabinoid receptors in the brain, CB_1 and CB_2 receptors [15;98]. CB_1 and CB_2 receptors bind endocannabinoids, synthetic cannabinoids, and cannabinoids found in nature (such as in *Cannabis sativa* L.) with high affinity [15;98]. Although only CB_1 receptors were initially identified in the brain [246], later studies have also identified CB_2 receptors in several brain areas including, PFCx, hippocampus, amygdala, substantia nigra, and cerebellum [18;21], triggering a reevaluation of the possible roles that CB_2 receptors might play in the brain.

We have previously reported that repeated exposure to either nonselective cannabinoid agonists or selective CB_2 receptor agonists upregulate and enhance the activity of 5-HT_{2A} receptors in rat PFCx and neuronal cell models [100;262;263;314]. CB_2 receptors can couple to $G_{i/o}$ class of G-proteins to regulate transient ERK1/2 signaling while β-Arrestin 2 may be involved in the long-term regulation of ERK1/2 signaling [88;99;100;293]. Recent evidence has highlighted that neuronal CB_1 receptors can modulate ERK1/2 signaling through $G_{i/o}$ and multiple tyrosine kinase receptors (RTKs) [99]. While G-protein mediated activation of $ERK_{1/2}$ is transient and peaks within 2-5 minutes [171;300], β-Arrestins can form a scaffolding complex with ERK1/2 to regulate long-term ERK1/2 activity [171;257;300]. Although the mechanisms of the cannabinoid-induced upregulation of 5-HT_{2A} receptors have not been completely identified, our results suggest that activation of β-Arrestin 2 and ERK1/2 signaling pathway mediates this phenomenon that is dependent on CB_2, but not CB_1, receptors [263;314]. The key role of β-Arrestin 2 in this upregulation seems to involve an enhanced cannabinoid-induced interaction between β-Arrestin 2 and ERK1/2 in rat PFCx [100].

Recent reports suggest that certain GRK proteins could trigger the activation of the β-Arrestin 2 and ERK1/2 signaling pathway [325]. The classical role described for GRK proteins is to trigger the desensitization of GPCRs [171;326]. Indeed, GRK2 and GRK3 proteins would phosphorylate the serine and threonine residues within the intracellular loops and carboxyl-terminal tail domains of GPCRs to uncouple them from their G-proteins, and hence, trigger the desensitization of their corresponding signaling pathway [171;326]. GRK2 and GRK3 proteins would also inhibit β-Arrestin signaling in a Gβγ-dependent pathway [325;327]. On the other hand, GRK5 and GRK6 proteins would have new roles in the signaling of GPCR that would relate to their ability to trigger the activation of the β-Arrestin2/ERK1/2 signaling pathway in a G-protein independent way [290;325;327]. Indeed, over-expression of GRK5 and/or GRK6 has been found to enhance β-Arrestin 2 mediated ERK1/2 activation while over-expression of GRK2 and/or GRK3 abolishes β-Arrestin 2 mediated ERK1/2 activation [325].

Here we report that repeated CP55940 treatment increases CB_2 receptor phosphorylation and selectively increases GRK5 mRNA and protein expression in rat PFCx and a neuronal cell model without changes in the mRNA or protein levels of GRK6. This was also associated with reduced levels of GRK2 mRNA and protein levels in this area of the limbic brain and in cultured cells.

A limited number of reports study the effect of cannabinoids on the regulation of GRK protein expression. For instance, multiple THC (tetrahydrocannabinol, CB_1/CB_2 receptor agonist) treatments, but not a single THC treatment, upregulates GRK2 and GRK4 in the striatum, GRK4 in the cerebellum, and GRK2 in the PFCx and hippocampus [308]. To the best of our knowledge there are currently no other reports detailing the effects of repeated cannabinoid agonist exposure on the expression of GRK proteins. This limited evidence would

suggest that chronic exposure to different classes of cannabinoids may have differential effects on expression of GRKs and subsequent regulation of signaling cascades throughout the brain. Furthermore, our previous evidence suggests that repeated CP55940 or GP1a treatment enhances β-Arrestin 2 mediated ERK1/2 signaling as we have previously reported that repeated cannabinoid treatment enhances pERK levels over a single cannabinoid exposure [100;314]. We have previously found the β-Arrestin 2 shRNA lentivirus transfection significantly reduces cannabinoid-induced increases in pERK levels [314]. The modulation of GRK protein expression by cannabinoids could be contributing to an intensification of this signaling cascade. Interestingly, different agonists and drugs of abuse have been shown to modulate changes in expression of GRKs and changes in GRK expression have been described in different pathophysiological conditions [328].

