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Cannabinoid Agonists Increase the Interaction between β -Arrestin 2 and ERK1/2 and Upregulate β -Arrestin 2 and 5-HT_{2A} Receptors

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Abstract

We have recently reported that selective cannabinoid 2 (**CB**₂) receptor agonists upregulate 5-HT_{2A} receptors by enhancing ERK1/2 signaling in prefrontal cortex (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**, 50 μ g/kg, 7 days, i.p.) 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 because of their minimal effect on serotonergic neurotransmission.

Keywords

Cannabinoid receptors; 5-HT_{2A} receptor; ERK1/2; β -Arrestin 2; prefrontal cortex

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Conflict of Interest Statement:

The Authors declare that there is no conflict of interest.

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1. 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 [1–4]. 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 serotonin 2A (5-HT_{2A}) receptor neurotransmission in prefrontal cortex (PFCx), such as schizophrenia, psychosis and anxiety [5–8]. 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* [9;10].

Cannabinoid agonists can produce their physiological effects through the activation of two G-protein coupled cannabinoid receptors in the brain, **CB₁** and **CB₂** receptors [11;12]. **CB₁** and **CB₂** receptors bind endocannabinoids, synthetic cannabinoids, and cannabinoids found in nature (such as *Cannabis sativa*) with high affinity [11;12]. Although only **CB₁** receptors were initially identified in the brain [13], later studies have identified **CB₂** receptors in several brain areas such as PFCx, hippocampus, amygdala, substantia nigra, and cerebellum [14;15], triggering a reevaluation of the possible roles that **CB₂** 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 a either G-protein or β -Arrestin dependent pathway [12;16]. While G-protein-mediated activation of ERK1/2 is transient and peaks within 2–5 minutes [17;18], 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 [17–19].

We recently reported that 5-HT_{2A} receptors are upregulated by repeated exposures to cannabinoid agonists through a mechanism that would involve **CB₂** receptor-mediated activation of ERK1/2 signaling, and that is independent of **CB₁** receptor activation [9;10]. Moreover, we presented experimental evidence that sustained treatment with a non-selective cannabinoid agonist (**CP55940**) or selective **CB₂** receptor agonists (**JWH-133** or GP1a) upregulate 5-HT_{2A} receptors in a neuronal cell line [10], an effect that was not replicated by selective **CB₁** agonists [10]. The **CB₂** receptor is a class A GPCR which means it would preferentially interact with β -Arrestin 2 to form a scaffolding complex with ERK1/2 [20]. **Accordingly**, we also reported that the cannabinoid receptor agonist-induced upregulation of 5-HT_{2A} receptors was prevented in cells stably transfected with either **CB₂** or β -Arrestin 2 shRNA lentiviral particles [10].

Here we examined mechanisms which could contribute to the **CB₂** receptor- and ERK1/2-mediated enhanced activation of 5-HT_{2A} receptors. We studied the involvement of selective **CB₁** and **CB₂** 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 **CB₂** receptors, activation of ERK1/2, and activation of the transcription factor AP-1. We also detected a strong **CB₂**, but not **CB₁**, 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.

2. Materials and Methods

2.1 Drugs

(-)-*cis*-3-[2-Hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol (**CP55940**), a **CB₁** and **CB₂** receptor agonist; (6*aR*,10*aR*)-3-(1,1-Dimethylbutyl)-6*a*,7,10,10*a*-tetrahydro-6,6,9-trimethyl-6*H*-dibenzo[*b,d*]pyran (**JWH-133**) a selective **CB₂** agonist; N-(Piperidin-1-yl)-1-(2,4-dichlorophenyl)-1,4-dihydro-6-methylindeno[1,2-*c*]pyrazole-3-carboxamide (GP1*a*) a highly selective **CB₂** receptor agonist; N-(2-Chloroethyl)-5*Z*,8*Z*,11*Z*,14*Z*-eicosatetraenamido (ACEA) a highly selective **CB₁** 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 **CB₁** receptor antagonist; *N*-(1,3-Benzodioxol-5-ylmethyl)-1,2-dihydro-7-methoxy-2-oxo-8-(pentyloxy)-3-quinolinecarboxamide (**JTE-907**) a selective **CB₂** 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 (**JWH-073**), a **CB₁/CB₂** receptor agonist, was synthesized in the laboratory of Dr. Thomas Prisinzano as described previously [21].

2.2 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); 1 mL/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 [22;23] and upregulation of 5-HT_{2A} receptors in rat PFCx [9]. 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.

2.3 Co-Immunoprecipitation

Co-immunoprecipitation (co-IP) was conducted with the Thermo Scientific Pierce co-IP kit following manufacturer's protocol and as previously described [9]. 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 μ g) 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 [10;24;25].

2.4 Western Blot

Membrane-associated fractions were isolated using the ProteoExtract™ 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 [26]. pERK antibody was purchased from Santa Cruz, CA. The specificity of the antibody has been verified in the literature [27]. 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 [26;28]. All samples were standardized to controls and normalized to their respective actin levels.

2.5 CP55940-induced ERK1/2 activation in PFCx tissue

This assay was based upon a PLC β assay that has been previously described [26]. 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 4°C for 10 minutes. The supernatant was removed, 500 μ L of homogenate buffer was added to the pellet, samples were vortexed and centrifuged at 13,000 rpm at 4°C 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.

2.6 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 was analyzed using the comparative cycle threshold (Ct) method as previously described [9]. The primers used in this manuscript were: 5-HT_{2A} (F:5'-AACGGTCCATCCACAGAG-3' and R:5'-AACAGGAAGAACACGATGC-3'), β -Arrestin 2 (F:5'-AGCACCGCGCAGTACAAGT-3', 5'-R:CACGCTTCTCTCGGTTGTCA-3') and GAPDH (F: 5'-TGGAGTCTACTGGCGTCTTCAC-3' and R:5'-GGCATGGACTGTGGTCATGA-3'). These primers have been previously validated in the literature [9;10;29;30].

2.7 Cell Culture Protocol

CLU213 cells, a **rat** neuronal cell line that co-expresses 5-HT_{2A}, D₂, **CB₁** and **CB₂** receptors, were purchased from Cedarlane Laboratories (Burlington, NC). CLU213 were grown on 100-mm² plates treated with polystyrene (Corning Incorporated, Corning, NY) and maintained in 5% CO₂ at 37°C, in Dulbecco's modified eagle medium (DMEM; Mediatech Inc, Manassas, VA) containing 10% fetal bovine serum (FBS; Thermo Scientific, Logan, UT).

