

Characterization of Cannabidiol-Mediated 5-HT_{2A} Receptor Modulation Under Variable Receptor Expression Conditions

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SUMMARY

The 5-HT_{2A} receptor is a G_{q/11}-coupled G protein-coupled receptor involved in processes such as cognition, perception, mood regulation and implicated in neuropsychiatric disorders. It is also the main receptor through which classical serotonergic psychedelics exert their effects, making it an important pharmacological target. In parallel, (-)-*trans*-cannabidiol (CBD), a non-intoxicating phytocannabinoid, has attracted increasing attention due to its polypharmacology and potential therapeutic effects. Recent evidence suggests that CBD may act as a negative allosteric modulator (NAM) of the 5-HT_{2A} receptor. However, allosteric modulation is often assay- and context-dependent. Consequently, the robustness and functional relevance of the proposed NAM activity of CBD at the 5-HT_{2A} receptor remain incompletely characterized.

This thesis aimed to further characterize the proposed modulatory effects of CBD on 5-HT_{2A} receptor signalling using an inducible receptor expression system and to expand current knowledge by evaluating structurally related compounds. HEK293 Flp-In™ T-REx™ cells stably expressing the human 5-HT_{2A} receptor were used, enabling controlled manipulation of receptor expression levels through tetracycline induction. Responses were assessed using Ca²⁺ mobilization as a downstream readout of receptor activation.

Under high receptor expression conditions, neither CBD, its enantiomer (+)-CBD, nor the major metabolites 6 α -OH-CBD and 7-COOH-CBD detectably modulated 5-HT-induced Ca²⁺ responses. Likewise, the reported positive allosteric modulators (PAMs), oleamide and JPC0323, failed to alter signalling under these conditions. Reducing receptor expression revealed a modest inhibitory effect on 5-HT-induced responses following 1-hour pretreatment with 10 μ M CBD, whereas 1 μ M CBD and shorter pretreatment periods remained inactive. Mescaline-induced responses appeared more sensitive to CBD-mediated inhibition than 5-HT-induced responses under high receptor expression conditions. Again, this effect was restricted to 1-hour pretreatment with 10 μ M CBD. Collectively, these findings suggest that receptor reserve, signal amplification and agonist efficacy influence the detectability of subtle modulatory effects of CBD in Ca²⁺ mobilization assays.

Overall, the observed effects of CBD at the 5-HT_{2A} receptor appear modest and strongly context-dependent, raising uncertainty regarding their physiological relevance. Future studies using complementary signalling readouts, such as a proximal G α_q BRET-biosensor or equilibrium-based IP₁ accumulation assays, will be necessary to clarify the mechanistic and translational relevance of this interaction. Aggregation controls should also be included to distinguish specific allosteric modulation from non-specific effects related to CBD. Further assessment of structurally related compounds under reduced receptor expression or mescaline as the agonist probe may provide additional insight into the structural determinants of this interaction.

SAMENVATTING

De 5-HT_{2A}-serotonine-receptor is een G_{q/11}-gekoppelde G-proteïne-gekoppelde receptor die betrokken is bij processen zoals cognitie, stemming, perceptie en neuropsychiatrische aandoeningen. De receptor vormt bovendien het voornaamste aangrijpingspunt voor serotonerge psychedelica en is daardoor een belangrijke farmacologische target. (-)-*Trans*-cannabidiol (CBD), een niet-intoxicerende fytocannabinoïde, krijgt toenemende aandacht vanwege zijn complexe polyfarmacologie en mogelijke therapeutische effecten. Een recente studie suggereert dat CBD zou kunnen optreden als een negatieve allosterische modulator (NAM) van de 5-HT_{2A} receptor. Aangezien allosterische modulatie vaak sterk assay- en contextafhankelijk kan zijn, blijven de robuustheid en relevantie van deze interactie onduidelijk.

Deze masterproef had als doel de effecten van CBD op de 5-HT_{2A} receptor verder te karakteriseren met behulp van een induceerbaar receptorexpressiesysteem. HEK293 Flp-In™ T-REx™-cellen die de humane 5-HT_{2A} receptor stabiel tot expressie brengen, werden gebruikt. De receptorexpressie werd via tetracycline-inductie geregeld en intracellulaire Ca²⁺-mobilisatie werd gebruikt als uitlezing van receptoractivatie.

Onder omstandigheden van hoge receptorexpressie veroorzaakten noch CBD en (+)-CBD, noch de belangrijke metabolieten 6 α -OH-CBD en 7-COOH-CBD een detecteerbare modulatie van 5-HT-geïnduceerde Ca²⁺-responsen. Evenmin werd een effect waargenomen voor de positieve allosterische modulatoren, oleamide en JPC0323. Verlaging van de receptorexpressie bracht daarentegen een bescheiden reductie van 5-HT-geïnduceerde responsen door CBD teweeg. Dit effect was concentratie- en tijdsafhankelijk en trad uitsluitend op na één uur voorbehandeling met 10 μ M CBD; 1 μ M CBD en kortere incubatietijden hadden geen effect. Mescaline-geïnduceerde responsen waren gevoeliger voor CBD-gemedieerde inhibitie dan 5-HT-geïnduceerde responsen onder condities van hoge receptorexpressie. Ook dit effect bleef beperkt tot een voorbehandeling van één uur met 10 μ M CBD. Deze bevindingen suggereren dat receptorreserve, signaalamplificatie en efficaciteit van de agonist de detectie van CBD-gemedieerde effecten kunnen beïnvloeden.

Samengevat zijn de effecten van CBD op de 5-HT_{2A} receptor eerder bescheiden en sterk contextafhankelijk, waardoor de fysiologische relevantie ervan onzeker blijft. Toekomstig onderzoek met complementaire assays, zoals G α_q -BRET-biosensoren of equilibrium gebaseerde IP1-accumulatie assays, is nodig om de translationele betekenis van deze interactie verder te verduidelijken. Daarnaast zijn aggregatiecontroles noodzakelijk om allosterische modulatie te onderscheiden van niet-specifieke effecten gelinkt aan CBD. Verdere evaluatie van structureel verwante verbindingen onder verlaagde receptorexpressiecondities of met mescaline als partiële agonist kan bijkomende inzichten opleveren over de invloed van structurele modificaties.

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LIST OF ABBREVIATIONS

(+)-CBD	(+)- <i>trans</i> -cannabidiol
5-HT	5-hydroxytryptamine
6 α -OH-CBD	6 α -hydroxy-cannabidiol
7-COOH-CBD	7-carboxy-cannabidiol
7-OH-CBD	7-hydroxy-cannabidiol
ANOVA	Analysis of variance
AtT20	Mouse pituitary tumour cell line 20
BRET	Bioluminescence resonance energy transfer
BSA	Bovine serum albumin
CB1	Cannabinoid receptor type 1
CB2	Cannabinoid receptor type 2
CBD	(-)- <i>trans</i> -cannabidiol
CRC	Concentration-response curve
DAG	Diacylglycerol
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EC ₅₀	Half-maximal effective concentration, potency
E _{max}	Maximal agonist response, maximal effect
FBS	Fetal bovine serum
GFP2	Green fluorescent protein 2
GIRK	G protein-gated inwardly rectifying potassium
GPCR	G protein-coupled receptor
GTP	Guanosine triphosphate
HBSS	Hank's balanced salt solution
HEK293	Human embryonic kidney 293
IP ₁	Inositol-1-phosphate
IP ₃	Inositol-1,4,5-trisphosphate
JPC0323	N-[2-hydroxy-1,1- <i>bis</i> (hydroxymethyl)ethyl]-9Z-octadecenamide

LSD	Lysergic acid diethylamide
MPA	Membrane potential assay
NAM	Negative allosteric modulator
PAM	Positive allosteric modulator
PAR-1	Protease-activated receptor 1
PBS	Phosphate-buffered saline
PEI	Polyethyleneimine
RFU	Relative fluorescence units
RLuc8	Renilla luciferase 8
S1P	Sphingosine-1-phosphate
SEM	Standard error of the mean
THC	Δ^9 -tetrahydrocannabinol
T-REx™	Tetracycline-Regulated Expression
TRUPATH	Transducer pathway

1. INTRODUCTION

1.1. G PROTEIN-COUPLED RECEPTORS (GPCRs) AND ALLOSTERIC PHARMACOLOGY

1.1.1. GPCR structure and signalling

G protein-coupled receptors (GPCRs) represent one of the largest and most important target classes in modern pharmacology. These seven-transmembrane receptors detect a plethora of extracellular signals, including neurotransmitters, peptides, hormones, ions and photons and transduce them into intracellular responses by undergoing conformational changes that activate associated heterotrimeric G proteins ($G\alpha\beta\gamma$) [1]. Receptor activation promotes the exchange of guanosine diphosphate for guanosine triphosphate (GTP) on the $G\alpha$ subunit, resulting in dissociation into $G\alpha$ -GTP and $G\beta\gamma$. Both $G\alpha$ -GTP and $G\beta\gamma$ subsequently regulate downstream signalling pathways [1,2].