Our evidence indicates that the cannabinoid-induced changes in GRK5 expression could be mediated by changes in transcription as we report here that repeated CP55940 treatment increases GRK5 mRNA and protein in rat PFCx and in our neuronal cell culture model. In neuronal cells we found that a selective CB_2 receptor agonist (GP1a), but not a selective CB_1 receptor agonist (ACEA), significantly increased GRK5 mRNA levels compared to vehicle treated controls, suggesting that CB_2 receptors mediate the cannabinoid-induced upregulation of GRK5. Confirmatory evidence of the role of CB_2 receptors in the GRK5 upregulation was provided by studies with either CB_1 or CB_2 shRNA lentiviral particles (Fig. 24) where the cannabinoid-induced upregulation of GRK5 was prevented only in CB_2 shRNA lentiviral treated cells. Although the detailed mechanism of cannabinoid-induced upregulation of GRK5 was not identified in this manuscript, we speculate that transcription factor such as Nuclear Factor immunoglobulin K chain enhancer- B cell (NF-kB) could mediate this GRK5 upregulation. The

CB_2 receptor is positively coupled to the ERK1/2 signaling pathway that regulates NF-kB [15;232]. Rat, human, and mouse GRK5 promoter contains a consensus sequence for NF-kB and activation of NF-kB increases GRK5 expression [329].

Here we also investigated the role of GRK5 in the cannabinoid-induced upregulation of 5-HT_{2A} receptors. Through the use of GRK5 shRNA lentiviral particles we identified that GRK5 is involved in the cannabinoid-induced upregulation and enhanced activity of 5-HT_{2A} receptors. Indeed, treatment with GRK5 lentiviral particles significantly reduced the CP55940 and GP1a induced upregulation of 5-HT_{2A} receptors without significantly altering basal levels of 5-HT_{2A} receptor mRNA. Yet treatment with CP55940 or GP1a significantly increased 5-HT_{2A} mRNA levels in GRK5 shRNA lentivirus treated cells compared to vehicle treated controls. This evidence suggests that CB_2 receptor can mediate 5-HT_{2A} receptor upregulation, a least in part, through GRK5. Here the CP55940- and GP1a-induced increases in 5-HT_{2A} mRNA levels could be attributed to new rates in synthesis and degradation of GRK5 protein due to the GRK5 shRNA lentivirus particles transduction.

Additionally, we found that repeated CP55940 and GP1a treatment significantly increased serotonin stimulated 5-HT_{2A} receptor mediated Ca^{2+} release (Fig. 26D and 26E). We have previously reported that repeated CP55940 treatment significantly increases 5-HT_{2A} receptor mediated PLCβ activity in rat PFCx and 5-HT_{2A} receptor mediated phosphoinositol hydrolysis in a neuronal cell culture model [314]. Interestingly, here we provide more evidence that the enhanced 5-HT_{2A} receptor activity would involve the cannabinoid-induced upregulation of 5-HT_{2A} receptors. As shown in Figure 26E, repeated GP1a treatment significantly increased the Emax (two-fold increase) without significantly affecting the EC50 which could be explained, at least in part, by the enhanced cannabinoid-induced over-expression (two-fold) of 5-HT_{2A}

receptors in neuronal cells and in rat PFCx. The role of CB_2 receptors in mediating this phenomenon was identified by either CB_2 or GRK5 shRNA treatment. Indeed, CB_2 or GRK5 shRNA lentiviral particle treatment prevented CP55940 and GP1a induced increases in serotonin stimulated 5-HT_{2A} receptor mediated Ca^{2+} release (Fig 27A and 27B).