2.7.1 Effect of Non-Selective and Selective CB₁ and CB₂ 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 (**CB₁** and **CB₂** agonist, K_i: 0.58

nM and 0.68 nM for **CB₁** and **CB₂** receptors, respectively) [31]; **JWH-133** 30 nM (selective **CB₂** agonist, K_i : 3.4 nM and 677 nM for **CB₂** and **CB₁** receptors, respectively) [32], GP1a 1 nM (highly selective **CB₂** agonist, K_i : 0.037 nM and 353 nM for **CB₂** and **CB₁** receptors, respectively) [33], ACEA 15 nM (selective **CB₁** agonist, K_i : 1.4 nM and 3.1 μ M for **CB₁** and **CB₂** receptors, respectively) [34] or **JWH-073** 40 nM (**CB₁** and **CB₂** agonist, K_i : 8.9 nM and 38 nM for **CB₁** and **CB₂** receptors, respectively) [21;35] 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), **JWH-133** (30 nM) or GP1a (1 nM) for 72 hours. Cells were washed (3x) with PBS every 24 hours and fresh vehicle, **CP55940**, **JWH-133** or GP1a were added. Expression of membrane-associated β -Arrestin 2 was determined by Western blot as previously described.

2.7.2 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 (**CB₁** antagonist, K_i : 1 nM and >10,000 nM for **CB₁** and **CB₂** receptor, respectively) [36]; or **JTE-907** 10 nM (**CB₂** antagonist, K_i : 0.38 and 1,050 nM for **CB₂** and **CB₁** receptors, respectively) [37]. 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.

2.7.3 Effect of selective CB₂ 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 **CB₂** receptor antagonist) [37]. 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.

2.7.4 Lentivirus and stable transduction of shRNAs in CLU213 cells

— β -Arrestin 2 shRNA (r), **CB₂** shRNA (r), **CB₁** shRNA (r), copGFP control, control shRNA lentiviral particles, polybrene, and puromycin 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 **CB₂** shRNA lentiviral particles and then transduction was conducted as previously described [10]. Cells were analyzed for β -Arrestin 2, **CB₂**, or **CB₁** knockdown one week after initiation of puromycin selection.

2.7.5 Effect of CB₂ or CB₁ shRNA lentivirus transfection on cannabinoid-induced upregulation of β -Arrestin 2

—After confirming that treatment with the **CB₂** or **CB₁** shRNA lentivirus reduced **CB₂** or **CB₁** mRNA 80% and 70%, respectively, control, **CB₂**, or **CB₁** shRNA treated cells were treated with either vehicle (ethanol 0.01% final concentration), **CP55940** 1 nM, **JWH-133** 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.

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

—Cells were pretreated with either vehicle or 250 μ g/mL ConA for 20 minutes [10;38]. 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.

2.7.7 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) [10]. 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.

2.7.8 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.

2.7.9 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 μ M) [39] or SR11302 (1 μ M) [40] 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.

2.7.10 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 **JWH-073** 40 nM for 24 hours. mRNA was isolated and qRT-PCR for β -Arrestin 2 mRNA was performed.

2.8 Statistics

All data are expressed as the mean \pm 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. Results

3.1 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 CB₂ receptor regulation of ERK1/2 activation. [9;10]. Cannabinoid receptors could produce a long-term ERK1/2 activation by a mechanism that may involve a β -Arrestin-ERK1/2 scaffolding complex [17–19]. Specifically, CB₂ 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 [20]. 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. 1. A). 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. 1. A, 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. 1. A, 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. 1. A, lanes 5 & 6).

3.2 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 *in vitro* 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 ($78 \pm 5\%$ increase in CP55940 compared to controls, Fig. 1. B). No significant differences ($p > 0.05$) in total ERK1/2 protein levels were detected between both experimental groups.

3.3 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. 1.A); and (2) ERK1/2 signaling pathway (Fig. 1.B) in PFCx of CP55940-treated rats compared to vehicle controls. Here, we examined the effect of repeated CP55940 ($50\mu\text{g}/\text{kg}$ 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 ($108 \pm 13\%$ increase compared to controls, Fig. 1.C). Interestingly, CP55940 treatment did not significantly alter ($p > 0.05$) total ERK1/2 protein levels in rat PFCx (Fig. 1.D). Actin was used as a protein loading controls 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 ($107 \pm 15\%$ increase compared to controls, Fig. 1.E).

3.4 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 previous shown that GP1a (a selective CB₂ receptor agonist) upregulates 5-HT_{2A} receptors while treatment with ACEA (a selective CB₁ receptor agonist) does not significantly alter 5-HT_{2A} receptor mRNA levels [9]. Treatment with β -Arrestin 2 shRNA lentiviral particles significantly ($p < 0.01$) reduced β -Arrestin 2 mRNA and protein levels by approximately 85% [10]. 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 $109 \pm 2\%$ (Fig. 1.F). 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. 1.F). The two-way ANOVA showed main effects of transfection ($F_{1,63.37} = 0.39$, $p < 0.0001$), CB₂ agonist ($F_{3,71.59} = 0.44$, $p < 0.0001$), and a main interaction between these two factors ($F_{3,61.22} = 0.38$, $p < 0.0001$) 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 later protein is necessary for the

cannabinoid-induced upregulation of 5-HT_{2A} receptors as indicated in studies conducted in our neuronal cell model.

3.5 CP55940 and selective CB₂ 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** (CB₁/CB₂ receptor agonist, 1nM), selective CB₂ receptor agonists **JWH-133** (30nM) or GP1a (1nM), or a selective CB₁ 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 ($67 \pm 5\%$ increase, Fig. 2.A). Noteworthy, sustained treatment with either **JWH-133** or GP1a, but not ACEA, significantly increased ($p < 0.01$) β -Arrestin 2 mRNA levels ($72 \pm 5\%$ and $64 \pm 4\%$ increase compared to controls, respectively)(Fig. 2.A). We also determined what effect **CP55940**, **JWH-133**, and GP1a treatment had on β -Arrestin 2 protein levels. We found that treatment with **CP55940** (1nM), **JWH-133** (30nM), and GP1a (1nM) also significantly increased ($p < 0.01$) β -Arrestin 2 protein levels compared to vehicle treated controls ($105 \pm 26\%$, $120 \pm 12\%$, and $143 \pm 21\%$ increase compared to vehicle controls, respectively)(Fig. 2B).