However, GPCR signalling is no longer viewed as a simple transition between inactive and active receptor states. GPCRs can adopt multiple conformational states associated with distinct signalling outcomes. Consequently, different ligands may stabilize distinct receptor conformations and preferentially engage specific intracellular pathways, a phenomenon known as biased signalling or functional selectivity [3].

1.1.2. Allosteric modulation of GPCRs

Historically, most approved GPCR-targeting drugs have acted at orthosteric binding sites, which are recognized by endogenous ligands. Because orthosteric sites are typically highly conserved across receptor subtypes, opportunities for subtype-selective targeting may be limited. In contrast, allosteric ligands bind to topographically distinct sites that are often less conserved across receptor subtypes and therefore structurally more diverse. This structural diversity can facilitate the development of ligands with improved receptor subtype selectivity and has contributed to the growing interest in allosteric modulators [4–6].

Allosteric modulators are commonly classified into three categories. Positive allosteric modulators (PAMs) enhance the potency and/or efficacy of orthosteric agonists. In contrast, negative allosteric modulators (NAMs) attenuate agonist-induced effects. Neutral allosteric ligands bind to allosteric sites without altering the efficacy or potency of orthosteric agonists, although they may still influence receptor pharmacology by competing with other allosteric ligands. In addition, some allosteric modulators may display intrinsic agonist or inverse agonist activity alongside their modulatory effects [4,7].

Interest in allosteric modulators as therapeutic agents has increased because they may offer several advantages over orthosteric ligands. These include the potential for improved receptor subtype selectivity and the saturability of their modulatory effect, meaning that increasing modulator concentrations do not necessarily produce unlimited enhancement or inhibition of receptor signalling. This property may reduce the risk of excessive modulation and could contribute to improved safety compared with orthosteric agonists. Furthermore, the activity of modulators without intrinsic efficacy depends on the presence and local concentration of endogenous agonists, potentially allowing preservation of physiological signalling by providing a degree of spatiotemporal selectivity [8–11].

Despite these advantages, the clinical translation and pharmacological characterization of allosteric modulators remain challenging. Interspecies differences in receptor sequence may complicate clinical translation, as variability is often more pronounced at allosteric sites than at orthosteric sites [12]. In addition, allosteric modulators frequently exhibit “probe dependence”, whereby the observed modulatory effect varies according to the orthosteric ligand used. Different probes may therefore reveal distinct pharmacological profiles for the same allosteric modulator [13,14]. Moreover, observed pharmacological effects may differ depending on cellular background, assay system and receptor expression levels [13]. Consequently, the detection and characterization of allosteric modulation are highly dependent on the experimental context, including the selected signalling readout [7,15]. These considerations are particularly relevant when evaluating weak or incompletely characterized allosteric interactions, where the apparent presence or absence of modulation may depend strongly on the experimental system used.

1.2. CANNABIDIOL (CBD) PHARMACOLOGY AND METABOLISM

1.2.1. Pharmacology and clinical development of CBD

Cannabis sativa produces a diverse range of phytochemicals, including terpenes, flavonoids and more than 100 phytocannabinoids [16]. Phytocannabinoids are naturally occurring compounds produced by *Cannabis sativa*, several of which interact with cannabinoid receptors [17]. In the plant, cannabinoids mainly occur as acidic precursors, such as cannabidiolic acid, which decarboxylate upon heating [18,19]. One of the major phytocannabinoids, (-)-*trans*-cannabidiol (CBD), was first isolated in 1940 and its structure was later elucidated in 1963 [20,21]. Despite CBD's earlier identification, research historically focused more strongly on Δ^9 -tetrahydrocannabinol (THC), which was recognized as the principal psychoactive constituent of *Cannabis* [17,22].

Unlike THC, CBD is non-intoxicating and has been investigated for several potential therapeutic effects, including anxiolytic, antipsychotic and neuroprotective effects [16,22–26]. In 2018, this therapeutic potential

translated into clinical application with the approval of Epidiolex®, a purified CBD formulation. Epidiolex® is indicated for the treatment of Dravet syndrome and Lennox-Gastaut syndrome in patients aged two years and older [27]. In addition, Sativex®, an oromucosal spray containing CBD and THC in a ratio of 1:1, has been approved in several countries for the treatment of multiple sclerosis-associated spasticity [28].

CBD contains two chiral centres, giving rise to four stereoisomers [29]. The naturally occurring form in *Cannabis sativa* is (-)-*trans*-CBD. The remaining stereoisomers, (+)-*trans*-cannabidiol ((+)-CBD), (+)-*cis*-cannabidiol and (-)-*cis*-cannabidiol, can be synthesized and may exhibit distinct biological activities [29]. For example, (+)-*trans*-CBD has been reported to act as an agonist at sphingosine-1-phosphate (S1P) receptor types 1 and 3, whereas (-)-*trans*-CBD lacks this activity [30]. This distinction is important because experimental studies do not always clearly specify the stereochemical form of CBD used. Plant-derived CBD predominantly consists of (-)-*trans*-CBD, whereas synthetic preparations may contain (+)-*trans*-CBD, (-)-*trans*-CBD or racemic mixtures.

1.2.2. CBD as an allosteric modulator

CBD displays pronounced polypharmacology, interacting with multiple molecular targets including GPCRs, ion channels and enzymes [31]. Depending on the receptor system and experimental context, CBD has been reported to exert agonistic, antagonistic and allosteric effects. Table 1.1 summarizes the reported positive or negative allosteric modulatory effects of CBD across different receptor systems. However, these effects are often assay- and context-dependent.

Table 1.1. Overview of reported allosteric effects of CBD. CB1, cannabinoid receptor 1; CB2, cannabinoid receptor 2; MOR, μ -opioid receptor; DOR, δ -opioid receptor; 5-HT, 5-hydroxytryptamine; A_{2A}, adenosine receptor type 2A; GlyR, glycine receptor; α_7 -nAChR, α_7 -nicotinic acetylcholine receptor; GABA_A, γ -aminobutyric acid receptor type A.

Target	Reported modulation	Reference
CB1	NAM	[32–35]
CB2	NAM; partial agonist; antagonist	[35,36]
MOR, DOR	NAM (acceleration of agonist dissociation)	[37,38]
5-HT _{1A}	PAM; partial agonist	[39,40]
5-HT _{2A}	NAM	[41]
5-HT _{3A}	NAM	[42,43]
A _{2A} , A _{2A} -CB2 heteromer	NAM; biased NAM with reduced β -arrestin signalling at the heteromer	[44–46]
GlyR	PAM	[47]
α_7 -nAChR	NAM	[48]
GABA _A	PAM	[49]

1.2.3. Metabolic pathways and major metabolites of CBD

Understanding CBD metabolism is important because biotransformation may alter receptor interactions and pharmacological activity. Although CBD pharmacokinetics have been relatively well characterized, the receptor pharmacology of its metabolites remains much less understood [50]. This is relevant because circulating concentrations of certain metabolites may equal or exceed those of parent CBD. Metabolites with pharmacological activity could therefore contribute to, modify or even counteract the effects observed after CBD administration [50,51].

