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Cannabinoid-Induced Enhanced Interaction and Protein Levels of Serotonin 5-HT_{2A} and Dopamine D_2 Receptors in Rat Prefrontal Cortex

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Abstract

Recent evidence suggests that non-selective cannabinoid receptor agonists may regulate serotonin 2A (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 dopamine D2 (D_2) receptors. Here, we present experimental evidence that Sprague-Dawley rats treated with a non-selective cannabinoid receptor agonist (CP55,940, 50μg/kg, 7days, i.p.) showed 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 prefrontal cortex (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, CB1 and CB2 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 CB2 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.

Keywords

Serotonin; prefrontal cortex; 5-HT_{2A} receptor; D_2 receptor; G proteins; cannabis sativa

Introduction

Serotonin 2A (5-HT_{2A}) and dopamine D2 (D_2) receptors are molecular targets in the treatment of various neuropsychiatric disorders such as depression, anxiety, and schizophrenia (Carrasco and Van de Kar 2003; Celada et al. 2004; De Almeida et al. 2008; Lawford et al. 2006; Schiller et al. 2006; Weisstaub et al. 2004). 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

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Conflict of Interest Statement
The Authors declare that there is no conflict of interest.

several brain areas, including prefrontal cortex (PFCx) (De Almeida et al. 2008; Singh et al. 2007). Noteworthy, recent evidence indicates that post-synaptically located 5-HT_{2A} and D_2 receptors can assemble into functionally interacting heteromers in PFCx (Albizu et al. 2011; Borroto-Escuela et al. 2010). 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 (Lukasiewicz et al. 2011)

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 (Albizu et al. 2011). 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 (Borroto-Escuela et al. 2010; Fuxe et al. 2010). 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 (Hill et al. 2006). It was reported that rats treated with HU-210, a non-selective cannabinoid receptor agonist, exhibited enhanced the 5-HT_{2A} receptor mediated-head twitches (Hill et al. 2006). This behavioral test has been widely used as a model of activity of 5-HT_{2A} receptors in PFCx (Darmani and Reeves 1996; Willins and Meltzer 1997). If exposure to cannabinoids modifies the expression of cortical 5-HT_{2A} receptors in PFCx, they 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 brain are produced mainly through G-protein coupled cannabinoid receptors, CB1 and CB2 receptors (Howlett 2005). While CB1 receptors were initially identified in brain, early reports identified CB2 receptors only in immune cells (Barrio et al. 2011; Fribourg et al. 2011; Galiegue et al. 1995; Munro et al. 1993). However, recent studies have established the expression of CB2 receptors in normal neurons in cortex, amygdala, hypothalamus, hippocampus, etc. (Barrio et al. 2011; Fribourg et al. 2011; Garcia-Gutierrez et al. 2010; Gong et al. 2006). CB1 and CB2 receptors couple to $G\alpha_{i/o}$ G-proteins (Felder et al. 2006; Gong et al. 2006; Herkenham 1991), and would activate ERK in a protein kinase C (PKC)-dependent manner (Bouaboula et al. 1996; Onaivi et al. 2008). 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.

Materials and Methods

Drugs

(−)-*cis*-3-[2-Hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol (CP 55,940), a CB1 and CB2 receptor agonist, N-(Piperidin-1-yl)-1-(2,4-dichlorophenyl)-1,4-dihydro-6-methylindeno[1,2-c]pyrazole-3-carboxamide (GP 1a), a highly selective CB2 receptor agonist; 3-(1,1-Dimethylbutyl)-1-deoxy-Δ^8-tetrahydrocannabinol (JWH 133), a selective CB2 receptor agonist;[6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1H-indol-3-yl](4-methoxyphenyl)-methanone (AM 630), a selective CB2 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 (NKH 477), a potent activator of adenyl cyclase; 2-[1-(3-Dimethylaminopropyl)indol-3-yl]-3-(indol-3-yl) maleimide (GF 109203X), 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 (Go 6967), 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 (SR 11302), an inhibitor of activating protein-1 transcription factor activity and N-(2-Chloroethyl)-5Z,8Z,11Z,14Z-eicosatetraenamide (ACEA), a highly selective CB1 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. 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 CP 55,940 (0.05 mg/kg, i.p.) once a day for 7 days. Rats were sacrificed by decapitation 48 h after the last CP 55,940 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 prefrontal cortex lysate (300 μg) from vehicle and CP 55,940 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 prefrontal cortex lysates from vehicle- and CP55,940-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 (Montezinho et al. 2006; Nam and Kim 2008; Singh et al. 2007).

Western Blot

Membrane-associated proteins were isolated using the ProteoExtract™ 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 4°C 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 (Baltzis et al. 2004; Boundy et al. 1993; Montezinho et al. 2006; Nam and Kim 2008; Singh et al. 2007). 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 prefrontal cortex 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 (Kindlundh-Hogberg et al. 2006; Roessner et al. 2010; Singh et al. 2009; Zhang et al. 2008).

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, CB1 and CB2 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.2E and 2F). Although many in vitro cannabinoids studies use transformed cells that overexpress neurotransmitter receptors, we chose this neuronal cell line because it endogenously expresses 5-HT_{2A}, D_2, CB1 and CB2 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 Non-Selective and Selective CB1 and CB2 Receptor Agonists on 5-HT_{2A} and D_2 Receptor mRNA

CLU213 cells were incubated with either vehicle (ethanol 0.01% final concentration), CP55,940 (CB1 and CB2 agonist, 1 nM) (Thomas et al. 1998; Wiley et al. 1995); ACEA (CB1 agonist, 15 nM) (Hillard et al. 1999; Rutkowska and Jachimczuk 2004); or GP 1a (CB2 agonist, 1nM) (Gorantla et al. 2010; Murineddu et al. 2006) for 24 hours. mRNA was isolated and qRT-PCR for 5-HT_{2A} and D_2 mRNA were performed as described above.

Effect of Highly Selective CB2 Receptor Agonists on 5-HT_{2A} Receptor mRNA in Cultured Cells

CLU213 cells were pretreated with either vehicle (ethanol 0.01% final concentration) or 1 μM AM 630 (Barrio et al. 2011), a highly selective CB2 receptor antagonist. Twenty minutes later cells were treated with either vehicle or one of the following highly selective CB2 agonists, 30 nM JWH 133 (Barrio et al. 2011; Zarruk et al. 2011) or 1nM GP 1a (Gorantla et al. 2010; Murineddu et al. 2006). 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 GP 1a-Induced Increases in 5-HT_{2A} receptor mRNA and Protein Levels

CLU213 cells were treated with either vehicle (PBS) or PTX (100 ng/ml)(Bokoch et al. 1983; Casey et al. 1989). Twenty minutes later cells were treated with either vehicle (ethanol 0.01% final concentration) or GP 1a (1 nM) 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 GP 1a (1 nM) for 72 hours. Cells were washed (3x) with PBS every 24 hours and fresh vehicle or GP 1a were added. Expression of membrane-associated 5-HT_{2A} receptors was determined by Western blot as previously described.