3.6 A selective CB₂ 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 CB₂ receptors. In order to examine the relative roles of CB₁ and CB₂ receptors in the upregulation of β -Arrestin 2, cells were pretreated with either vehicle, a selective CB₁ receptor antagonist **PF-514273** (20nM), or a selective CB₂ 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 ($75 \pm 2\%$ increase compared to controls, Fig. 2.C). 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 CB₂ receptor antagonist, **JTE-907**, prevented the **CP55940**-induced increases in β -Arrestin 2 mRNA levels ($p < 0.01$, Fig. 2.C). 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 ($F_{2,37.3} = 0.28$, $p < 0.0001$), cannabinoid agonists ($F_{1,143.3} = 1.08$, $p < 0.0001$), and a main interaction between these two factors ($F_{2,36.9} = 0.28$, $p < 0.0001$) on β -Arrestin 2 mRNA levels.

In order to more thoroughly investigate the role of CB₂ receptors in the cannabinoid-induced upregulation of β -Arrestin 2 mRNA levels, we pretreated cells with either vehicle or selective CB₂ antagonist **JTE-907** and then treated cells with vehicle or a highly selective CB₂ receptor agonist GP1a. As expected, GP1a treatment significantly ($p < 0.01$) increased β -Arrestin 2 mRNA levels ($74 \pm 5\%$ increase compared to controls, Fig. 2.D). This GP1a-induced upregulation of β -Arrestin 2 was prevented ($p < 0.01$) in cells pretreated with **JTE-907** (Fig. 2.D). The two-way ANOVA showed main effects of **JTE-907** pretreatment ($F_{1,35.4} = 0.28$, $p < 0.0003$), GP1a treatment ($F_{1,68.3} = 0.54$, $p < 0.0001$), and a main interaction between these two factors ($F_{1,37.1} = 0.29$, $p < 0.0003$) on β -Arrestin 2 mRNA levels.

3.7 CB₂, but not CB₁, shRNA lentiviral particle treatment prevents cannabinoid-induced increases in β -Arrestin 2 mRNA level

We also used cells stably transfected with control, CB₂, or CB₁ shRNA lentiviral particles to study whether the effect of cannabinoid agonists on β -Arrestin 2 mRNA levels. CB₁ and

CB₂ mRNA levels were significantly reduced by approximately 75–80% in neuronal cells treated with either **CB₁** or **CB₂** shRNA lentiviral particles, respectively (Fig. 3.A). We have previously reported similar reductions (75–80%) in protein levels of these receptors after transfection with these shRNA lentiviral particles [10].

Control or **CB₂** 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 ($76 \pm 3\%$, $65 \pm 2\%$, and $72 \pm 5\%$ increase, respectively)(Fig 3B). Noteworthy, **CB₂** shRNA treatment prevented ($p < 0.01$) the **CP55940**, **JWH-133**, and GP1a induced increases in β -Arrestin 2 mRNA levels. **CB₂** 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 ($F_{3,97.9} = 0.83$, $p < 0.001$) and cannabinoid agonist treatment ($F_{1,33.2} = 0.28$, $p < 0.0001$). There was a significant interaction between transfection and cannabinoid agonist treatment ($F_{3,23.1} = 0.19$, $p < 0.0001$).

We also determined what effect **CP55940**, **JWH-133** and GP1a treatment had on β -Arrestin 2 upregulation in control or **CB₁** 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 ($68 \pm 3\%$, $74 \pm 5\%$, and $71 \pm 5\%$ increase, respectively)(Fig. 3.C). Noteworthy, treatment with **CB₁** 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. 3. C). The two-way ANOVA for β -Arrestin 2 mRNA showed significant main effects of transfection ($F_{1,7.05} = 0.03$, $p < 0.0173$) and cannabinoid agonist treatment ($F_{3,180.67} = 0.75$, $p < 0.0001$). There was no significant interaction between transfection and cannabinoid agonist treatment ($F_{3,0.11} = 0.0005$, $p > 0.9526$). This evidence indicates that **CB₂**, but not **CB₁**, receptors, would mediate the cannabinoid-induced upregulation of β -Arrestin 2.

3.8 Effect of cannabinoid receptor internalization on the **CB₂**-mediated upregulation of β -Arrestin 2 mRNA

Next, we investigated whether **CB₂** 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 **CB₂** receptors [41]. Specifically, **CB₂**-induced ERK1/2 activation may require the internalization of membrane-associated **CB₂** receptors [41]. Here, we used two different approaches to study the role of internalization of **CB₂** receptors on β -Arrestin 2 upregulation: (1) ConA treatment to prevent **CB₂** receptor internalization [10]; and (2) different cannabinoid agonists that are either good or poor internalizers of **CB₂** receptors [41].

Currently, there are several protocols that are commonly used to prevent clathrin-mediated internalization of GPCR which include: ConA, hypertonic sucrose, or depletion of intercellular potassium [38]. 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 **CB₂** receptors from the membrane to the cytosol and prevented the cannabinoid-induced ERK1/2 activation [10]. **CP55940** and GP1a significantly ($p < 0.01$) increased β -Arrestin 2 mRNA levels compared to vehicle treated cells ($76 \pm 3\%$, and $77 \pm 3\%$ increase, respectively) (Fig. 4. A). ConA pretreatment significantly ($p < 0.01$) blocked the effect of **CP55940** and GP1a on β -Arrestin 2 mRNA levels compared to controls (Fig. 4. A). The two-way ANOVA showed a main effect of ConA ($F_{1,107} = 0.93$, $p < 0.0001$), cannabinoid

agonists ($F_{2,35} = 0.31$, $p < 0.0001$), and a main interaction between these two factors ($F_{2,32} = 0.276$, $p < 0.0001$) 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 [41]. Indeed, **CP55940** and GP1a would be efficacious CB_2 receptor internalizers while aminoalkylindoles, such as **JWH-073**, were classified as poor CB_2 receptor internalizers [41]. 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 4B). These results obtained seems to indicate that CB_2 internalization of CB_2 receptors is a critical step in the upregulation of β -Arrestin 2 mRNA.