CBD shows substantial pharmacokinetic variability depending on the route of administration, with pronounced inter- and intraindividual differences. Reported plasma concentrations are typically in the low nanomolar range, although high-dose administration can yield concentrations in the high nanomolar to low micromolar range. Following absorption, CBD undergoes extensive hepatic metabolism, resulting in a broad range of oxidative and conjugated metabolites [50,52–55]. Early *in vivo* studies identified unchanged CBD, its *O*-glucuronide and multiple oxidized metabolites in urine, reflecting extensive biotransformation. These studies also suggested that C-7 oxidation represents a key initial metabolic step, followed by further hydroxylation of the pentyl side chain and modifications in the terpene region of CBD [50,51,56].

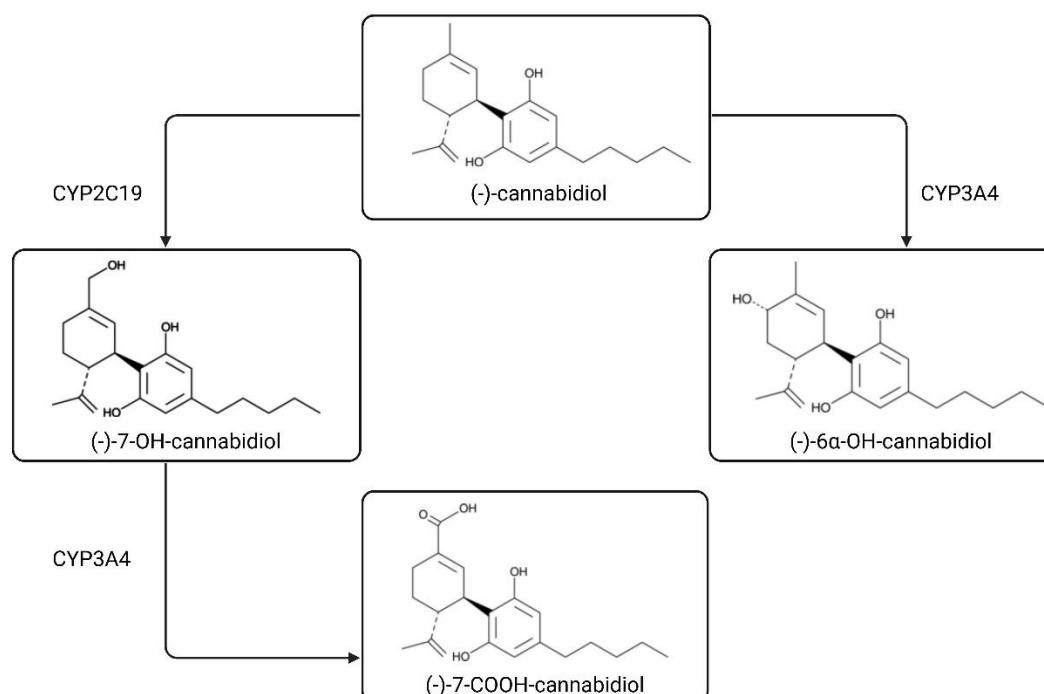


Figure 11. Structures of CBD and three major metabolites. Metabolism by cytochrome P450 enzymes produces 7-hydroxy-cannabidiol (7-OH-CBD), 7-carboxy-cannabidiol (7-COOH-CBD) and 6α-hydroxy-cannabidiol (6α-OH-CBD). Structures were adapted from Cayman Chemical and the figure was created in BioRender.

In plasma samples from healthy volunteers following intravenous CBD administration, 7-carboxy-CBD (7-COOH-CBD; Figure 1.1) and related hydroxylated derivatives were identified as the major circulating metabolites [50,57,58]. Oxidative metabolism of CBD is primarily mediated by cytochrome P450 enzymes, particularly CYP2C19 and CYP3A4 (Figure 1.1). *In vitro* studies using human liver microsomes identified eight monohydroxylated metabolites, of which 6 α -hydroxy-CBD (6 α -OH-CBD), 6 β -hydroxy-CBD, 4''-hydroxy-CBD and 7-hydroxy-CBD (7-OH-CBD) were the most abundant [50,56]. Among these, 7-OH-CBD has been identified as an active metabolite [59]. Regarding phase II metabolism, glucuronidation at the phenolic oxygen of CBD is the dominant pathway [50,60].

1.3. THE SEROTONERGIC SYSTEM

1.3.1. The serotonin receptor family and classification

Serotonin (5-hydroxytryptamine; 5-HT) is a monoamine neurotransmitter originally identified as a peripheral vasoconstrictor but later recognized as an important regulator of central nervous system function. In the central nervous system, 5-HT contributes to numerous behavioural and physiological processes, including mood, cognition and perception [61–63].

The serotonin receptor family comprises seven receptor subfamilies, 5-HT₁ to 5-HT₇, making it one of the most diverse receptor families activated by a single neurotransmitter. These subfamilies can be further divided into distinct subtypes, such as 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C}. With the exception of the ionotropic 5-HT₃ receptor, which forms a ligand-gated ion channel, all serotonin receptors belong to the GPCR superfamily [64–67].

G protein-coupled 5-HT receptors are commonly grouped according to their predominant G protein coupling. The 5-HT₂ receptor family, comprising 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C} receptors, primarily couples to G_{q/11} proteins, leading to activation of phospholipase C signalling pathways. The G_s-coupled 5-HT receptors include the subtypes 5-HT₄, 5-HT₆ and 5-HT₇, which stimulate adenylyl cyclase and increase cyclic adenosine monophosphate levels. In contrast, the 5-HT₁ and 5-HT₅ receptor families predominantly couple to G_{i/o} proteins, leading to inhibition of adenylyl cyclase and reduced cyclic adenosine monophosphate levels [64].

1.3.2. 5-HT_{2A} receptor signalling pathways

Activation of the 5-HT_{2A} receptor can initiate multiple downstream signalling pathways, with the canonical pathway involving G $\alpha_{q/11}$ -mediated signal transduction [67–69]. Activation of G $\alpha_{q/11}$ stimulates phospholipase C- β , which hydrolyses the membrane phospholipid phosphatidylinositol-4,5-bisphosphate into

inositol-1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG). IP₃ subsequently triggers the release of intracellular Ca²⁺ from the endoplasmic reticulum. DAG activates protein kinase C and both IP₃- and DAG-dependent pathways regulate downstream cellular processes [65,67–70].

Beyond the canonical pathway, the 5-HT_{2A} receptor engages several non-canonical pathways, as illustrated in Figure 1.2. These include coupling to alternative G proteins and interactions with scaffolding proteins such as β -arrestins, which contribute to receptor internalization, desensitization and signalling bias [63,67–69,71]. Additional pathways include phospholipase A₂ activation and subsequent arachidonic acid release, processes implicated in inflammatory signalling [72]. This signalling complexity also includes activation of extracellular signal-regulated kinase 1/2, which may occur through G $\beta\gamma$ subunits released following activation of G_{i/o}. In parallel, G $\alpha_{i/o}$ signalling can inhibit adenylyl cyclase. Furthermore, receptor coupling to G $\alpha_{12/13}$ has been implicated in phospholipase A₂ activation through Ras homolog family member A and p38 mitogen-activated protein kinase [73,74].