Effect of a selective ERK1/2 inhibitor (PD 198306) or Adenylyl Cyclase Activator (NKH 477) on GP 1a-Induced Increases in 5-HT_{2A} Receptor mRNA

CLU213 cells were treated with either vehicle (ethanol 0.01% final concentration), NKH 477 (20 μM) (Sobolewski et al. 2004; Toya et al. 1998) or PD 198306 (200 nM) (Ciruela et al. 2003; Pelletier et al. 2003). Twenty minutes later cells were incubated with either vehicle (ethanol 0.01% final concentration) or GP 1a (1 nM) for 24 hours. mRNA was isolated and qRT-PCR for 5-HT_{2A} was performed as described.

Effect of PKC Inhibitors on GP 1a-Induced Increases in 5-HT_{2A} Receptor mRNA

CLU213 cells were treated with either vehicle (ethanol 0.01% final concentration), GF 109203X (5 μM)(Jacobson et al. 1995; Toullec et al. 1991), or Go 6967 (10 nM)(Martiny-Baron et al. 1993) for 20 min. Cells were then treated with either vehicle (ethanol 0.01% final concentration) or GP 1a (1 nM) for 24 hours. mRNA was isolated and qRT-PCR for 5-HT_{2A} was performed as described above.

Effect of PKC Activator on GP 1a-Induced Increases in 5-HT_{2A} Receptor mRNA

CLU213 cells were treated with either vehicle (DMSO 0.01% final concentration), PDBu (1 μM), or PDBu (30 nM)(Burns et al. 1990; Kim et al. 2005) for 20 minutes. Cells were then treated with either vehicle (ethanol 0.01% final concentration) or GP 1a (1 nM) 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 (10 μM)(Best et al. 2004) or SR 11302 (1 μM)(Fanjul et al. 1994; Huang et al. 1997) 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 GP 1a-Induced Increases in Nuclear levels of c-Fos Protein

CLU213 cells were treated with either vehicle (ethanol 0.01% final concentration) or PD 198306 (200 nM)(Ciruela et al. 2003; Pelletier et al. 2003)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 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.

Results

Effect of CP 55,940 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 CP55,940 on the physical interaction between 5-HT_{2A} and D_2 receptors in rat PFCx (Fig.1). PFCx lysate of rats treated with either vehicle or CP 55,940 (a non-selective CB1/CB2 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.1A, lanes 1 and 2). We also used inactive columns, unable to bind D_2 receptor antibody as control (Fig.1A, 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 PCx of CP55,940-treated rats compared with vehicle controls (approx. 200% increase, Fig.1A lanes 1 and 2 for vehicle or CP55,940 samples, respectively). No co-precipitation of 5-HT_{2A} and D_2 receptors was detected when using inactive columns (Fig.1A, lanes 3 and 4). Similarly, we found an approx. two-fold increased co-precipitation of D_2 receptors with 5-HT_{2A} receptors in PFCx lysate of CP55,940-treated rats compared to controls when we used 5-HT_{2A} receptor as a bait (Fig.1B, lanes 5 and 6 for vehicle of CP55,940 samples, respectively). No co-precipitation of 5-HT_{2A} and D_2 receptors was detected when using inactive columns (Fig.1B, lanes 7 and 8). This evidence suggests that CP55,940 treatment enhances formation of a 5-HT_{2A}-D_2 receptor heteromer in rat PFCx.

Effect of Chronic CP 55,940 Treatment on the Protein Expression of D_2 and 5-HT_{2A} Receptors in Rat PFCx

CP55,940 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.1. In our next experiments, we studied the effect of CP55,940 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 codified for the same gene (Doly et al. 2004; Khan et al. 1998; Usiello et al. 2000). 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 (Dal Toso et al. 1989). 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 (Doly et al. 2004; Khan et al. 1998; Usiello et al. 2000).

Chronic administration of CP55,940 produced significant increases in membrane-associated levels of D_2S receptors (Fig.2A), D_2L receptors (Fig. 2B), and 5-HT_{2A} receptors (Fig. 2C) 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). Actin was used as a control for protein loading in all these Western blots. We also determined the effect of chronic CP 55,940 treatment on 5-HT_{2A} and D_2 mRNA levels in rat PFCx. 5-HT_{2A} receptor mRNA was significantly ($p<0.05$) increased (approx.90% increase) in PFCx of CP55,940-treated rats

compared to vehicle-treated controls (Fig. 2D). Interestingly, D_2 receptor mRNA was significantly ($p<0.05$) reduced (approx. 45% reduction) in PFCx of CP 55,940 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, CB1 and CB2 receptors. In these experiments we tested the effect of either a non-selective cannabinoid agonist (CP55,940) (Thomas et al. 1998; Wiley et al. 1995); a selective CB1 receptor agonist (ACEA) (Hillard et al. 1999; Rutkowska and Jachimczuk 2004); or a selective CB2 receptor agonist (GP 1a) (Gorantla et al. 2010; Murineddu et al. 2006).

We found that either CP55,940 or GP 1a produced a significant ($p<0.01$) upregulation of 5-HT_{2A} receptor mRNA levels in CLU213 cells (Fig. 2E). Cells treated with either CP55,940 or GP 1a exhibited an approx. 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 CP55,940 or GP 1a. The CB1 agonist ACEA did not have significant effects on 5-HT_{2A} receptor mRNA levels (Fig. 2E). On the other hand, cells treated with either CP55,940 or ACEA exhibited a significant ($p<0.05$) downregulation of D_2 mRNA levels in CLU213 cells. Cells treated with CP55,940 exhibited an approx. 60% reduction ($P<0.05$) in D_2 mRNA levels while cells treated with ACEA exhibited an approx. 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 CP55,940 or ACEA.

Since we detected a very strong regulation of 5-HT_{2A} receptor mRNA induced by GP 1a, a highly selective CB2 receptor agonist, we also studied the effect of other selective CB2 agonist and antagonist on 5-HT_{2A} upregulation. In this experiment cells were pretreated with either vehicle or AM 630, a selective CB2 antagonist. Twenty minutes later the cells were incubated with either vehicle, JWH 133 or GP 1a as described in Methods. We found that both JWH 133 and GP 1a produced a significant ($p<0.01$) upregulation of 5-HT_{2A} receptor mRNA in CLU213 cells (Fig.2F). There were no significant ($p>0.05$) differences between the 5-HT_{2A} upregulation induced by JWH 133 or GP 1a. This strong 5-HT_{2A} mRNA upregulation induced by these CB2 receptor agonists was significantly ($p<0.01$) inhibited in cells pretreated with a selective CB2 antagonist, AM 630 (Fig.2F). No significant ($p>0.05$) differences in 5-HT_{2A} mRNA were found between vehicle treated cells and cells pretreated with AM 630 and later treated with either vehicle, JWH 133, or GP 1a (Fig.2F). The two-way ANOVA for 5-HT_{2A} mRNA showed a significant main effect of AM 630 pretreatment ($F_{1,134.75}$, $p<0.0001$) and CB2 agonists treatment ($F_{1,65.98}$, $p<0.0001$). There was also a significant interaction between AM 630 pretreatment and CB2 agonists treatment ($F_{1,40.03}$ $p<0.0001$).