We also examined the role of GRK5 in the cannabinoid-induced phosphorylation of the CB_2 receptor and enhanced β-Arrestin 2/ERK interaction (Fig. 28). We found that GRK5 shRNA lentiviral particle treatment reduced the cannabinoid-induced enhanced phosphorylation of the CB_2 receptor and the enhanced β-Arrestin 2/ERK interaction in a neuronal cell culture model. Here the GP1a induced increases in CB_2 receptor phosphorylation and β-Arrestin/ERK interaction in GRK5 shRNA lentivirus treated cells could be attributed to new rates in synthesis and degradation of GRK5 after GRK5 shRNA lentivirus treatment and/or shifts in GRK6 activity. This evidence indicates that GRK5 is necessary for the cannabinoid-induced upregulation of 5-HT_{2A} receptors. Although further evidence is required, it is possible to speculate that GRK5 induced phosphorylation of the CB_2 receptor and subsequent formation of the β-Arrestin 2/ERK scaffolding complex could be an initiating mechanism contributing to the upregulation and enhanced activity of 5-HT_{2A} receptors. Further experiments are needed to demonstrate this hypothesis.

In conclusion, this study provides new insight into the cannabinoid agonist regulation of GRK proteins in rat PFCx and neuronal cell culture. Furthermore, this study is the first to show that GRK5 is involved in the cannabinoid-induced upregulation and enhanced activity of 5-HT_{2A} receptors in neuronal cells. We also identified mechanisms contributing to the upregulation of GRK5 in a neuronal cell model. Recent and independent clinical studies have provided evidence indicating that sustained use of nonselective cannabinoid agonists may precipitate the onset of

mental disorders associated with dysfunction of 5-HT_{2A} receptor neurotransmission in PFCx, such as anxiety, schizophrenia, and psychosis [10-13;110;111;330]. Yet a definitive mechanism by which repeated cannabinoid agonist exposure may be precipitating neuropsychiatric disorders has not been identified. The results presented here and in previous studies [100;262;263;314] suggest that GRK5 mediated enhanced phosphorylation of the CB_2 receptor and enhanced β-Arrestin 2/ERK interaction would drive the upregulation of 5-HT_{2A} receptors and GRK5. Interestingly, a recent report has linked enhanced function and expression of 5-HT_{2A} receptors in PFCx to enhanced anxiety-like behaviors in rodents [31]. Furthermore, the therapeutic benefits of atypical antipsychotics are proposed to be modulated desensitization of 5-HT_{2A} receptor signaling in PFCx particularly pyramidal neurons which are enriched in 5-HT_{2A} receptors [29;179]. Therefore, this study may facilitate a better understanding of mechanisms underlying the etiology of some neuropsychiatric disorders and adverse effects of chronic exposure to cannabinoids. Understanding the mechanisms underlying the adverse effects of repeated cannabinoid exposure is especially critical as accumulating evidence is showing that selective CB_2 receptor agonists have wide therapeutic application in the treatment of a variety of different conditions [2-5]. This evidence could provide insight into mechanisms that can be targeted to prevent the potential adverse effect while deriving the therapeutic benefits of cannabinoids.

Chapter 8: Conclusions

Marijuana (*Cannabis sativa* L.) is the most commonly abused illicit drug in the United States [7;8]. According to the 2009 National Survey on Drug Use and Health, more than 28.5 million Americans age 12 and older have tried marijuana at least once [331]. Of note, more than 60% of marijuana users are under the age of 18 [7;8;332]. Furthermore, recent epidemiological data of U.S. high school students showed that marijuana use has increased since 2008 while perceived risk of marijuana use continues to decrease [333;334]. However, recent and independent clinical studies provide strong evidence indicating that sustained use of non-selective cannabinoid agonists may precipitate the onset of mental disorders associated with dysfunction of 5-HT_{2A} receptor neurotransmission in the PFCx, such as anxiety, schizophrenia, and psychosis [10-13;110;111]. Currently, the precise mechanism by which sustained cannabinoid exposure may precipitate these neuropsychiatric disorders is unknown. A recent behavioral report has provided evidence suggesting that sustained cannabinoid agonist use can enhance 5-HT_{2A} receptor signaling [32]. Yet this study did not identify the molecular mechanisms by which cannabinoid receptors would modify 5-HT_{2A} receptor signaling. Cannabinoid-induced increases in 5-HT_{2A} receptor function and/or expression in the PFCx would be of particular interest since enhanced 5-HT_{2A} receptor function and/or expression in this area of the brain have been linked with the pathophysiology of neuropsychiatric disorders such as anxiety and schizophrenia [31;143-145;197].