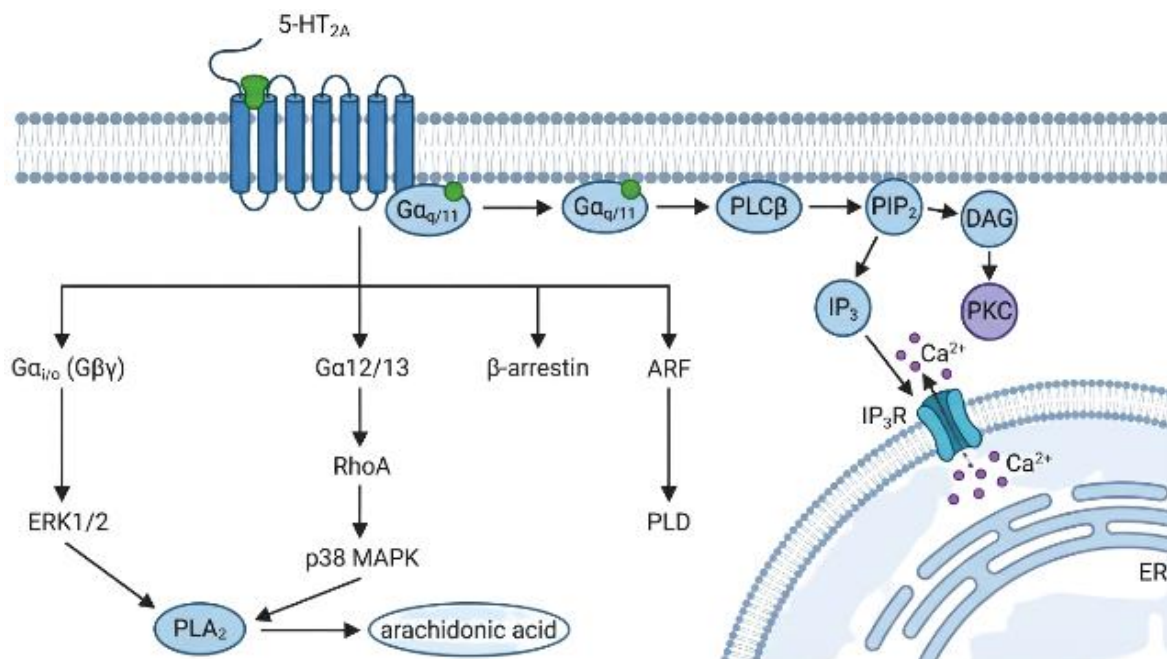


Figure 1.2. Overview of selected signalling pathways associated with 5-HT_{2A} receptor activation. The canonical pathway involves G $\alpha_{q/11}$ -mediated signalling. Additional pathways include G $\beta\gamma$ subunits associated with G_{i/o}, G $\alpha_{12/13}$, β -arrestin and ARF. Inhibition of adenylyl cyclase by G $\alpha_{i/o}$ is not depicted. ERK 1/2, extracellular signal-regulated kinase 1/2; ARF, adenosine diphosphate ribosylation factor; RhoA, Ras homolog family member A; MAPK, mitogen-activated protein kinase; PLA₂, phospholipase A₂; PLD, phospholipase D; PLC β , phospholipase C- β ; ER, endoplasmic reticulum; PIP₂, phosphatidylinositol-4,5-bisphosphate; PKC, protein kinase C. Figure was created with BioRender.

The 5-HT_{2A} receptor can also activate phospholipase D via mechanisms involving adenosine diphosphate ribosylation factor, a small G protein capable of directly interacting with GPCRs. Collectively, these pathways highlight the pleiotropic nature of 5-HT_{2A} receptor signalling and provide a mechanistic basis for functional selectivity at this receptor [75].

1.3.3. Functional relevance of the 5-HT_{2A} receptor

The 5-HT_{2A} receptor is widely expressed throughout the central and peripheral nervous systems [63,67]. In the brain, it is particularly implicated in cognition, perception and mood regulation. Consequently, the 5-HT_{2A} receptor has become an important pharmacological target in neuropsychiatric disorders, including depression, schizophrenia and anxiety [69,76–78]. In addition, 5-HT_{2A} receptor signalling has been associated with peripheral pathologies, including cardiovascular disease and gastrointestinal disorders [69].

Although 5-HT_{2A} receptor agonists have long attracted therapeutic interest, their development was historically limited due to their pronounced psychoactive effects. The classification of several serotonergic psychedelics as Schedule I controlled substances by the U.S. Drug Enforcement Administration further restricted clinical and pharmacological research [69,79]. More recently, however, interest in the therapeutic application of serotonergic psychedelics has re-emerged. Compounds such as psilocybin, the natural prodrug of psilocin, are currently being investigated in a growing number of clinical trials for conditions including depression, addiction, anxiety and obsessive-compulsive disorder [77]. This renewed interest is partly driven by evidence that serotonergic psychedelics can promote neuroplasticity-related processes, which may contribute to sustained clinical effects [76,80].

In contrast, 5-HT_{2A} antagonists and inverse agonists have been more extensively developed and are well established in clinical practice. 5-HT_{2A} antagonism or inverse agonism constitutes a key pharmacological component of several atypical antipsychotics. Agents combining 5-HT_{2A} antagonism with dopamine D2 receptor inhibition are widely used in the treatment of schizophrenia. Additionally, some of these antagonists have also demonstrated efficacy in mood disorders, including mania and depression [81,82].

Together, the central role of the 5-HT_{2A} receptor in neuropsychiatric pharmacology and the potential advantages of allosteric modulation make this receptor an attractive target for allosteric ligand discovery. In this context, CBD is of particular interest because it has recently been proposed to act as a NAM of the 5-HT_{2A} receptor [41].

1.4. CBD AND SEROTONERGIC MODULATION

1.4.1. CBD interactions with serotonergic receptors

CBD has been reported to interact with multiple components of the serotonergic system, including both GPCRs and ligand-gated ion channels. Among these interactions, the 5-HT_{1A} receptor is one of the most extensively investigated. Early studies reported that CBD enhanced [³⁵S]-guanosine 5'-O-(3-thio)-triphosphate binding, a non-hydrolysable GTP analogue, consistent with agonist-like activity or facilitation of receptor activation [39,40]. This interaction has been proposed to contribute to the anxiolytic, antipsychotic and antidepressant-like effects associated with CBD [83–85].

In addition to GPCR targets, CBD modulates the ligand-gated 5-HT_{3A} receptor. In *Xenopus laevis* oocytes expressing the mouse 5-HT_{3A} receptor, CBD reduced 5-HT efficacy without displacing the radioligand, supporting an allosteric mode of modulation [42,43]. Collectively, these findings suggest that CBD can influence serotonergic signalling through multiple receptor systems and mechanisms.

1.4.2. CBD and the 5-HT_{2A} receptor

Early studies suggested a possible interaction between CBD and the 5-HT_{2A} receptor by demonstrating reduced binding of the antagonist ketanserin in the presence of CBD [39]. CBD has also been shown to reduce 2,5-dimethoxy-4-iodoamphetamine-induced head-twitch responses, a behavioural proxy for 5-HT_{2A}-mediated hallucinogenic activity [41,86]. However, these early observations provided only indirect evidence and did not establish whether CBD directly modulates 5-HT_{2A} receptor signalling.

More recently, Billard *et al.* proposed that CBD may act as a NAM of the 5-HT_{2A} receptor. Using bioluminescence resonance energy transfer (BRET)-based biosensors for DAG production, Gα_q activation and β-arrestin 2 recruitment, Billard *et al.* observed that CBD reduced 5-HT_{2A} agonist efficacy without substantially altering agonist potency [41]. These findings may provide an additional mechanism contributing to CBD's proposed antipsychotic profile, alongside previously implicated targets such as transient receptor potential vanilloid 1 and 5-HT_{1A} receptors [26,85,87,88].

Although allosteric modulation is well established across many GPCR families, few well-characterized allosteric modulators of the 5-HT_{2A} receptor have been described. One reported example is oleamide, an endogenous lipid identified in cerebrospinal fluid that has been described as a 5-HT_{2A} receptor PAM [89,90]. Synthetic analogues of oleamide have also been developed, including N-[2-hydroxy-1,1-

bis(hydroxymethyl)ethyl]-9Z-octadecenamide (JPC0323), which has been described as a dual 5-HT_{2A}/5-HT_{2C} receptor modulator [91]. Computational site identification by ligand competitive saturation has suggested a putative shared binding site for CBD and oleamide [41].