Effect of G-Protein and ERK1/2 Signaling Inhibitors on the GP 1a-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 CB2 receptor agonist GP 1a. Previous reports suggested that CB1 and CB2 cannabinoid 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 (Bouaboula et al. 1996). Here we used PTX to prevent the GP 1a-induced activation of $G\alpha_{i/o}$ G-proteins (Bouaboula et al. 1996). PTX-induced ADP-ribosylation of

$G\alpha_{i/o}$ subunits mediates the inactivation of their signaling by interfering with Gα/receptor coupling (Bokoch et al. 1983; Casey et al. 1989).

Figure 3 illustrates the effect of PTX pretreatment on GP 1a-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 GP 1a (1nM) was added to the media. We found that in vehicle pretreated cells, GP 1a significantly ($p<0.01$) increased 5-HT_{2A} mRNA levels (two-fold increase) over controls (Fig.3A). This effect of GP 1a 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 ($F_{1,23.52}$, $p<0.0004$) and GP 1a treatment ($F_{1,34.11}$ $p<0.0001$). There was a significant interaction between PTX pretreatment and GP 1a treatment ($F_{1,47.74}$ $p<0.0001$).

In Figure 3B, CLU213 cells were treated with either vehicle or PTX (100 ng/ml) then vehicle or GP 1a (1 nM) 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 GP 1a, as described in Methods. We found that in vehicle pretreated cells, GP 1a significantly ($p<0.01$) increased 5-HT_{2A} receptor protein levels (approx. 60% increase) over controls (Fig. 3B). The effect of GP 1a 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 ($F_{1,23.18,}$ $p<0.0001$) and GP 1a treatment ($F_{1,19.34,}$ $p<0.0003$). There was a significant interaction between PTX pretreatment and GP 1a treatment ($F_{1,7.14,}$ $p<0.0151$). These data suggest that the GP 1a-induced upregulation of 5-HT_{2A} receptors is mediated by a $G\alpha_{i/o}$ G-protein mechanism.

Coupling of CB2 cannabinoid receptors to $G\alpha_{i/o}$ G-proteins mediates the increases in ERK signaling and also the inhibition adenylate cyclase that results in reduced cAMP levels (Felder et al. 2006). In our next experiment, we studied the effect of a ERK1/2 inhibitor (PD 198306) (Pelletier et al. 2003) and an adenylyl cyclase activator (NHK 477)(Sobolewski et al. 2004) on the GP 1a-induced upregulation of 5-HT_{2A} receptor mRNA. CLU213 cells were treated with either vehicle, PD 198306 (200nM) or NKH 477 (20 μM). Twenty min later cells were treated with either vehicle or GP 1a (1nM) for 24 hours. Consistent with our previous findings, GP 1a significantly ($p<0.05$) increased 5-HT_{2A} mRNA levels (approx. two-fold increase) over controls (Fig.3C). The effect of GP 1a was prevented ($p<0.05$) in cells pretreated with either PD 198306 or NKH 477. No significant ($p>0.05$) effect of PD 198306 or NKH 477 was found on basal 5-HT_{2A} receptor mRNA levels (Fig.3C). These results suggest that the GP 1a-induced upregulation of 5-HT_{2A} receptors is dependent on ERK1/2 activation and prevented by activation of adenylyl cyclase.

Effect of PKC on GP 1a-Induced Upregulation of 5-HT_{2A} Receptor mRNA

Figure 4A and 4B illustrate the effect of PKC inhibition on GP 1a-induced 5-HT_{2A} receptor upregulation. Bouaboula et al. (1996) proposed that cannabinoid receptors activate the ERK1/2 signaling cascade through PKC activation (Bouaboula et al. 1996). Additionally, they reported evidenced to suggest that Ca^{2+-} dependent PKC isoforms could be involved in CB2 signal transduction which are not involved in CB1 signal transduction (Bouaboula et al. 1996). Here we studied the effect of two different PKC inhibitors (GF 109203X and Go 6967) on GP 1a-induced increases in 5-HT_{2A} receptor mRNA levels. GF 109203X 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) (Toullec et al. 1991) while Go 6967 selectively inhibits Ca^{2+} -dependent isoforms PKCα and PKCβ1 (IC_{50} values are 2.3 and 6.2 nM, respectively) (Martiny-Baron et al. 1993).

In Figure 4A, CLU213 cells were pretreated with either vehicle or GF 109203X (5 μM) for 20 minutes and then treated with vehicle or GP 1a (1 nm). In this experiment, 5 μM GF 109203X should produce a substantial inhibition of most PKC isoforms. We found that GP 1a significantly ($p<0.05$) increased 5-HT_{2A} receptor mRNA levels (two-fold increase) in vehicle pretreated cells compared to vehicle treated controls (Fig 4A). Furthermore, GF 109203X 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 GP 1a-induced increases in 5-HT_{2A} receptor mRNA levels. The two-way ANOVA for 5-HT_{2A} mRNA showed significant main effects of GF 109203X pretreatment ($F_{1,6.68}$, $p<0.0324$) and GP 1a treatment ($F_{1,10.82}$ $p<0.011$). There was no significant interaction between GF 109203X pretreatment and GP 1a treatment ($F_{1,2.17}$ $p>0.05$).

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 Go 6967 (10 nm) for 20 minutes then treated with either vehicle or GP 1a (1 nm) for 24 h. GP 1a significantly ($p<0.01$) increased 5-HT_{2A} receptor mRNA levels (two-fold increase) compared to vehicle pretreated controls (Fig 4B). Pretreatment with Go 6967 significantly ($p <0.01$) increased basal 5-HT_{2A} receptor mRNA levels (58% increase) over vehicle pretreated controls while Go 6967 pretreatment did not have a significant effect ($p>0.05$) on GP 1a-induced upregulation of 5-HT_{2A} receptors mRNA. The two-way ANOVA for 5-HT_{2A} receptor mRNA showed a main effect of Go 6967 pretreatment ($F_{1, 5.85,}$ $p<0.0418$) and a main effect of GP 1a treatment ($F_{1,16.15}$, $p<0.0038$). There was no significant interaction between Go 6967 pretreatment and GP 1a treatment ($F_{1,2.03,}$ $p>0.05$).

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) (Burns et al. 1990), has on GP 1a-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 (30 nM) (Burns et al. 1990).

CLU213 cells were pretreated with either vehicle or PDBu (1μM) for 20 minutes.Cells were then treated with either vehicle or GP 1a (1 nm). GP 1a significantly ($p<0.05$) increased 5-HT_{2A} receptor mRNA levels (two-fold increase, Fig 4C). This effect of GP 1a 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 ($F_{1,6.12}$, $p<0.0385$) and GP 1a treatment ($F_{1,6.10}$ $p<0.0375$). There was a significant interaction between PDBu pretreatment and GP 1a treatment ($F_{1,5.38}$ $p<0.0489$).