3.9 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 [16]. We used PD198306, a selective ERK1/2 inhibitor [42], 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 (approx. 9,000- and 200-fold selectivity between CB_2/CB_1 receptors for GP1a and **JWH-133**, respectively) [32;33]. 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 ($104 \pm 10\%$ increase, Fig 5A). This GP1a-induced upregulation of β -Arrestin 2 was prevented ($p < 0.01$) in cells pretreated with PD198306 (Fig. 5A). The two-way ANOVA for β -Arrestin 2 mRNA showed significant main effects of PD198306 pretreatment ($F_{1,30,2} = 0.54$, $p < 0.0006$) and GP1a treatment ($F_{1,48,8} = 0.87$, $p < 0.0001$). There was a significant interaction between PD198306 and GP1a treatment ($F_{1,41,6} = 0.74$, $p < 0.0002$).

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 [43]. 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. 5.B). 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. 5.B). 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 ($F_{1,429084} = 39.41$, $p < 0.0001$) and **CP55940** treatment ($F_{1,444167} = 40.79$, $p < 0.0001$). There was a significant interaction between transfection and **CP55940** treatment ($F_{1,191213} = 175.7$, $p < 0.0001$).

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 5C). β -Arrestin 2 shRNA lentivirus transfection significantly ($p < 0.01$) reduced GP1a-induced increases in pERK levels (Fig

5C). 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 ($F_{1,131794} = 18.3$, $p < 0.0001$) and GP1a treatment ($F_{1,142369} = 19.8$, $p < 0.0008$). There was a significant interaction between transfection and GP1a treatment ($F_{1,252455} = 350.5$, $p < 0.0001$).

3.10 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 [43]. The transcription factors CREB and AP-1 have consensus sequences within the promoter region of the rat β -Arrestin 2 gene [44]. 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 [45]. 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 [46]. 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 ($10\mu\text{M}$) for 20 min and then treated with vehicle or GP1a (1nM). Naphthol AS-E phosphate blocks cAMP-induction of CREB-dependent gene transcription ($K_i = 10\mu\text{M}$) [39]. We found that Naphthol AS-E phosphate did not inhibit or significantly decrease GP1a-induced increases in β -Arrestin 2 mRNA (Fig. 6.A). 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 ($F_{1,0.004} = 0.038$, $p > 0.0557$) and a significant main effect of GP1a treatment ($F_{1,1.29} = 116.6$, $p < 0.0001$). There was no significant interaction between Naphthol AS-E pretreatment and GP1a treatment ($F_{1,0.007} = 0.683$, $p > 0.683$) on.

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 ($1\mu\text{M}$) 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 ($E_{\text{max}} = 1\mu\text{M}$) [47]. As expected, GP1a induced a significant ($p < 0.01$) increase in β -Arrestin 2 mRNA levels (Fig. 6.B). SR11302 pretreatment significantly reduced (approximately 49% decrease, $p < 0.01$) the GP1a-induced upregulation of the β -Arrestin 2 mRNA (Fig. 6B). 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 ($F_{1,0.12} = 4.46$, $p > 0.0676$) but did show a significant effect of GP1a treatment ($F_{1,0.88} p < 0.0005$). There was a significant interaction between SR11302 pretreatment and GP1a treatment ($F_{1,0.24} = 8.71$, $p < 0.0184$). These results suggest that AP-1, but not CREB, would mediate at least in part the GP1a-induced upregulation of β -Arrestin 2.

4. Discussion

β -Arrestin 1 and 2 were initially found to hinder G protein coupling of agonist-activated G-protein coupled receptors (GPCR) 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 [17–19]. 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 [48;49]. It was found that G protein-coupled receptor kinases (GRKs) could terminate this agonist-induced signaling response through phosphorylation of the receptor [17]. 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 [17]. 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 [48;49].

Interestingly, previous reports showed that chronic exposure to THC (tetrahydrocannabinol, **CB₁/CB₂** receptor agonist) upregulates β -Arrestin1 in striatum and β -Arrestin 2 in cerebellum and hippocampus [50] but did not upregulate total ERK1/2 levels in hippocampus [51]. 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 [48;49]. After internalization of the GPCR, evidence indicates that the scaffolding complex can continue to regulate signaling cascades [19].

Our results indicate that exposure to **CP55940**, a **CB₁/CB₂** receptor agonist, increases the interaction between β -Arrestin 2 and ERK1/2 in rat PFCx (Fig. 1.A). Co-immunoprecipitation has been successfully used by some groups to demonstrate an interaction between β -Arrestin 2 and its scaffolding proteins [52–54]. 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. 1.B). 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. 1.C and 1D). 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. 1.E) suggesting that increases in β -Arrestin 2 expression most likely occurs 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. 1.F). 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 **CB₁** and **CB₂** receptor agonists or antagonists, and **CB₁** and **CB₂** shRNA lentiviral particles to determine the contribution of **CB₁** and **CB₂** receptors to the regulation of β -Arrestin 2 mRNA levels. In our neuronal cell line which expresses **CB₁** and **CB₂** receptors, we found that treatment with the non-selective **CB₁/CB₂** agonist **CP55940** and treatment with the selective **CB₂** receptors agonists, **JWH-133** and **GP1a**, increased β -Arrestin 2 mRNA and protein levels (Fig. 2.A and 2B). This cannabinoid-induced upregulation of β -Arrestin 2 was not mimicked by a selective **CB₁**

agonist (ACEA) (Fig. 2.A) suggesting that **CB₂** receptors may be mediating this response. Additionally, we found that a selective **CB₂** receptor antagonist **JTE-907**, but not a selective **CB₁** receptor antagonist **PF-514273**, prevented the **CP55940**-induced increases in β -Arrestin 2 mRNA levels (Fig. 2.C). Moreover, treatment with **CB₂** shRNA lentiviral particles, but not **CB₁** shRNA lentiviral particles, prevented the **CP55940**, **JWH-133**, and GP1a-induced increases β -Arrestin 2 mRNA levels (Fig. 3.B and 3C). This evidence indicates the **CB₂** receptors are required for the cannabinoid-induced upregulation of β -Arrestin 2.