Despite the recent evidence for CBD-mediated modulation of the 5-HT_{2A} receptor, the robustness and functional relevance of this interaction remain incompletely understood. Existing evidence is largely based on proximal signalling readouts, whereas it is unclear whether this modulation is sufficiently robust to influence downstream functional responses such as intracellular Ca²⁺ mobilization. This is particularly relevant because allosteric modulation can be influenced by the experimental context, including the cellular assay system, receptor expression level and orthosteric agonist used. Therefore, it remains uncertain whether CBD-mediated modulation represents a broadly detectable pharmacological effect or a context-dependent phenomenon. In addition, it is unknown whether structurally related CBD compounds share this activity.

2. OBJECTIVES

The 5-HT_{2A} receptor is a GPCR centrally involved in cognition, perception, mood regulation and neuropsychiatric disorders. It is also the primary receptor through which serotonergic psychedelics exert many of their characteristic effects, making the 5-HT_{2A} receptor an important pharmacological target. In parallel, the phytocannabinoid CBD attracts increasing interest due to its polypharmacology and proposed therapeutic applications, including in several neuropsychiatric conditions. Recent evidence suggests that CBD may act as a NAM of the 5-HT_{2A} receptor. Because allosteric modulators can potentially offer advantages over orthosteric ligands, such as improved subtype selectivity, spatiotemporal selectivity and saturability of effect, they represent an attractive strategy for modulating GPCR signalling. However, GPCR allosteric modulation often depends strongly on receptor expression, assay readout and the ligand used. Consequently, the robustness and functional relevance of CBD-mediated 5-HT_{2A} modulation remain incompletely understood.

The main objective of this study is to further characterize the proposed modulatory effect of CBD on 5-HT_{2A} receptor-mediated signalling using a controlled receptor expression system. Specifically, the study assesses intracellular Ca²⁺ mobilization as a downstream functional readout of G_q-coupled signalling in human embryonic kidney 293 (HEK293) Flp-In™ tetracycline-regulated expression system (T-REx™) cells stably expressing the human 5-HT_{2A} receptor. By varying tetracycline concentrations, receptor expression levels are experimentally manipulated to investigate the influence of receptor density on potential CBD-mediated modulation. Effects of CBD are assessed using the endogenous agonist 5-HT as well as selected partial agonists, mescaline and psilocin.

A secondary objective is to determine whether structurally related CBD compounds share modulatory activity at the 5-HT_{2A} receptor. For this purpose, the effects of the CBD enantiomer (+)-CBD and two major metabolites, 7-COOH-CBD and 6 α -OH-CBD are evaluated. This provides insight into the influence of stereochemistry and metabolic transformation on CBD's pharmacological activity. In addition, the reported 5-HT_{2A} PAMs oleamide and JPC0323 are screened as comparator compounds to evaluate whether previously described allosteric modulators can be detected under the same assay conditions. To support interpretation of negative or modest effects by CBD at the 5-HT_{2A} receptor, its bioactivity is verified using a CBI membrane potential assay (MPA).

Finally, an exploratory objective is to investigate proximal signalling events using TRUPATH BRET biosensors to assess G-protein activation downstream of the 5-HT_{2A} receptor, including G_{12/13} and G_q. Collectively, these objectives broaden the understanding of CBD's effects on 5-HT_{2A} receptor signalling and evaluate the influence of receptor density, agonist probe and structural modifications on this modulation.

3. MATERIALS AND METHODS

3.1. CELL CULTURE AND INDUCIBLE EXPRESSION SYSTEM

3.1.1. HEK293 Flp-In™ T-REx™ 5-HT_{2A} inducible expression cell line

HEK293 Flp-In™ T-REx™ cells (Life Technologies, Mulgrave, VIC, Australia) stably expressing the human 5-HT_{2A} receptor (GenScript, Piscataway, NJ, USA) were used. The Flp-In™ system enables site-specific recombination, allowing stable genomic integration of the human 5-HT_{2A} receptor gene [92,93]. The T-REx™ system provides inducible receptor expression through tetracycline-regulated transcription. 5-HT_{2A} receptor expression was induced by tetracycline addition prior to experiments [93,94].

3.1.2. Cell culture and maintenance

Cells were maintained in T75 culture flasks (Corning Inc., Kennebunk, ME, USA) containing 8 mL Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Carlsbad, CA, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; Bovogen, Melbourne, VIC, Australia), 100 U/mL penicillin (Life Technologies, Carlsbad, CA, USA), 100 µg/mL streptomycin (Life Technologies, Carlsbad, CA, USA), 80 µg/mL hygromycin B gold (InvivoGen, San Diego, CA, USA) and 15 µg/mL blasticidin (InvivoGen, San Diego, CA, USA). Cultures were maintained at 37 °C in a humidified atmosphere containing 5% CO₂ and medium was replaced every two to three days.

Cells were passaged at 80-90% confluency. Culture medium was aspirated, cells were rinsed with phosphate-buffered saline (PBS; Life Technologies, Carlsbad, CA, USA) and detached using trypsin-ethylenediaminetetraacetic acid (Life Technologies, Carlsbad, CA, USA). Trypsin activity was neutralized with complete DMEM, after which cells were centrifuged for 5 minutes at 105 × g. Cell pellets were resuspended in complete medium and reseeded at the required density. All cell culture procedures were performed under sterile conditions and cells were used up to passage 25.

3.1.3. Poly-D-lysine coating of 96-well plates

Poly-D-lysine-coated 96-well plates were used to enhance the attachment of HEK293 cells. A 10 µg/mL poly-D-lysine solution (Sigma-Aldrich, Melbourne, VIC, Australia) was prepared by diluting 100 µL of a 1 mg/mL stock solution in 10 mL of sterile-filtered Milli-Q water. Plates were incubated with 50 µL of 10 µg/mL poly-D-lysine per well for 10 minutes, followed by two wash steps with 50 µL of sterile Milli-Q water. Plates were allowed to dry under sterile conditions and were used within a month.

3.2. Ca²⁺ MOBILIZATION ASSAY

3.2.1. Assay principle

The Ca²⁺ mobilization assay is a cell-based method used to assess the activation of G_q-coupled receptors by measuring intracellular Ca²⁺ levels. Cells are loaded with a membrane-permeable Ca²⁺-sensitive dye in its acetoxymethyl ester form. Intracellular hydrolysis of the ester generates a membrane-impermeable form of the dye. Upon receptor activation, Ca²⁺ released from the endoplasmic reticulum binds the dye, resulting in increased fluorescence intensity (Figure 3.1). The dye kit additionally contains a masking dye to reduce background fluorescence and eliminate the need for washing steps. Fluorescence was measured using a FlexStation 3 microplate reader (Molecular Devices, Sunnyvale, CA, USA) [95].