Our results show that repeated treatment with non-selective CB_1/CB_2 receptor agonist, CP55940, can significantly enhance 5-HT_{2A} receptor-mediated PLCβ signaling, increase 5-HT_{2A} receptor-membrane associated protein levels (two-fold), and increase 5-HT_{2A} receptor mRNA levels (two-fold) in the PFCx of rodents [263;314]. Cannabinoid-induced increases in the

transcription of 5-HT_{2A} receptor gene and translation of 5-HT_{2A} receptor mRNA could explain the increases in 5-HT_{2A} receptor protein levels which could contribute to the enhanced 5-HT_{2A} receptor function. To examine this mechanism, we wanted to identify suitable neuronal cell culture models for studying cannabinoid-induced upregulation and enhanced activity of 5-HT_{2A} receptors. We identified two neuronal cell culture models, CLU213 and A1A1v cells, which endogenously co-express the CB_1, CB_2, and 5-HT_{2A} receptors [314]. Furthermore, we found that repeated CP55940 (non-selective CB_1/CB_2 agonist) treatment, significantly increased 5-HT_{2A} receptor PLCβ activity, increased 5-HT_{2A} membrane-associated and total protein levels, and increased 5-HT_{2A} mRNA levels (two-fold) in CLU213 and A1A1v cells [263;314]. Moreover, we found that repeated CP55940 treatment, but not a single CP55940 treatment, significantly increased 5-HT_{2A} receptor protein levels [262]. This evidence indicated that these neuronal models would be suitable to study the molecular mechanisms underlying cannabinoid-induced upregulation and enhanced activity of 5-HT_{2A} receptors. However, we primarily used CLU213 cells to study the molecular mechanisms by which cannabinoid agonists induce the upregulation and enhanced activity of 5-HT_{2A} receptors because: (1) they mimic our *in vivo* results, (2) they do not show signs of aging in the first 20 passages (such as vacuole formation), and (3) they are easy to transfect so that so that we are able to use biochemical approaches to further validate pharmacological findings.

Since CP55940 is a non-selective CB_1/CB_2 agonist, we moved forward to examine the relative roles of CB_1 and/or CB_2 receptors in cannabinoid-induced upregulation and enhanced activity of 5-HT_{2A} receptors in CLU213 cells. To accomplish this we used selective CB_1 or CB_2 receptor agonists, selective CB_1 or CB_2 antagonists, and CB_1 or CB_2 shRNA lentiviral particles to determine the contribution of these cannabinoid receptors to the regulation of 5-HT_{2A} receptor

expression and function. Our results indicate that this cannabinoid-induced upregulation and enhanced activity of 5-HT_{2A} receptors is mediated by CB_2 receptors. Indeed, selective CB_2 receptor agonists (GP1a and JWH133), but not selective CB_1 receptor agonist (ACEA), significantly increased 5-HT_{2A} receptor mRNA levels, protein levels, and PLCβ activity (two-fold) [263;314]. We also found that repeated GP1a treatment significantly increased serotonin stimulated 5-HT_{2A} receptor mediated Ca^{2+} release (two-fold increase) without significantly affecting the EC50 which could be explained, at least in part, by the enhanced cannabinoid-induced over-expression (two-fold) of 5-HT_{2A} receptors [335]. Furthermore, selective CB_2 receptors antagonists (JTE907 and AM630), but not selective CB_1 receptor antagonist (PF514273), prevented the cannabinoid-induced upregulation of 5-HT_{2A} receptors [263;314]. Moreover, we found that CB_2 shRNA lentiviral particles, but not CB_1 shRNA lentiviral particles, prevented the cannabinoid-induced upregulation and enhanced activity of 5-HT_{2A} receptors in CLU213 cells [262]. Taken together, this evidence strongly implicates the CB_2 receptor in the cannabinoid-induced upregulation of 5-HT_{2A} receptors and would suggest that cannabinoid induced upregulation of the 5-HT_{2A} receptor could contribute to the enhanced function of the 5-HT_{2A} receptors.