Recent evidence indicates that **CB₂** receptor ligands can distinctly regulate the signal transduction mechanisms associated with the **CB₂** receptors, a phenomenon known as functional selectivity [19;41;55]. Atwood et al. have identified different classes of cannabinoid receptor agonists which differ in their ability induce internalization of **CB₂** receptors [41]. They generalize that bicyclic cannabinoid such as **CP55940** would be efficacious **CB₂** receptor internalizers while aminoalkylindoles would be poor **CB₂** receptor internalizers. We found that **CP55940**, but not **JWH-073**, induced β -Arrestin 2 upregulation (Fig. 4.B), suggesting that upregulation of β -Arrestin 2 is dependent on the ability of the agonists to promote internalization of **CB₂** receptors. To further explore this possibility, we pretreated cells with ConA which is commonly used to prevent clathrin mediated endocytosis of GPCRs [38;56] 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. 4.A). This evidence further suggests that internalization of the **CB₂** 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 **CB₂**-induced upregulation of β -Arrestin 2 [12;42]. Previous studies have identified that 200 nM of PD198306 can prevent ERK1/2 activation in breast cancer cells [42]. We found the PD198306 pretreatment prevented the selective **CB₂** receptor agonist induced increases in β -Arrestin 2 mRNA levels (Fig. 5.A). This evidence suggests that **CB₂** receptors can induce the upregulation of β -Arrestin 2 through a mechanism that involves ERK activation. Currently, the mechanism by which **CB₂** 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 [19]. 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. 5.B and 5C). Yet treatment with **CP55940** and GP1a significantly increased nuclear pERK levels in β -Arrestin 2 shRNA lentivirus treated cells compared vehicle treated controls. This evidence suggests that **CB₂** receptors can mediate ERK1/2 activation, at least in part, through β -Arrestin 2. Here the **CP55940** and GP1a induced increased 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 [49]. However, internalization of GPCRs is typically mediated primarily by one isoform of β -Arrestin [49].

Our results suggest that inhibition of AP-1, but not CREB, significantly decreased the GP1a-induced upregulation of 5-HT_{2A} receptors (Fig. 6.A and 6B). 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 have been associated with several neuropsychiatric disorders such as schizophrenia, anxiety, and depression [6–8;57]. Interestingly, dysregulation of 5-HT_{2A} and D₂ receptor signaling has been associated with

schizophrenia, stress response, anxiety, and depression [58–61]. 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 [6–8;57]. 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 [62]. More importantly, recent evidence indicates that repeated exposure to a selective **CB₂** receptor agonist, **JWH-133**, induces anxiety-like behaviors in rodents that are blocked by a selective **CB₂** receptor antagonist [63]. 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 **CB₂** receptor-mediated upregulation of 5-HT_{2A} receptors [10] could contribute to the cannabinoid-induced enhanced interaction between 5-HT_{2A} and D₂ receptors in PFCx [9]. 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 **CB₂** 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 **CB₂** receptor-mediated upregulation of β -Arrestin 2 would be mediated, at least in part, by an ERK1/2-dependent activation of AP-1.

5. Conclusion

Emerging studies are identifying that selective **CB₂** receptor agonists such as **JWH-133** have wide therapeutic application in the treatment of conditions such as stroke, neurodegenerative diseases, and neuropathic pain [1–4]. However, the mechanism that we are currently defining could represent a potential adverse effect of the long-term use of selective **CB₂** receptor agonists such as **GP1a** and **JWH-133**. Repeated exposure to these agonists, that are efficacious **CB₂** receptor internalizers, could be associated with potential adverse effects as mentioned above. However, our evidence suggest that **CB₂** receptor agonists such as **JWH-073**, that are categorized as poor **CB₂** 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.

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Non-standards abbreviations

5-HT	5-hydroxytryptamine
5-HT_{2A}	Serotonin 2A
Δ^9-THC	Δ^9 -Tetrahydrocannabinol
ERK	extracellular regulated kinase
PFCx	prefrontal cortex
GPCR	G-protein coupled receptor
MAP	Mitogen Activated Protein kinase

pERK phosphorylated extracellular regulated kinase

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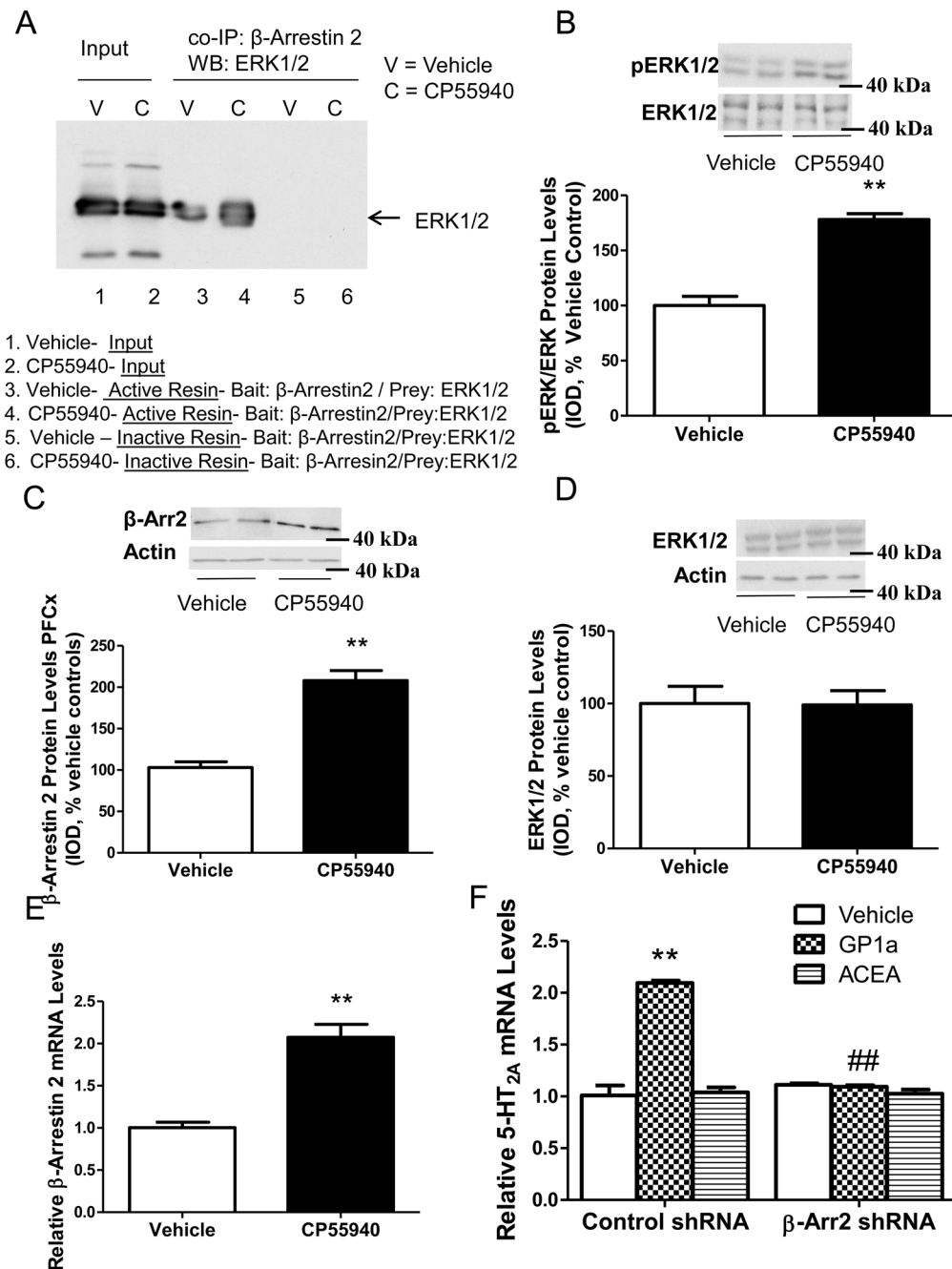