In order to examine the effect Ca^{2+}-dependent PKC isoforms on GP 1a-induced increases in 5-HT_{2A} receptor mRNA, we used a concentration of PDBu (30 nm) that activated the Ca^{2+}-dependent isoforms (Burns et al. 1990). GP 1a significantly ($p<0.01$) increased 5-HT_{2A} receptor mRNA levels (two-fold increase, Fig 4D). 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 (approx. 20% decrease, $p<0.05$) the GP 1a-induced upregulation of 5-HT_{2A} receptors. The two-way ANOVA for 5-HT_{2A} receptor mRNA showed significant main effects of PDBu pretreatment ($F_{1,6.26}$ $p<0.0368$) and GP 1a treatment ($F_{1,79.39}$ $p<0.0001$). There was no significant interaction between PDBu pretreatment and GP 1a treatment ($F_{1,1.62}$ $p<0.2385$). The use of PKC activators seems to suggest that both Ca^{2+}-dependent and Ca^{2+}-independent PKC isoforms play a role preventing the GP 1a-induced upregulation of 5-HT_{2A} receptor mRNA.

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

Figure 5 illustrates the effect of CREB or AP-1 transcription factor inhibitor pretreatment on GP 1a-induced upregulation of 5-HT_{2A} receptor mRNA in CLU213 cells. Here we wanted to identify possible transcription factor(s) that would contribute to GP 1a-induced increases of 5-HT_{2A} receptor mRNA. In our previous experiments we showed that the GP 1a-induced upregulation of 5-HT_{2A} receptors is prevented by PD 198306, an inhibitor of ERK1/2 activation. Activation of ERK involved the phosphorylation of this protein in the cytoplasm and its translocation to the nucleus (Campbell et al. 1995; Chang et al. 2003; Seger and Krebs 1995). In the nucleus, phosphorylated ERK (pERK) can activate several transcription factors such as CREB, c-Fos, ELK-1, SP-1, and EGR-1 (Campbell et al. 1995; Chang et al. 2003; Seger and Krebs 1995). The transcription factors CREB and AP-1 have consensus sequences within the promoter region of the rat 5-HT_{2A} receptor gene (Chalecka-Franaszek et al. 1999; Du et al. 1994; Ferry and Molinoff 1996). Therefore, we decided to test the effects of inhibitors of these transcription factors on the GP 1a-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 (Chrivia et al. 1993; Kwok et al. 1994). 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 (Karin 1995; Karin et al. 1997). In our first experiment, we study the effect of CREB inhibitor pretreatment on the GP 1a-induced upregulation of 5-HT_{2A} receptors.

CLU213 cells were treated with either vehicle or Naphthol AS-E phosphate (10 μM) for 20 minutes and then treated with vehicle or GP 1a (1 nm). Naphthol AS-E phosphate blocks cAMP-induction of CREB-dependent gene transcription (K_i 10 μM) (Best et al. 2004). We found that Naphthol AS-E phosphate did not inhibit or decrease GP 1a-induced increases in 5-HT_{2A} receptor mRNA (Fig. 5A). 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 ($F_{1,0.006}$, $p>0.9384$) and a significant main effect of GP 1a treatment ($F_{1,28.91}$ $p<0.0007$). There was no significant interaction between Naphthol AS-E pretreatment and GP 1a treatment ($F_{1,0.23}$ $p>0.6453$). These data indicate that CREB is not involved in GP 1a-induced 5-HT_{2A} receptor upregulation.

We then studied the effect of AP-1 inhibition on GP 1a-induced increases in 5-HT_{2A} receptor mRNA. CLU213 cells were treated with either vehicle or SR 11302 (1 μM) for 20 min then vehicle or GP 1a (1nM) was added to the incubation media. SR 11302 is retinoid that transrepresses AP-1 without transactivating the retinoic acid response element (E_{max} 1 μM) (Fanjul et al. 1994). As expected, GP 1a induced a significant ($p<0.05$) increase in 5-HT_{2A} mRNA levels (approx. two fold increase in 5-HT_{2A} mRNA) (Fig.5B). SR 11302 pretreatment significantly reduced (approximately 55% decrease, $p<0.05$) the GP 1a-induced upregulation of the 5-HT_{2A} receptor mRNA (Fig. 5B). No significant ($p>0.05$) effect of SR 11302 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 SR 11302 pretreatment ($F_{1,2.89}$, $p>0.1271$) and did show a significant effect of GP 1a treatment ($F_{1,32.80}$ $p<0.0004$). There was a significant interaction between SR 11302 pretreatment and GP 1a treatment ($F_{1,7.48}$ $p<0.0256$).

Our data seems to indicate that GP 1a-induced upregulation of 5-HT2A 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 GP 1a-induced increases in the nuclear-associated

protein levels of c-fos. CLU213 cells were treated with either vehicle or PD 198306 (200 nm) for 20 minutes and then treated with either vehicle or GP 1a (1 nm) for 15 minutes. As mentioned above, PD 198306 is a potent inhibitor of ERK1/2 (IC_{50} 100 nM) (Pelletier et al. 2003). We found that in vehicle pretreated cells GP 1a significantly ($p<0.05$) increased c-fos levels over controls (Fig. 5C). Indeed, GP 1A induced an approx. 40% increase in the nuclear-associated protein levels of c-fos. This effect of GP 1a was prevented ($p<0.01$) in cells pretreated with PD 198306 (Fig.5C). No significant ($p>0.05$) effect of PD 198306 was found on basal 5-HT_{2A} mRNA levels. The two-way ANOVA for 5-HT_{2A} mRNA showed significant main effects of PD 198306 pretreatment ($F_{1,15.74}$, $p<0.0008$) and GP 1a treatment ($F_{1,6.09}$ $p<0.0147$). There was a significant interaction between PD 198306 pretreatment and GP 1a treatment ($F_{1,5.95}$ $p<0.0241$).

Discussion

G protein-coupled receptors (GPCRs) can exist as dimers or part of larger oligomeric complexes (Lohse 2006; Milligan 2004). 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 (Albizu et al. 2011; Borroto-Escuela et al. 2010; Lukasiewicz et al. 2011; Lukasiewicz et al. 2010). 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, substantia nigra, etc (Lukasiewicz et al. 2010). Our results suggest that exposure to CP55,940, a non-selective cannabinoid CB1/CB2 receptor agonist (Thomas et al. 1998; Wiley et al. 1995), increases the interaction between 5-HT_{2A} and D_2 receptors in rat PFCx (Fig.1). Indeed, we found increased co-immunoprecipitation of 5-HT_{2A} and D_2 receptors in PFCx samples of CP55,940 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 (Albizu et al. 2011; Lukasiewicz et al. 2010). 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 (Lukasiewicz et al. 2010).