Our next experiments were conducted to identify some signaling components that would mediate the upregulation of 5-HT_{2A} receptors. Previous studies indicate that the CB_2 receptor can couple to Gαi and Gαo G-proteins to negatively regulate adenylyl cyclase/cAMP signaling cascade and positively regulate the ERK1/2 signaling cascade [246;247]. We used pertussis toxin (PTX) to examine the role of the Gαi and Gαo G-proteins in the selective CB_2 agonist (GP1a)-induced upregulation of 5-HT_{2A} receptors as pertussis toxin has been shown to inactivate the Gαi and Gα0 subunits via interference with Gα/receptor coupling [213;214]. We found that pertussis

toxin significantly reduced GP1a-induced increased in 5-HT_{2A} receptor mRNA and protein levels in CLU213 cells [263]. This evidence suggests that the GP1a-induced upregulation of 5-HT_{2A} receptors may involve a $G\alpha_{i/o}$ G-protein mechanism. Additionally, we studied the effect of an adenylyl cyclase activator (NHK 477) on GP1a-induced upregulation of 5-HT_{2A} receptors. We found that pretreatment with the adenylyl cyclase activator significantly reduced cannabinoid-induced increases in 5-HT_{2A} receptor mRNA levels [263]. We also tested the effect of a potent ERK1/2 inhibitor (PD198306) on the selective CB_2 agonist (GP1a)-induced increases in 5-HT_{2A} receptor mRNA levels and membrane-associated 5-HT_{2A} protein levels in neuronal cells. We found that ERK1/2 inhibitor pretreatment prevented the GP1a-induced increases in 5-HT_{2A} receptor mRNA levels and membrane-associated protein levels in neuronal cells [314]. This would indicate that the GP1a-induced upregulation and increased protein expression of 5-HT_{2A} receptors requires ERK1/2 activation and GP1a-induced upregulation of 5-HT_{2A} receptors. Since ERK1/2 can be activated by phosphorylation (pERK1/2) in the cytoplasm and then translocate to the nucleus to regulate transcription factors, we measured nuclear-associated levels of pERK1/2 as an index of CB_2 receptor-induced ERK activation [98;288]. We found that ERK1/2 inhibitor (PD198306) pretreatment also prevented GP1a-induced increases in nuclear associated pERK protein levels [314]. This evidence might indicate that CB_2 receptor agonists would induce an increase in the nuclear levels of pERK that would mediate the upregulation of 5-HT_{2A} receptor mRNA and increase synthesis of the 5-HT_{2A} receptor protein.

The ERK1/2 signaling cascade could be modulating the cannabinoid-induced upregulation of 5-HT_{2A} receptor through regulation of transcription factors. Activation of the ERK1/2 signaling cascade has been shown to regulate transcription factors such as CREB, c-Fos, SP-1 and EGR-1 [231;232]. Proteins belonging to the c-Fos and c-Jun family can heterodimerize

to form the AP-1 transcription factor. AP-1 and CREB have consensus sequences within the rat 5-HT_{2A} receptor promoter region [235;236]. We used inhibitors of AP-1 and CREB to examine their role in the cannabinoid-induced upregulation of 5-HT_{2A} receptors in CLU213 cells. Our results show that inhibition of AP-1, but not the CREB, significantly decreased the GP1a-induced upregulation of 5-HT_{2A} receptors in CLU213 cells [263]. This evidence would indicate that AP-1 is involved in the cannabinoid-induced upregulation of 5-HT_{2A} receptors. Additionally, we also found that inhibition of ERK1/2 with PD198306 prevented the GP1a-induced increases in nuclear-associated c-Fos levels in CLU213 cells [263].