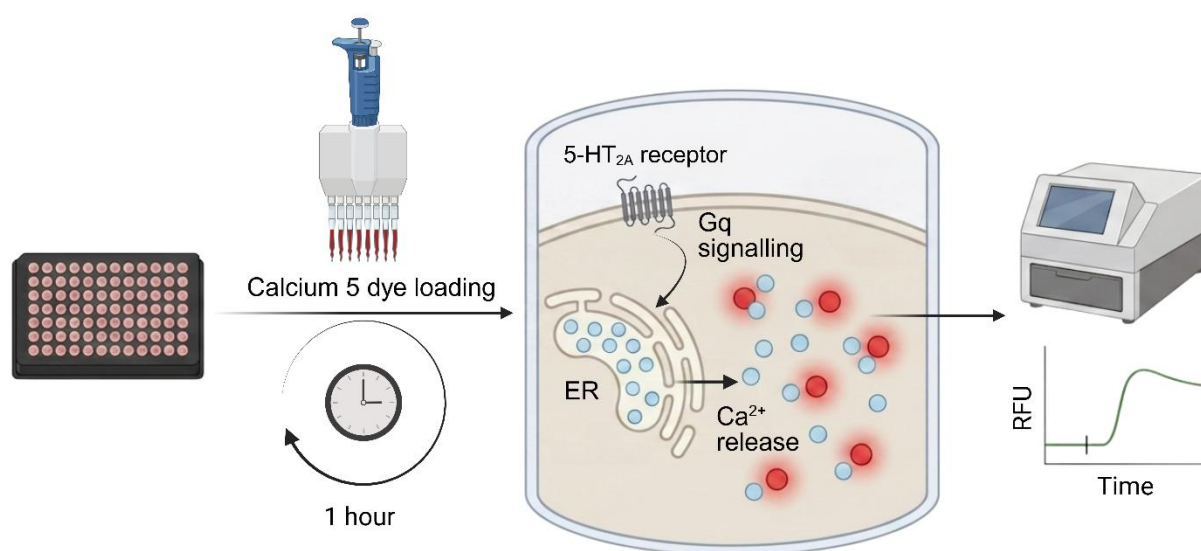


Figure 3.1. Schematic overview of the Ca²⁺ mobilization assay used to measure G_q-coupled receptor activation. Receptor stimulation induces Ca²⁺ release from the endoplasmic reticulum, resulting in increased fluorescence of the Ca²⁺-sensitive dye. Fluorescence is measured over time using a FlexStation 3 microplate reader. ER, endoplasmic reticulum; RFU, relative fluorescence units. The figure was created with BioRender.

3.2.2. Cell plating and receptor induction

Cells were harvested from one confluent T75 flask as described in Section 3.1.2 and resuspended in 10 mL Leibovitz's L-15 medium (Life Technologies, Carlsbad, CA, USA) supplemented with 1% (*v/v*) FBS, 100 U/mL penicillin, 100 µg/mL streptomycin and 15 mM glucose (Sigma-Aldrich, Melbourne, VIC, Australia). For standard Ca²⁺ mobilization experiments, tetracycline (Sigma-Aldrich, Melbourne, VIC, Australia) was added to a final concentration of 1 µg/mL to induce receptor expression. Cells were seeded into black-walled, clear-bottom, poly-

D-lysine-coated 96-well plates (Corning Inc., Kennebunk, ME, USA) at 90 μ L per well using an automated P1200 multichannel pipette (Gilson Inc., Middleton, WI, USA). Plates were incubated for 24 hours at 37 °C under ambient atmospheric conditions in a humidified incubator.

3.2.3. Ca^{2+} -sensitive dye loading

On the day of the experiment, Calcium 5 dye (Molecular Devices, Sunnyvale, CA, USA) was prepared in modified Hanks' balanced saline solution (HBSS) composed of (in mM): NaCl 145, CaCl_2 1.26, MgCl_2 0.493, HEPES 22, Na_2HPO_4 0.338, NaHCO_3 4.17, KH_2PO_4 0.441, MgSO_4 0.407 and glucose 1 mg/mL. The pH of HBSS was adjusted to 7.4 and osmolarity to 315 ± 15 mOsmol. Calcium 5 dye was used at 50% of the manufacturer's recommended concentration. Probenecid (Biotium, Fremont, CA, USA) was added at a final concentration of 1.25 mM to enhance intracellular dye retention by inhibiting organic anion transporters. Cells were loaded with 90 μ L dye per well and incubated for 1 hour at 37 °C in the FlexStation 3 microplate reader.

3.2.4. Drug preparation

All drug solutions were prepared at $10 \times$ the desired final assay concentration. Stock solutions of 5-HT (10 mM; Sigma-Aldrich, Melbourne, VIC, Australia) and other agonists, including lysergic acid diethylamide (LSD), mescaline and psilocin (all from Cayman Chemical, Ann Arbor, MI, USA), were serially diluted in HBSS containing 0.1% (*v/v*) bovine serum albumin (BSA; Sigma-Aldrich, Melbourne, VIC, Australia). Following 1:10 dilution upon addition to assay wells, final agonist concentrations ranged from 100 pM to 30 μ M.

CBD (National Measurement Institute, Sydney, NSW, Australia), (+)-CBD, 7-COOH-CBD, 6 α -OH-CBD, oleamide and JPC0323 (all from Cayman Chemical, Ann Arbor, MI, USA) were prepared from 30 mM stock solutions in dimethyl sulfoxide (DMSO; Sigma-Aldrich, Melbourne, VIC, Australia). Stock solutions were diluted 1:3 with DMSO to obtain 10 mM intermediate solutions and were subsequently diluted to 100 μ M in HBSS containing 0.1% (*v/v*) BSA. Further dilutions were prepared in HBSS containing 0.1% (*v/v*) BSA and 1% (*v/v*) DMSO to maintain compound solubility. The final DMSO concentration in assay wells was 0.1% (*v/v*). Ritanserin (Cayman Chemical, Ann Arbor, MI, USA), a selective 5-HT_{2A/2C} receptor antagonist, was prepared similarly. Detailed dilution schemes are provided in Appendix Table A8.4.

3.2.5. Assay procedure

For assays involving 5-minute pretreatment, baseline fluorescence was recorded for 60 seconds prior to the addition of 20 μ L pretreatment. Following a 5-minute pretreatment period, 22 μ L of agonist solution was added and fluorescence measurements were continued for an additional 120 seconds.

For 1-hour pretreatment experiments, pretreatment was added manually at 20 μ L per well during the 1-hour dye loading period. Pretreatment additions were staggered at 10-minute intervals across duplicate wells to ensure a consistent 1-hour pretreatment duration. Baseline fluorescence was measured for 60 seconds, followed by addition of 22 μ L agonist solution and continued fluorescence measurements for 220 seconds.

In all experiments, HBSS containing 0.01% (*m/v*) BSA and 0.1% (*v/v*) DMSO was used as the vehicle control. Fluorescence was measured using excitation and emission wavelengths of 485 nm and 525 nm, respectively. Assay performance was validated using activation of the endogenously expressed protease-activated receptor 1 (PAR-1) with its agonist Thr-Phe-Leu-Leu-Arg-NH₂ (AusPep, Tullamarine, VIC, Australia). The ionophore ionomycin (5 μ M) was used to confirm that dye saturation did not occur. All conditions were tested in duplicate wells and experiments were independently repeated three to six times unless stated otherwise. Additional experimental details are provided in Appendix Table A8.3.

3.2.6. Tetracycline titration of receptor expression

To investigate the impact of receptor expression levels on assay sensitivity, tetracycline titration experiments were performed using the T-REx™ inducible expression system. This approach allowed 5-HT_{2A} receptor activation to be assessed across varying receptor expression levels within the same cellular background. Cells were left uninduced or induced with tetracycline at 1, 5, 10, 100 and 1000 ng/mL. Ca²⁺ mobilization experiments were subsequently performed as described above. As a control for general Ca²⁺ signalling capacity, responses mediated by the endogenously expressed PAR-1 receptor, which is not regulated by the T-REx™ system, were assessed across tetracycline concentrations.

3.3. TRANSDUCER PATHWAY (TRUPATH) BRET ASSAY

3.3.1. Assay principle

The TRUPATH BRET assay monitors G protein activation by measuring changes in the proximity between tagged G protein subunits. The G α subunit is labelled with a bioluminescent donor, Renilla luciferase 8 (RLuc8),

while the $G\gamma$ subunit is fused to the fluorescent acceptor, green fluorescent protein 2 (GFP2). In the inactive state, the $G\alpha\beta\gamma$ heterotrimer remains assembled, positioning the donor and acceptor in close proximity. Addition of a coelenterazine substrate triggers bioluminescence from the RLuc8 donor, which transfers energy to the nearby GFP2 through BRET. This excites GFP2, causing it to emit fluorescence. Receptor activation induces conformational rearrangement and dissociation of the tagged subunits. This increased distance between donor and acceptor reduces energy transfer efficiency, resulting in a decreased BRET signal that reflects G protein activation [96–98].