The CP55,940-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 CP55,940 treated rats compared to control (Fig.2A, 2B and 2C). Moreover, we found increased 5-HT_{2A} receptor mRNA in PFCx of CP55,940 treated rats compared to controls (Fig.2D) 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 CP55,940 induced decreases in D_2 mRNA levels in PFCx (Fig.2D). This latter study suggests that the CP55,940-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 (Albizua et al. 2011; Cottet et al. 2011). 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 (Wang et al. 2004). Δ^9-THC, the main psychoactive component of cannabis sativa (marijuana) is a nonselective CB1 and CB2

receptor agonist (Dresen et al. 2010). 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 (Wang et al. 2004), suggesting that stimulation of cannabinoid receptors mediates a (Wang et al. 2004) reduction in D_2 mRNA levels in mesocorticolimbic neural systems (Wang et al. 2004).

In this manuscript, we used a neuronal cell line and selective CB1 and CB2 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.2E). D_2 receptor mRNA levels were decreased in neuronal cells treated with either CP55,940 a nonselective CB1/CB2 agonist or ACEA a selective CB1 agonist (Hillard et al. 1999; Rutkowska and Jachimczuk 2004) (Fig.2E). GP 1a a selective CB2 agonist (Gorantla et al. 2010; Murineddu et al. 2006) did not modify D_2 mRNA levels in cultured cells (Fig.2E). These studies suggests that the effect of CP55,940 on D_2 mRNA would be mediated by activation of CB1 receptors. On the other hand, activation of CB2 receptors seems to mediate the CP55,940-mediated upregulation of 5-HT_{2A} receptor mRNA (Fig.2E and 2F). Cells treated with either of the following highly selective CB2 agonists, JWH 133 (Barrio et al. 2011; Zarruk et al. 2011) or GP 1a (Gorantla et al. 2010; Murineddu et al. 2006), 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, AM 630 a highly selective CB2 antagonist (Barrio et al. 2011) prevented the JWH 133- or the GP 1a-induced upregulation of 5-HT_{2A} mRNA in CLU213 cells. AM 630 shows an approximate 165-fold selectivity over CB2 receptors compared to CB1 receptors (Barrio et al. 2011).

The results presented here suggest that CB2, but not CB1, receptor agonists mediate the upregulation of 5-HT_{2A} receptors. Interestingly, there has been some controversy regarding the expression of CB2 receptors in brain. Indeed, CB2 receptors were initially identified in the periphery but not in the brain (Abood and Martin 1996; Demuth and Molleman 2006). Brain expression of CB2 receptors has been much less well established and characterized in comparison to the expression of brain CB1 receptors. Later studies have identified CB2 receptors in several brain areas including: cortex, hippocampus, amygdala, substantia nigra, cerebellum etc (Garcia-Gutierrez et al. 2010; Gong et al. 2006). Furthermore, recent studies reported that there are functional CB2 receptors in the medial prefrontal cortex and that CB2 receptors are mainly localized in post-synaptic neurons (Brusco et al. 2008; den Boon et al. 2012; Onaivi et al. 2008). These findings have led to a re-evaluation of the possible roles that CB2 receptors may play in the brain. Interestingly, deletion of the CB2 receptor induces schizophrenia-related behaviors in mice and chronic treatment with a selective CB2 agonist (JWH 133) increases anxiety in mice (Garcia-Gutierrez et al. 2011; Onaivi et al. 2008; Ortega-Alvaro et al. 2011). Here we found that a selective CB2 receptor agonist induced increases in 5-HT_{2A} receptor mRNA and protein expression in a neuronal cell model. It is possible that CB2 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 CP 55,940. However, it is currently unknown whether CB2 receptors co-localize with 5-HT_{2A} receptors in PFCx.

GP 1a and JWH 133, two CB2 receptor agonists, induce an approximate two-fold increase in 5-HT_{2A} receptor mRNA and protein (Fig.2F). Similar increases in expression of 5-HT_{2A} receptor protein levels have been associated with exposure to drugs of abuse and estrogen (Akash et al. 2008; Cyr et al. 2000; Horner et al. 2011). In our next experiments we used GP 1a to study some of the molecular mechanisms involved in the upregulation of 5-HT_{2A} receptors by CB2 receptor agonists. For these experiments, we selected GP 1a because it shows higher CB2/CB1 receptor selectivity compared to JWH 133 (>5,000- and 165-fold CB2/CB1 selectivity, respectively) (Gorantla et al. 2010; Murineddu et al. 2006). 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. CB2 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 (Bouaboula et al. 1996). PTX catalyses the ADP-ribosylation of specific $G\alpha_i$ subunits preventing the receptor–G-protein interaction (Bouaboula et al. 1996). Our results indicate that PTX prevented the GP 1a-induced increases in 5-HT_{2A} mRNA and protein levels (Fig. 3A and 3B). Additionally, we found that the GP 1a-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 NKH 477 (Fig.3C) in cultured cells. Our results seem to indicate that the GP 1a-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 GP 1a-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 GP 1a-induced activation of ERK signaling (Bouaboula et al. 1996). We found that non-selective PKC inhibitors and selective calcium-dependent PKC inhibitors did not prevent or significantly reduce the GP 1a-induced 5-HT_{2A} upregulation (Fig.3A and 3B). 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 (Burns et al. 1990) (Fig.3C and 3D) significantly reduced GP 1a-induced increases in 5-HT_{2A} mRNA levels. Specifically, activation of both calcium dependent and independent isoforms completely inhibited the GP 1a-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 GP 1a-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 (Mischak et al. 1991). Moreover, activation of certain PKC isoforms inhibits gene transcription (Esteve et al. 2002; Newton 1995; Page et al. 2002) and that could prevent the GP 1a-induced increases in 5-HT_{2A} receptor mRNA levels. In summary, it appears that the GP 1a-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 (Lefkowitz and Shenoy 2005).

Activation of ERK signaling stimulates several transcription factors such as CREB, c-Fos, ELK-1, SP-1, and EGR-1 (Campbell et al. 1995; Chang et al. 2003; Seger and Krebs 1995). 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 (Chalecka-Franaszek et al. 1999; Du et al. 1994; Ferry and Molinoff 1996). Therefore, we tested the effects of inhibitors of these transcription factors on the GP 1a-induced upregulation of 5-HT_{2A} receptor mRNA. Our results suggest that inhibition of AP-1, but not the CREB, activation significantly decreased the GP 1a-induced upregulation of 5-HT_{2A} receptors (Fig.5A and 5B). The partial inhibition of the GP 1a-induced increases in 5-HT_{2A} mRNA levels by SR 11302 suggest 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 PD 198306 prevented the GP 1a-induced activation of c-fos (Fig.5C). Although further research is needed, SP-1 could also mediate the GP 1a-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 (Ferry et al. 1993; Seger and Krebs 1995).

Exposure to cannabinoids has been associated in the pathophysiology of several neuropsychiatric disorders such as anxiety, depression and schizophrenia (Crippa et al. 2009; Henquet et al. 2005; Kuepper et al. 2011; Large et al. 2011). 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 (Crippa et al. 2009; Kuepper et al. 2011; Large et al. 2011). 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 (Dresen et al. 2010), 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 CB2, but not CB1 agonists, seems to mediate this increase in 5-HT_{2A} mRNA. We hypothesize that this CB2 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.