GPCRs have been shown to activate ERK1/2 signaling in either a G-protein or β-Arrestin dependent pathway [15;88]. G-protein regulated activation of ERK1/2 has been shown to be transient and peaks within 2-5 minutes [171;300] while β-Arrestins can form a scaffolding complex with Raf-1, MEK, and ERK1/2 which can regulate the long-term activation of ERK1/2 [171;257;300]. The CB_2 receptor could regulate long-term ERK1/2 activation through a mechanism that may involve the β-Arrestin-ERK1/2 scaffolding complex [171;257;300]. Specifically, CB_2 receptors, which are a class A GPCR, would preferentially interact with β-Arrestin 2 and may regulate long-term ERK1/2 activation through β-Arrestin 2 [301]. Interestingly, we detected a two-fold increase in the co-immunoprecipitation of β-Arrestin 2 and ERK1/2 in PFCx of CP55940-treated rats compared to vehicle treated controls [100]. Furthermore, in cells transfected with β-Arrestin 2 shRNA lentiviral particles the CP55940, JWH133, and GP1a-induced upregulation of 5-HT_{2A} receptors was significantly reduced [262]. Additionally, we found that β-Arrestin 2 shRNA lentiviral particle treatment significantly reduced cannabinoid-induced increases in nuclear-associated pERK compared to vehicle treated controls [100]. This evidence implicates β-Arrestin 2 in the cannabinoid-induced upregulation of

5-HT_{2A} receptor and suggests that β-Arrestin 2 may be modulating these effects through enhanced β-Arrestin 2/ERK scaffolding complex formation and subsequent regulation of the ERK1/2 signaling cascade.

Of note, recent studies demonstrate that some GRKs, such as GRK5 and/or GRK6, can regulate β-Arrestin 2 signaling-mediated ERK1/2 activation [317]. GRK5 and/or GRK6 could regulate the formation of the β-Arrestin 2 and ERK scaffolding complex through phosphorylation of specific residues on the CB_2 receptor [316;317]. Furthermore, over-expression of GRK5 and/or GRK6 has been found to lead to an intensification of β-Arrestin 2 mediated ERK1/2 [325]. Interestingly, we found that repeated CP55940 treatment increases CB_2 receptor phosphorylation and selectively increases GRK5 mRNA and protein expression in rat PFCx and in CLU213 cells without changes in the mRNA or protein levels of GRK6 [335]. Through the use of GRK5 shRNA lentiviral particles we identified that GRK5 is involved in the cannabinoid-induced upregulation and enhanced activity of 5-HT_{2A} receptors in CLU213 cells [335]. Indeed, treatment with GRK5 lentiviral particles significantly reduced the cannabinoid-induced upregulation of 5-HT_{2A} receptors [335]. Furthermore, GRK5 shRNA lentiviral particle treatment prevented cannabinoid-induced increases in serotonin stimulated 5-HT_{2A} receptor mediated Ca^{2+} release [335]. We also found that GRK5 shRNA lentiviral particle treatment reduced the cannabinoid-induced enhanced phosphorylation of the CB_2 receptor and enhanced β-Arrestin 2/ERK interaction in our neuronal cell culture model [335]. Although further experimental evidence is needed, we speculate that upregulation of GRK5 and enhanced phosphorylation of the CB_2 receptor and subsequent formation of the β-Arrestin 2/ERK scaffolding complex could be contributing to the upregulation and enhanced activity of 5-HT_{2A} receptors. We also speculate that the cannabinoid-induced upregulation of GRK5 could be

contributing to an intensification of the β-Arrestin 2/ERK signaling mechanism. Interestingly, we have found that two GP1a treatments enhance pERK levels significantly over a single GP1a treatment without significantly modifying total ERK1/2 levels in CLU213 cells [314].

Currently, the exact role of cannabinoid-induced changes in CB_2 receptor trafficking in upregulation of 5-HT_{2A} receptors is unknown. Some of our evidence would suggest that internalization of the CB_2 receptor would be needed for the cannabinoid-induced upregulation of 5-HT_{2A} receptors. Different approaches are commonly used to prevent clathrin-mediated internalization of receptors such as concanavalin A treatment, use of hypertonic sucrose, or depletion of intracellular potassium [297;298]. We have found that pretreatment with concanavalin A prevents cannabinoid-induced increases in 5-HT_{2A} receptor mRNA levels [262]. Furthermore, we found that cannabinoid treatment increased cytosolic levels of CB_2 receptors and concanavalin A pretreatment significantly reduced cannabinoid-induced increases in CB_2 cytosolic levels [262]. Moreover, cannabinoid-induced increases in nuclear-associated pERK levels were significantly reduced by concanavalin A pretreatment [262]. We have also examined the effects of efficacious CB_2 receptor internalizers and poor CB_2 receptor internalizers on the upregulation of 5-HT_{2A} receptors. Atwood et al. have recently identified different classes of cannabinoids agonists that differ substantially in their ability to induce CB_2 receptor internalization [293]. Cannabinoid agonists such as CP55940 and JWH133 were classified as efficacious CB_2 receptor internalizers while cannabinoids such as JWH073 and WIN 55,212-2, aminoalkylindoles, were classified as poor CB_2 receptor internalizers [293]. We have found that CP55940 and JWH133-induced upregulation of 5-HT_{2A} receptors while JWH073 treatment did not significantly modify 5-HT_{2A} receptor expression [262]. Taken together, this evidence would