3.3.2. Transfection with TRUPATH plasmids and cell plating

Cells were harvested at 80-90% confluency, counted using an automated Countess II cell counter (Life Technologies, Carlsbad, CA, USA) and seeded into 10 cm tissue culture dishes (Corning Inc., Kennebunk, ME, USA) at a density of 8×10^6 cells per dish in 10 mL complete DMEM. Following a 4-hour incubation at 37 °C in a humidified atmosphere containing 5% CO₂, cells were transiently transfected with TRUPATH plasmids encoding either the $G\alpha_{13}$ or $G\alpha_q$ BRET biosensor ($G\alpha_{13}$ -RLuc8:G β 1 (untagged): $G\gamma$ -GFP2 or $G\alpha_q$ -RLuc8:G β 3 (untagged): $G\gamma$ -GFP2; Addgene, Watertown, MA, USA), as previously described by Olsen *et al.* [99].

For transfection, 3 μ g plasmid deoxyribonucleic acid (DNA) was diluted in 150 mM NaCl to a final volume of 500 μ L. Polyethyleneimine (PEI; Sigma-Aldrich, Melbourne, VIC, Australia) was prepared separately in a final volume of 500 μ L at a DNA:PEI mass ratio of 1:6. The PEI solution was added dropwise to the DNA solution, briefly vortexed and incubated for 10 minutes to allow complex formation. The resulting DNA-PEI complexes were added dropwise to the plated cells. Transfected cells were incubated for 24 hours at 37 °C in a humidified atmosphere containing 5% CO₂.

Cells were harvested 24 hours post-transfection and centrifuged for 5 minutes at $105 \times g$. Cell pellets were resuspended in phenol red-free L-15 medium supplemented with 1% (v/v) FBS, 100 U/mL penicillin, 100 μ g/mL streptomycin and 15 mM glucose and counted. Tetracycline was added to a final concentration of 1 μ g/mL to induce 5-HT_{2A} receptor expression. Cells were seeded at a density of 100,000 cells/well (80 μ L per well) into poly-D-lysine-coated, white-walled, clear-bottom, 96-well plates. Plates were incubated for 24 hours at 37 °C under ambient atmospheric conditions in a humidified incubator.

3.3.3. TRUPATH assay procedure

On the day of the experiment, drug solutions were prepared as described in Section 3.2.4. BRET2 measurements were performed using a CLARIOstar Plus microplate reader (BMG LABTECH, Ortenberg, Germany) maintained at 37 °C. Donor and acceptor emission were measured at 410 ± 40 nm and 515 ± 10 nm, respectively. Detailed settings are provided in Appendix Table A8.6.

The coelenterazine substrate Prolume Purple (Prolume Ltd, Pinetop, AZ, USA) was prepared at 50 μ M in HBSS and protected from light. A volume of 10 μ L substrate was added to each well to achieve a final concentration of 5 μ M. Baseline BRET signals were recorded for 10 cycles (~ 5 minutes), followed by addition of 10 μ L drug solution and continued measurements for an additional 10 cycles. Assay performance was evaluated using S1P (Cayman Chemical, Ann Arbor, MI, USA) as a positive control for $G\alpha_{13}$ and $G\alpha_q$ activation and PAR-1 agonist was included as a positive control for $G\alpha_q$ activation. Due to limited aqueous solubility, S1P was dissolved in 3 mM NaOH and corresponding vehicle controls were included.

3.4. CELL MEMBRANE POTENTIAL ASSAY

3.4.1. Assay principle

The MPA was used to assess activation of CB1 receptors in mouse pituitary tumour 20 (AtT20) cells. Activation of CB1 receptors engages the pertussis toxin-sensitive $G\alpha_{i/o}$ pathway, resulting in release of $G\beta\gamma$ subunits that directly activate G protein-gated inwardly rectifying potassium (GIRK) channels. Opening of GIRK channels leads to membrane hyperpolarization. The fluorescent dye initially partitions into the plasma membrane. Upon hyperpolarization, the dye redistributes from the plasma membrane into the extracellular space, where it is quenched. As a result, the fluorescence intensity decreases, providing a quantitative measure of CB1 receptor activation [100].

3.4.2. MPA assay procedure

AtT20 Flp-In™ T-REx™ cells stably expressing the CB1 receptor were used for this assay. The generation and characterization of analogous AtT20 Flp-In™ T-REx™ cells expressing the CB2 receptor were previously described by Foyzun *et al.* The CB1-expressing cell line used in the present study was previously generated by our laboratory group using the same approach [101]. These cells endogenously express GIRK channels necessary for the assay readout.

Cells were harvested from one confluent T75 flask as described in Section 3.1.2 and resuspended in 10 mL supplemented Leibovitz's L-15 medium as described in section 3.2.2. Tetracycline was added to a final concentration of 1 µg/mL to induce receptor expression. Cells were seeded into black-walled, clear-bottom, poly-D-lysine-coated 96-well plates at 90 µL per well using an automated P1200 multichannel pipette. Plates were incubated for 24 hours at 37 °C under ambient atmospheric conditions in a humidified incubator.

On the day of the assay, cells were loaded with 90 µL of fluorometric imaging plate reader membrane potential dye (Molecular Devices, Sunnyvale, CA, USA), prepared at 50% of the manufacturer's recommended concentration in HBSS. Cells were incubated for 1 hour to allow membrane partitioning of the dye. CBD pretreatment solutions and CP-55,940 solutions were prepared in the same manner as described in Section 3.2.4. Pretreatment was added manually during the dye-loading period at 20 µL per well. Additions were staggered at 10-minute intervals across duplicate wells to ensure a consistent pretreatment duration of 1 hour prior to stimulation with CP-55,940 (Cayman Chemical, Ann Arbor, MI, USA).

Fluorescence was measured using a FlexStation 3 microplate reader maintained at 37 °C. Baseline fluorescence was recorded for 60 seconds, followed by addition of 22 µL CP-55,940 and continued recording for 240 seconds. HBSS containing 0.01% (*m/v*) BSA and 0.1% (*v/v*) DMSO served as the vehicle control. Excitation and emission wavelengths were set to 530 and 565 nm, respectively. Additional experimental details are provided in Appendix Table A8.3. All conditions were tested in duplicate wells in three independent experiments.

3.5. DATA ANALYSIS AND STATISTICS

3.5.1. Ca²⁺ mobilization assay

Raw fluorescence data were acquired using a FlexStation 3 plate reader and exported as text files from SoftMax Pro software for analysis in Microsoft Excel. Relative fluorescence unit (RFU) values were baseline-normalized by dividing each time point by the mean baseline fluorescence measured prior to agonist addition. The resulting values were background-corrected by subtracting the mean vehicle signal across the plate. Finally, the normalized baseline value (100%) was subtracted from all values and data were expressed as the percentage change in fluorescence from baseline (Δ Fluorescence). Peak Ca²⁺ responses (maximum Δ Fluorescence following agonist addition) were extracted and averaged across duplicate wells for each condition. For experiments with low receptor expression, Δ Fluorescence values were normalized to the 10 µM 5-HT response of vehicle-pretreated cells to reduce interexperimental variability and expressed as Δ Fluorescence (% of 10 µM 5-HT). Responses obtained for mescaline and psilocin were normalized in the same manner.