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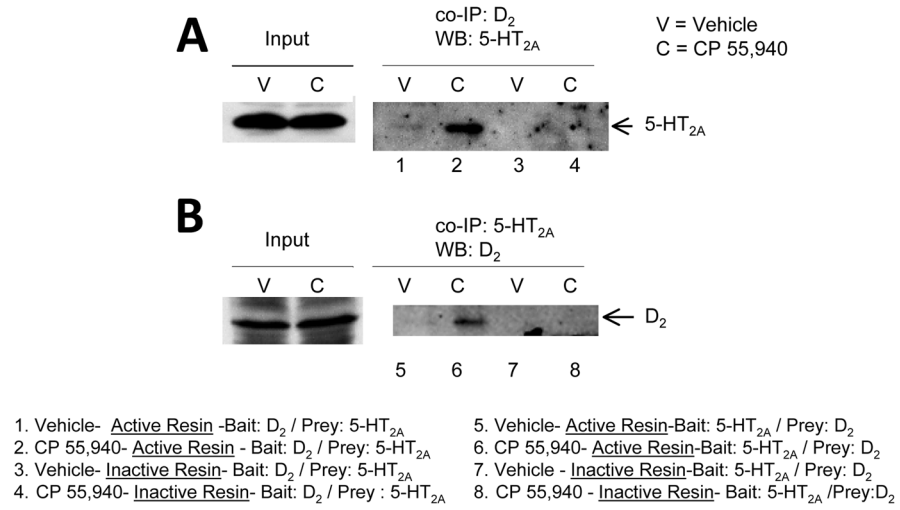

Figure 1. CP 55,940-induced enhanced co-immunoprecipitation of 5-HT$_{2A}$ and D$_2$ receptors in rat PFCx

(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 prefrontal cortex lysate (300 μg) from vehicle (Lanes 1,3,5, and 7) or CP 55,940 (2, 4, 6, and 8) treated rats. **Prefrontal cortex lysate (45 μg of protein) was used as an input control for both immunoprecipitations.**

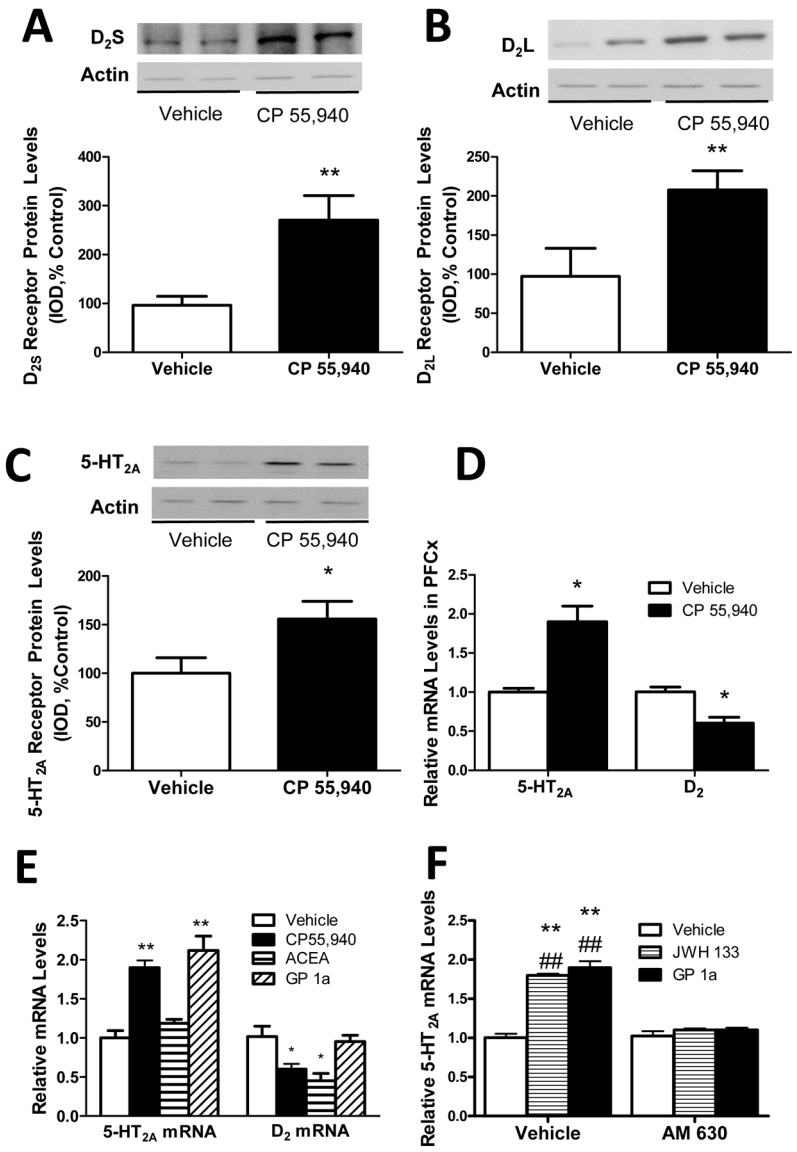

Figure 2. CP 55,940-induced increased membrane-associated expression of 5-HT$_{2A}$ and D$_2$ receptors in rat PFCx

(A) Increased membrane-associated D$_2$S receptor protein levels in PFCx of CP 55,940 treated rats. (**p<0.01 significant effect of CP 55,940 treatment compared to vehicle-treated controls). (B) Increased membrane-associated D$_2$L receptor protein levels in PFCx of CP 55,940 treated rats. (**p<0.01 significant effect of CP 55,940 treatment compared to vehicle-treated controls). (C) Increased membrane-associated 5-HT$_{2A}$ receptor protein levels in PFCx of CP 55,940 treated rats. (*p<0.05 significant effect of CP 55,940 treatment compared to vehicle-treated controls). (D) Increased 5-HT$_{2A}$ receptor mRNA levels and reduced D$_2$ receptor mRNA levels in PFCx of CP 55,940 treat rats. (*p<0.05 significant effect of CP 55,940 treatment compared to vehicle-treated controls). (E) Increased 5-HT$_{2A}$ receptor mRNA levels in CP 55,940 or GP 1a treated cells (**p<0.01 significant effect of CP 55,490 or GP 1a treatment compared to vehicle-treated controls) and reduced D$_2$ receptor mRNA levels in CP 55,940 or ACEA treated cells (*p<0.05 significant effect of CP 55,940 or ACEA treatment compared to vehicle-treated controls). (F) AM 630 pretreatment prevents GP 1a and JWH 133-induced increases in 5-HT$_{2A}$ receptor mRNA. **p<0.01,

significant effect of GP 1a or JWH 133 treatment on 5-HT_{2A} receptor mRNA levels compared to vehicle-treated controls. ##p<0.01, significant effect of AM 630 pretreatment on the GP 1a or JWH 133-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).