indicate that internalization of the CB_2 receptor may play a role in the cannabinoid-induced upregulation of 5-HT_{2A} receptors.

Recent evidence has highlighted that 5-HT_{2A} receptor can form functional heteromers with D_2 receptors in the PFCx that would synergistically enhance the activity of 5-HT_{2A} receptors [196;198]. Therefore we examined the effect of chronic CP55940 treatment on the co-immunoprecipitation of 5-HT_{2A} and D_2 in the rat PFCx. We identified that repeated CP55940 treatment increased the co-immunoprecipitation of 5-HT_{2A} and D_2 receptors compared to vehicle treated controls as well as significantly increased D_2 receptor membrane-associated protein levels in the PFCx [263]. The mechanisms underlying the enhanced interaction between 5-HT_{2A} and D_2 receptors in PFCx is still not well defined but it could be modulated by increased membrane-associated expression of 5-HT_{2A} and D_2 receptor in this brain area and has been shown to involve the third intracellular loop and the C-tail of the D_2 and 5-HT_{2A} receptors, respectively [145]. Indeed, Lukasiewicz et al. determined through site directed mutagenesis that specific motifs including arginine residues (217RRRRRKR222) in the third intracellular loop (ic3) of the D_2 receptor and acidic glutamate residues (454EE455) in the C-tail of the 5-HT_{2A} receptor are needed for the formation of the 5-HT_{2A} and D_2 receptor heteromer [145].

Although most of our work shows a single time point for the mechanisms involved and further future studies are warranted to test the model, we propose the following potential series of events based upon the literature and some of our own key findings. We propose that repeated CB_2 receptor stimulation induces the upregulation of GRK5 which would enhance the phosphorylation of the CB_2 receptor. The enhanced phosphorylation of the CB_2 receptor would result in the following series of events: (1) enhanced β-Arrestin 2 recruitment to the CB_2 receptor; (2) enhanced formation of the β-Arrestin 2/ERK scaffolding complex; and (3)

intensitification of the β-Arrestin 2 mediated ERK1/2 activation (Fig. 29). Indeed, over-expression of GRK5 has been shown to intensify β-Arrestin 2 mediated activation of ERK1/2 [325]. After formation of the β-Arrestin 2/ ERK scaffolding complex, there would be enhance translocation of activated ERK (pERK) from the cytosol to the nucleus (Fig. 29). Nuclear-associated pERK would then regulate the activation of AP-1 which our evidence indicates is involved in the cannabinoid-induced upregulation of 5-HT_{2A} receptors (Fig. 29). Cannabinoid-induced upregulation of 5-HT_{2A} receptor may then contribute to enhanced formation of the 5-HT_{2A}/D_2 heteromer which has been linked with an enhancement of 5-HT_{2A} receptor activity (Fig. 29). We also speculate that: (1) changes in D_2 receptor expression may involve both the CB_1 and CB_2 receptors and (2) cannabinoid-induced changes in D_2 receptor expression may contribute to enhanced 5-HT_{2A} and D_2 interaction (Fig 29). Interestingly, formation of this functional 5-HT_{2A}/D_2 receptor heteromer in the PFCx has been suggested to contribute to the pathophysiology of neuropsychiatric disorders such as schizophrenia [145;197] while atypical antipsychotics have been shown to reduce the formation of this heteromer [197]. Activity of cortical 5-HT_{2A} receptors has been associated with several physiological functions and neuropsychiatric disorders such as stress response, anxiety, and depression [24;26;27]. Therefore, the results presented in this dissertation might provide a molecular mechanism by which repeated cannabinoid exposure may contribute pathophysiology of some neuropsychiatric disorders in humans.