Figure 1. CP55940-induced enhanced co-immunoprecipitation of β -Arrestin 2 and ERK1/2 and increased β -Arrestin 2 protein expression in rat PFCx

(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 prefrontal cortex lysate (300 μ g) from vehicle (Lanes 3 and 5) or CP55940 (Lanes 4 and 6) treated rats. Prefrontal cortex lysate (30 μ g 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 lentivirs transfection prevents GP1a-induced increases in 5-HT_{2A} receptor mRNA. $**p<0.01$, significant effect of GP1a treatment on 5-HT receptor mRNA levels in control shRNA tranfected cells compared _{2A} 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 \pm SEM (n=6–8).

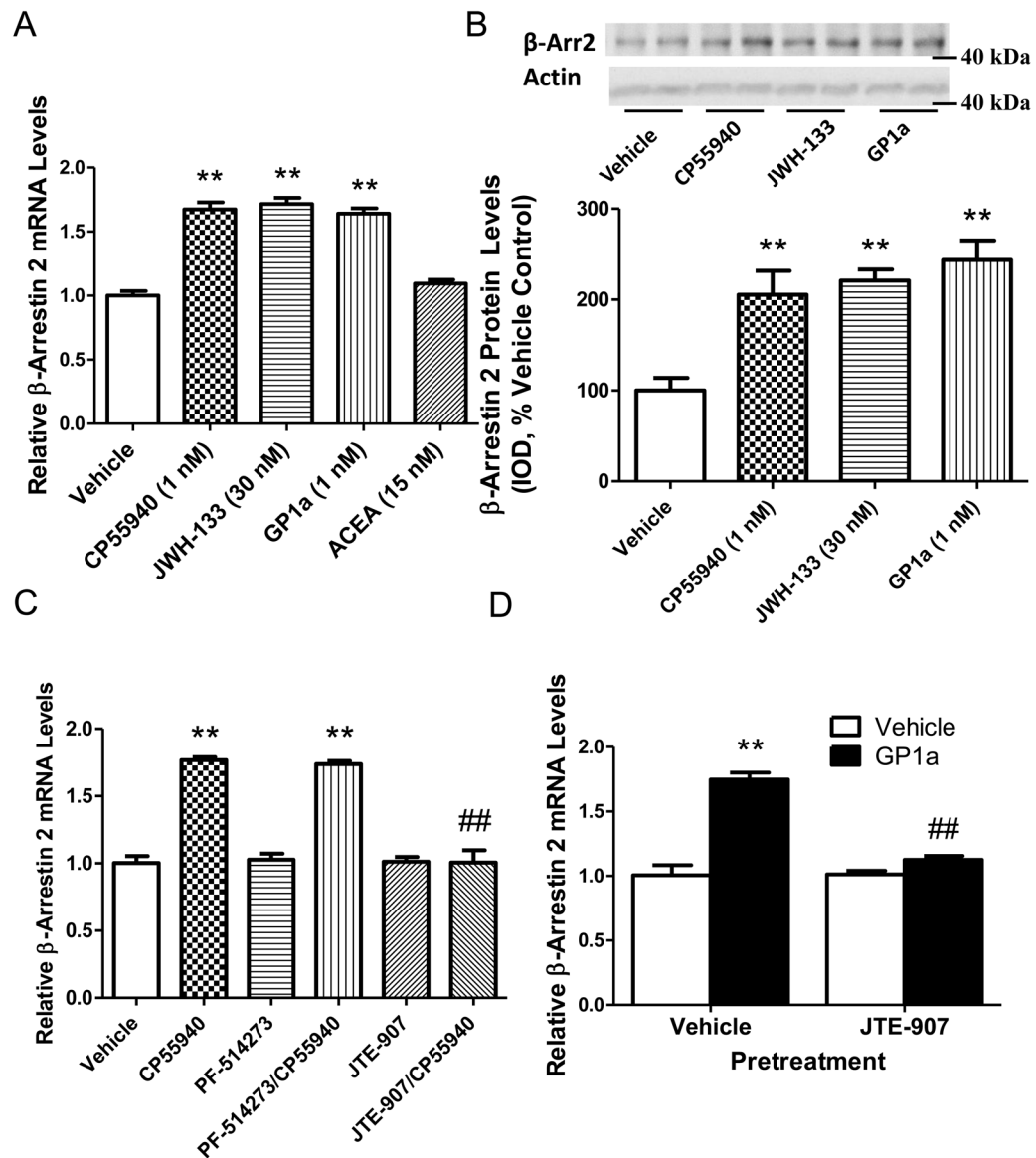


Figure 2. Selective CB2 receptor agonists, GP1a and JWH-133, upregulate β -Arrestin 2 and selective CB2 receptor antagonist, JTE-907, prevent cannabinoid-induced upregulation of β -Arrestin 2 in CLU213 cells

(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 CB2 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 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 \pm SEM (n=3).