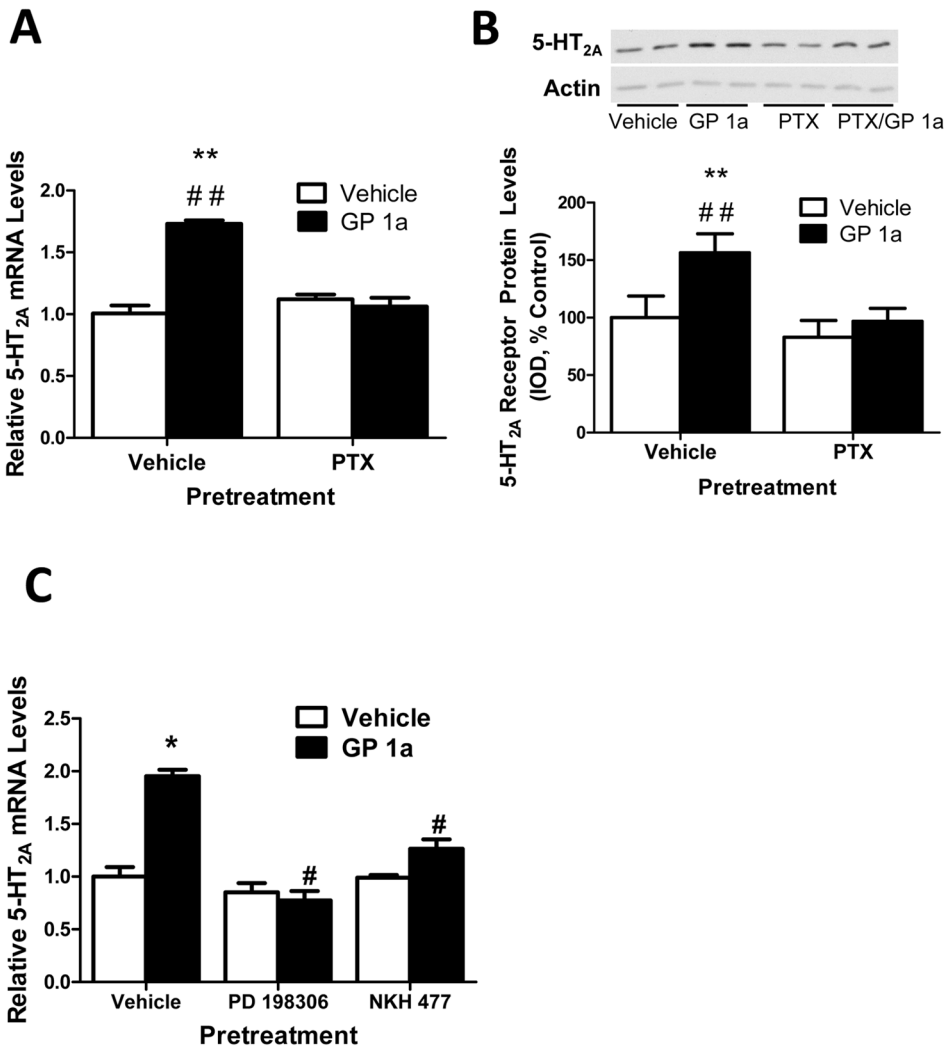

Figure 3. GP 1a, a selective CB2 receptor agonist, upregulated 5-HT$_{2A}$ receptors via Gα_i G-protein in CLU213 cells

(A) Pertussis toxin (100 ng/ml) prevents GP 1a-induced increases in 5-HT$_{2A}$ receptor mRNA. **p<0.01, significant effect of GP 1a treatment on 5-HT$_{2A}$ receptor mRNA levels compared to vehicle-treated controls. ##p<0.01, significant effect of pertussis toxin pretreatment on the GP 1a-induced upregulation of 5-HT$_{2A}$ receptors. (B) Pertussis toxin (100 ng/ml) prevents GP 1a-induced increases in membrane-associated 5-HT$_{2A}$ receptor protein expression. **p<0.01, significant effect of GP 1a treatment on 5-HT$_{2A}$ receptor protein levels compared to vehicle-treated controls. ##p<0.01, significant effect of pertussis toxin pretreatment on the GP 1a-induced increases in membrane-associated 5-HT$_{2A}$ receptor protein levels. (C) An inhibitor of ERK1/2 (PD 198306) prevents GP 1a-induced increases in 5-HT$_{2A}$ receptor mRNA and an activator of adenylyl cyclase (NHK 477) prevents GP 1a-induced increases in 5-HT$_{2A}$ receptor mRNA. *p<0.05, significant effect of GP 1a treatment on 5-HT$_{2A}$ receptor mRNA levels compared to vehicle-treated controls. #p<0.05, significant effect of PD 198306 or NKH 477 pretreatment on the GP 1a-induced increases in 5-HT$_{2A}$ receptor mRNA. **The data represent mean ± SEM (n=3).**

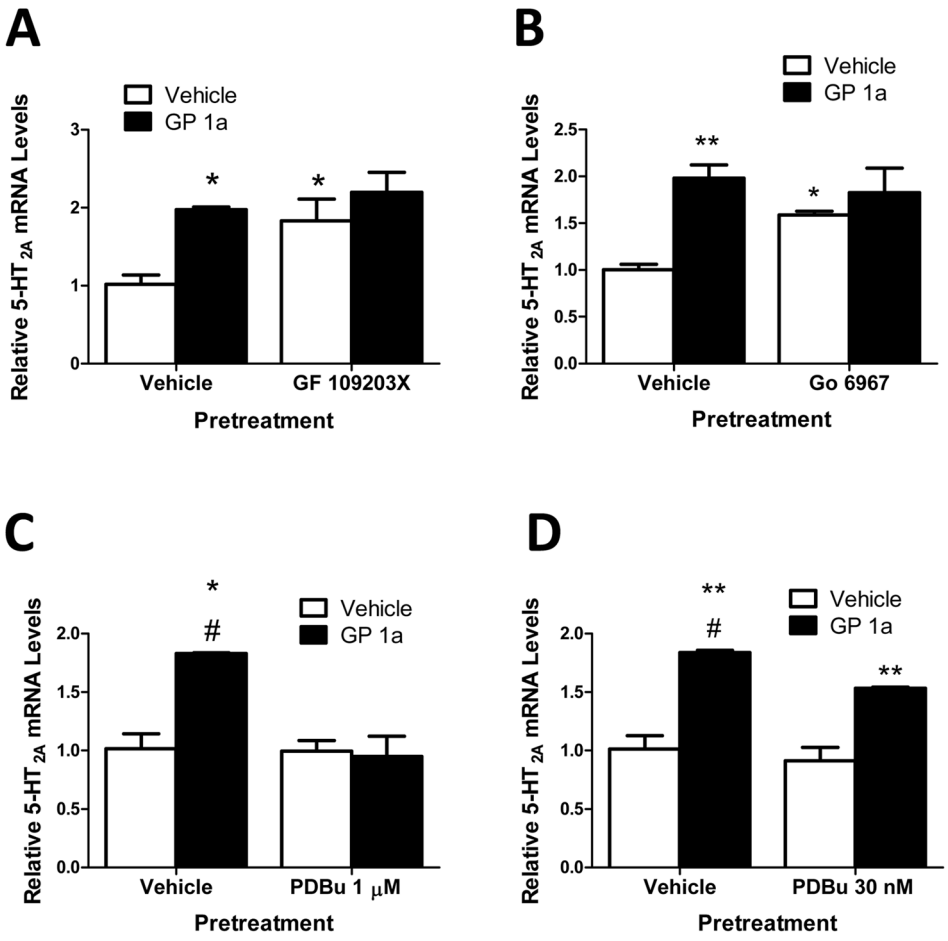

Figure 4. Ca^{2+} -independent and -dependent isoforms of PKC regulate 5-HT_{2A} receptor mRNA levels in CLU213 cells