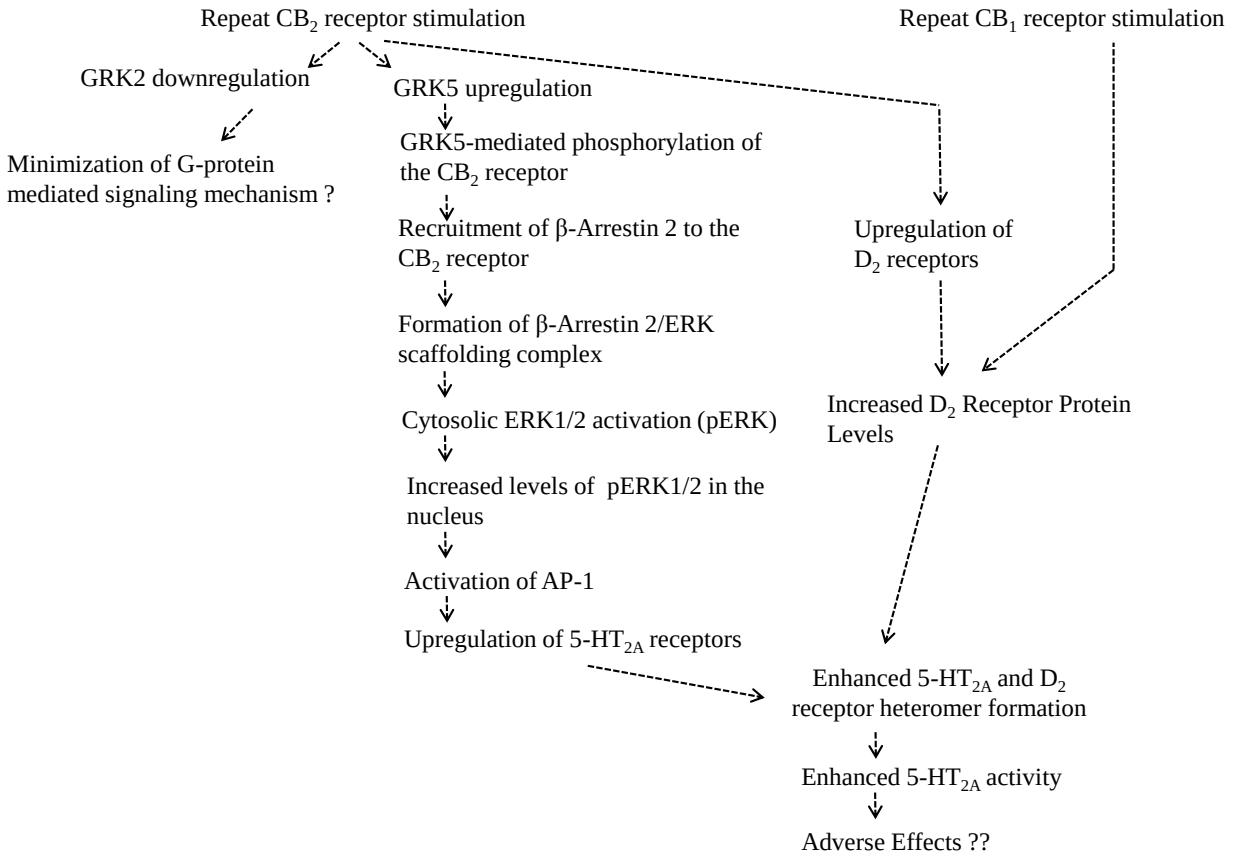


Figure 29. Proposed model for cannabinoid-induced upregulation and enhanced activity of 5-HT_{2A} receptors.

Chronic cannabinoid treatment induces the upregulation of 5-HT_{2A} receptors through a mechanism that involves GRK5 mediated enhanced phosphorylation of the CB_2 receptor, enhanced β-Arrestin 2 mediated activation of ERK1/2 signaling, and may involve internalization of the CB_2 receptor. This cannabinoid-induced upregulation and enhanced activity of 5-HT_{2A} receptor and enhanced 5-HT_{2A} and D_2 heteromer formation may contribute to some of the adverse effects associated with chronic cannabinoid exposure.

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