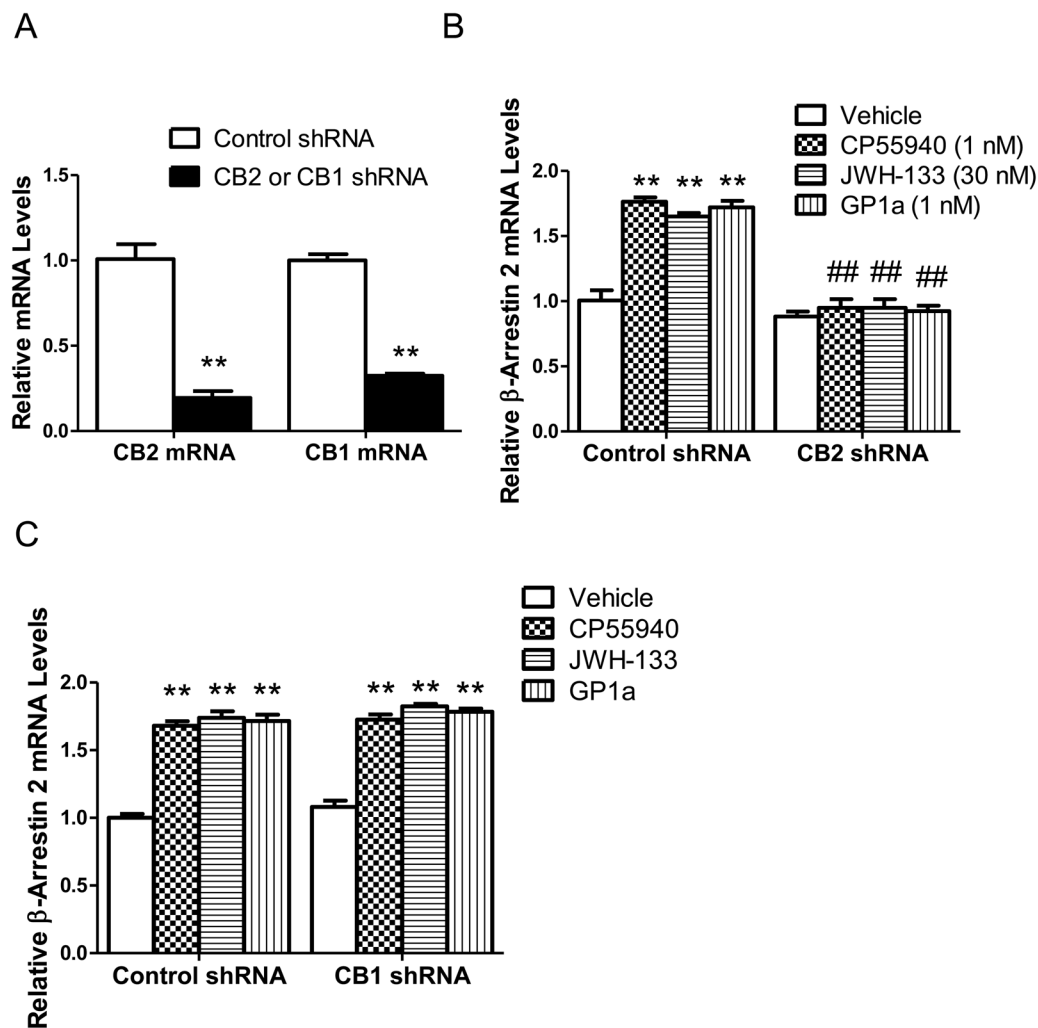


Figure 3. CB₂ receptors are necessary for CP55940, JWH-133, and GP1a-induced upregulation of β -Arrestin 2 in CLU213 cells

(A) Reduced CB₂ or CB₁ mRNA levels in cells treated with CB₂ or CB₁ shRNA lentiviral particles, respectively. ** $p < 0.01$ significant effect of CB₂ or CB₁ shRNA compared to control shRNA treated cells. (B) CB₂ shRNA lentiviral 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 CB₂ shRNA lentivirus transfection on the CP55940, JWH-133, and GP1a-induced upregulation of β -Arrestin 2. (C) CB₁ 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 CB₁ shRNA lentivirus treated cells compared to vehicle-treated controls. The data represent mean \pm SEM ($n=3$).

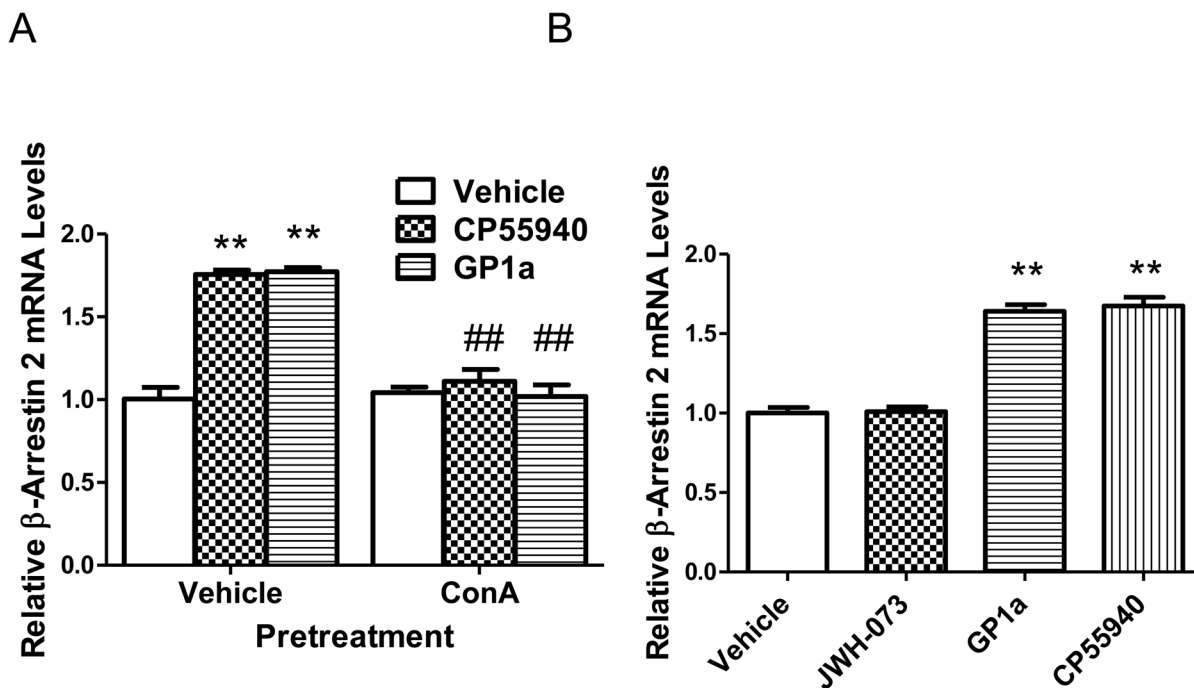


Figure 4. CP55940 and GP1a may upregulate β -Arrestin 2 via CB_2 receptor internalization in CLU213 cells

(A) Increased β -Arrestin 2 mRNA levels in cells treated with either **CP55940** or GP1a. 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 \pm SEM (n=3).