(A) Inhibition of Ca^{2+} -independent and –dependent isoforms of PKC (GF 109203X) enhanced basal levels of 5-HT_{2A} receptor mRNA. *p<0.05, significant effect of GP 1a treatment, GF 109203X pretreatment, and GP 1a/GF 109203X treatment compared to vehicle-treated controls. (B) Inhibition of Ca^{2+} -dependent isoforms of PKC (Go 6967) enhanced basal levels of 5-HT_{2A} receptor mRNA. **p<0.01, significant effect of GP 1a treatment, Go 6967 pretreatment, and GP 1a/Go 6967 treatment compared to vehicle-treated controls. (C) Activation of Ca^{2+} -independent and -dependent isoforms of PKC (PDBu) prevented GP 1a-induced increases in 5-HT_{2A} receptor mRNA. *p<0.05, significant effect of GP 1a treatment compared to vehicle-treated controls. #p<0.05, significant effect of PDBu pretreatment on GP 1a-induced increases in 5-HT_{2A} receptor mRNA. (D) Activation of Ca^{2+} -dependent isoforms of PKC did not prevent GP 1a-induced increases in 5-HT_{2A} receptor mRNA. **p<0.01, significant effect of GP 1a treatment compared to vehicle-treated controls. **The data represent mean ± SEM (n=3).**

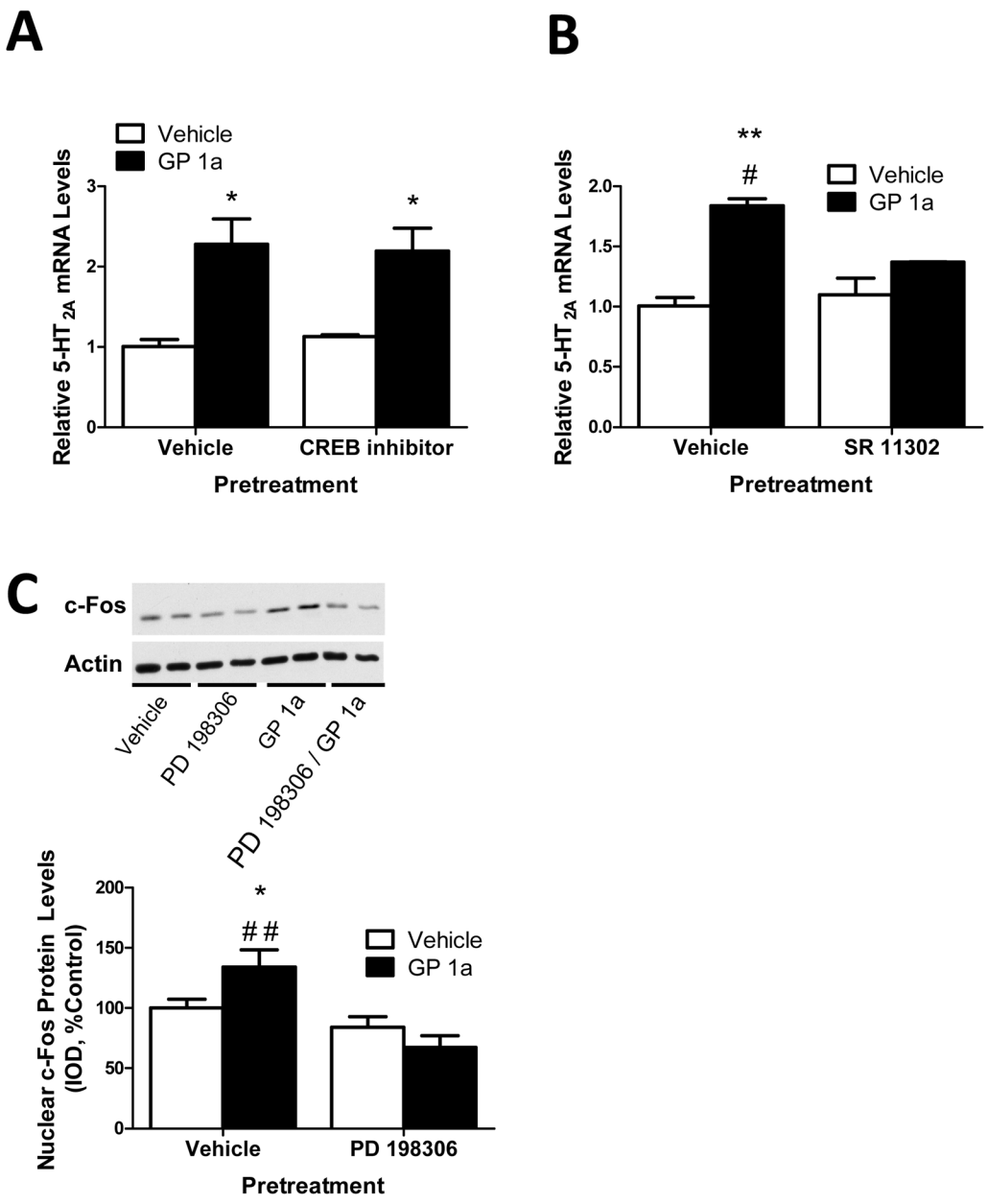

Figure 5. CB2 receptor-induced upregulation of 5-HT$_{2A}$ receptor involves AP-1 and c-fos, but not CREB, activation

(A) Inhibition of CREB activation did not prevent or significantly reduce GP 1a-induced increases in 5-HT$_{2A}$ receptor mRNA. *p<0.05, significant effect of GP 1a and CREB/GP 1a treatment on 5-HT$_{2A}$ receptor mRNA levels compared to vehicle-treated controls. (B) CB2 receptor-mediated upregulation of 5-HT$_{2A}$ receptor involves AP-1 transcription factor activity. **p<0.01, significant effect of GP 1a treatment compared to vehicle-treated controls. #p<0.05, significant effect of AP-1 transcription factor inhibitor pretreatment on GP 1a-induced increases in 5-HT$_{2A}$ receptor mRNA. (C) Inhibition of GP 1a mediated increases in nuclear c-fos protein levels via a selective ERK1/2 inhibitor (PD 198306). *p<0.05, significant effect of GP 1a treatment on nuclear c-fos levels compared to vehicle

treated controls. ##p<0.01, significant effect of PD 198306 pretreatment on GP 1a-induced increases in nuclear c-fos levels. **The data represent mean ± SEM (n=3).**