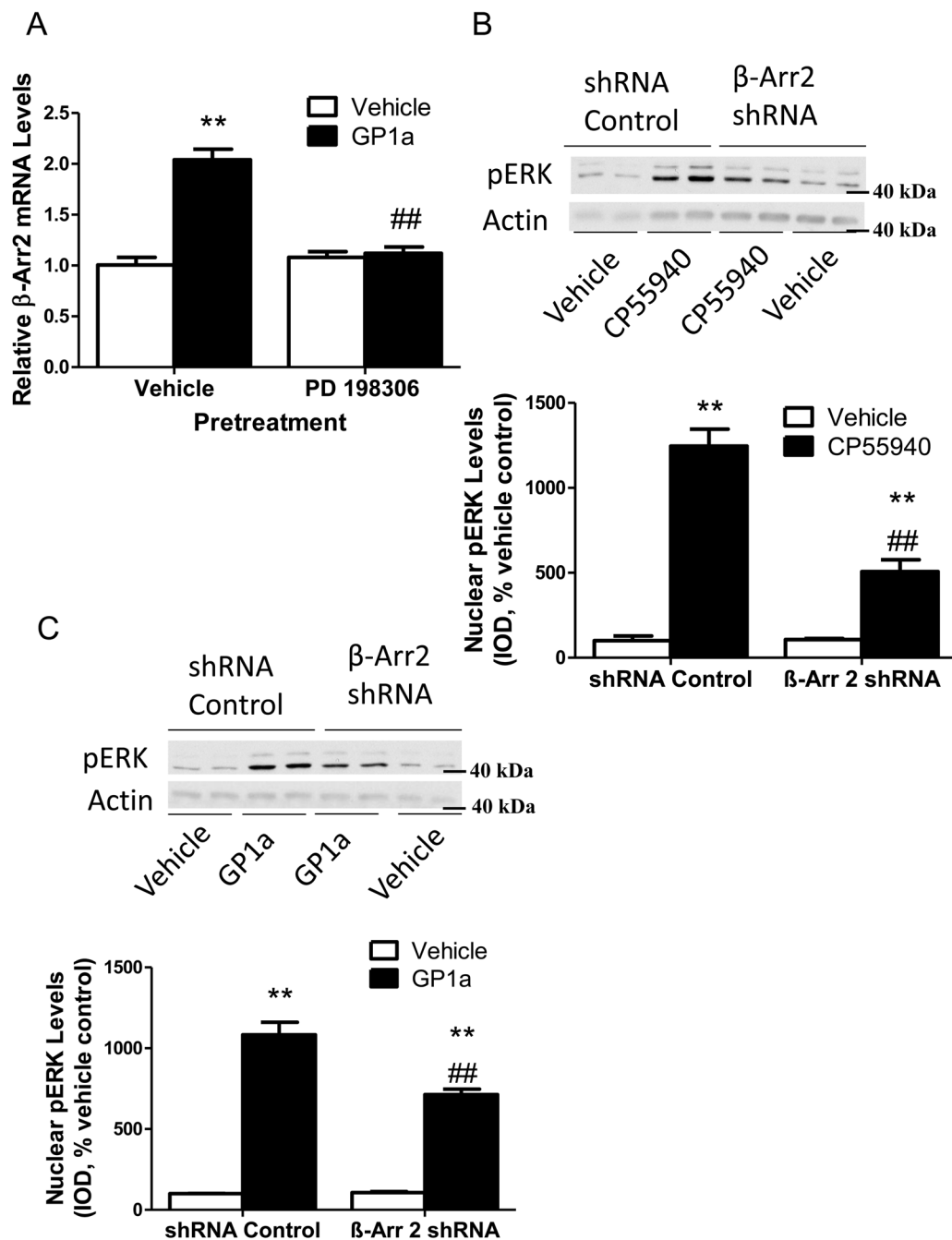


Figure 5. GP1a, a selective CB₂ receptor agonist, upregulate β -Arrestin 2 via ERK1/2 signaling CLU213 cells

(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 \pm SEM (n=3).

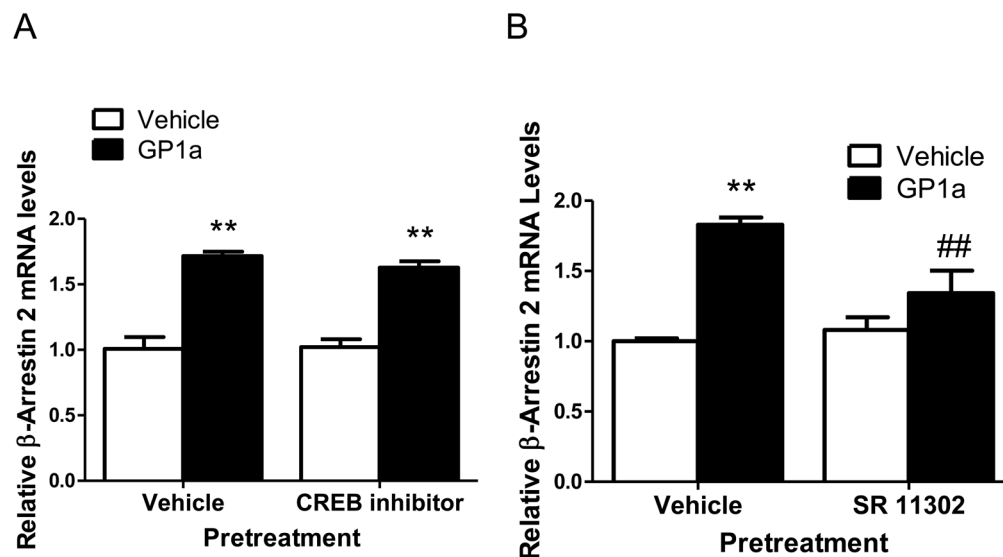


Figure 6. CB_2 receptor-induced upregulation of β -Arrestin 2 involves AP-1, but not CREB, activation

(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 \pm SEM ($n=3$).