Concentration-response curves (CRCs) from individual experiments were fitted in GraphPad Prism version 11.0.0 (GraphPad Software, San Diego, CA, USA) using a three-parameter logistic equation:

$$Y = Bottom + \frac{Top - Bottom}{1 + 10^{(\log[EC_{50}] - X)}} \quad [3.1]$$

where: *Bottom*: minimum *Y*-value (%)
Top: maximal *Y*-value (%; E_{max})
 Y : Δ Fluorescence (%)
 X : $\log_{10}$ -transformed agonist concentration (M)
 EC_{50} : Half-maximal effective concentration (M)

Because baseline fluorescence (100%) was subtracted, the *bottom* parameter was constrained to 0% during fitting. Maximal response (E_{max}) and potency (EC_{50} , half-maximal effective concentration) values were estimated for individual experiments. Before statistical analysis, EC_{50} values were converted to pEC_{50} values using Equation 3.2:

$$pEC_{50} = -\log_{10}(EC_{50}) \quad [3.2]$$

Parameter estimates were then pooled and presented as mean $\pm$ standard error of the mean (SEM) from three to six independent experiments, unless stated otherwise.

3.5.2. Membrane potential assay

Raw fluorescence data were exported as text files from SoftMax Pro software and analysed in Microsoft Excel. RFU values were normalized to the baseline by dividing each time point by the mean baseline fluorescence measured prior to agonist addition and expressing values as a percentage of baseline. The mean vehicle response was then subtracted. Responses were expressed as the peak percentage change from baseline fluorescence (Δ Fluorescence). Because activation results in a decrease in fluorescence intensity, responses were expressed such that a Δ Fluorescence of 20% corresponds to a 20% reduction relative to baseline.

Individual CRCs were fitted using Equation 3.1 in GraphPad Prism version 11.0.0 to estimate maximal response (E_{max}) and potency (EC_{50}). EC_{50} values were converted to pEC_{50} values using Equation 3.2 before statistical

analysis. Parameter estimates were then pooled and presented as mean $\pm$ SEM from three independent experiments.

3.5.3. BRET assay

Raw BRET data were exported and processed in Microsoft Excel. BRET ratios were calculated by dividing acceptor emission ($\lambda = 515$ nm) by donor emission ($\lambda = 410$ nm):

$$\text{BRET ratio} = \frac{\text{Acceptor emission } (\lambda = 515 \text{ nm})}{\text{Donor emission } (\lambda = 410 \text{ nm})} \quad [3.3]$$

BRET ratios were baseline-corrected by subtracting the average baseline BRET ratio recorded before drug addition. Baseline-corrected values were then averaged across duplicate wells and expressed as Δ BRET relative to vehicle controls:

$$\Delta\text{BRET} = \text{BRET ratio}_{\text{agonist}} - \text{BRET ratio}_{\text{vehicle}} \quad [3.4]$$

3.5.4. Statistical analysis

Statistical analyses were performed using GraphPad Prism version 11.0.0. Potency (pEC_{50}), maximal response (E_{max}) and submaximal 5-HT responses across pretreatment conditions were assessed using ordinary one-way analysis of variance (ANOVA), followed by Dunnett's post hoc test with vehicle-pretreated cells as the control group.

Ordinary one-way ANOVA followed by Tukey's multiple comparisons test was used to compare PAR-1 responses across tetracycline concentrations. For mescaline experiments, CRCs did not reach a plateau, preventing reliable curve fitting and estimation of E_{max} or pEC_{50} values. Therefore, Δ Fluorescence values normalized to the maximal 5-HT response (10 μM) were compared across conditions using ordinary two-way ANOVA, with mescaline concentration and pretreatment condition as the two factors. Dunnett's multiple comparisons test was used to compare each pretreatment condition with vehicle-pretreated cells at each mescaline concentration. Ionomycin- and 5-HT-induced responses were compared using an unpaired t-test. For all analyses, statistical significance was defined as $p < 0.05$.

4. RESULTS

4.1. EFFECTS OF CBD, RELATED COMPOUNDS AND PAMs ON 5-HT_{2A}-MEDIATED Ca²⁺ RESPONSES

4.1.1. Validation of the 5-HT_{2A} Ca²⁺ mobilization assay

Assay performance was validated prior to evaluating modulatory effects. Activation of the endogenously expressed PAR-1 receptor induced similar robust Ca²⁺ responses across all pretreatment conditions, indicating that G_q-mediated Ca²⁺ signalling remained intact (Figure 4.1). No significant differences in PAR-1 responses were observed between pretreatment conditions (ordinary one-way ANOVA, $n = 5-6$, $p > 0.05$). In addition, ionomycin-induced fluorescence responses exceeded those induced by 10 μ M 5-HT, confirming that the fluorescent dye was not saturated under the experimental conditions used (Figure 4.1B; unpaired t-test, $n = 6$, $p < 0.0001$).

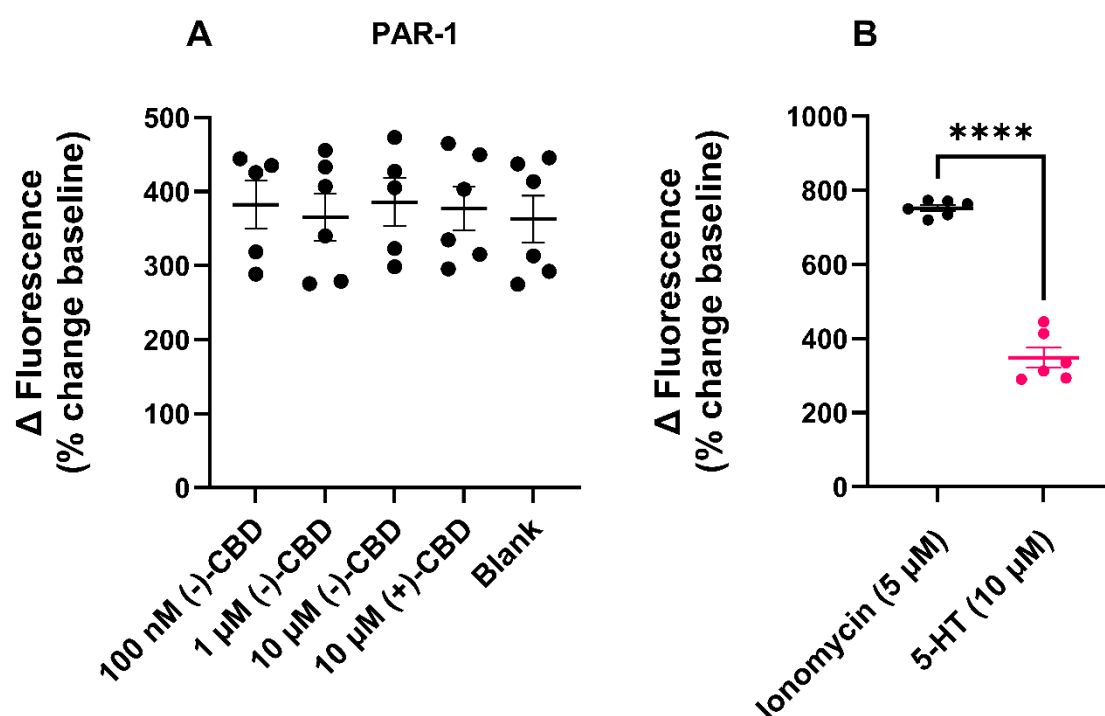


Figure 4.1. Validation of the Ca²⁺ mobilization assay. HEK293 Flp-In™ T-REx™ 5-HT_{2A} cells induced with 1000 ng/mL tetracycline. (A) Ca²⁺ responses induced by PAR-1 agonist (100 μ M) following 1 h pretreatment with CBD, (+)-CBD or vehicle (HBSS + 0.01% *m/v* BSA + 0.1% *v/v* DMSO). (B) Ca²⁺ responses induced by ionomycin (5 μ M) were significantly (****) higher than those of 10 μ M 5-HT (unpaired t-test, $n = 6$, $p < 0.0001$). Data are expressed as Δ Fluorescence (% change from baseline) and are presented as individual values with mean $\pm$ SEM from five to six independent experiments performed in duplicate.

To confirm that 5-HT-induced Ca^{2+} responses were mediated by the 5-HT_{2A} receptor, cells were pretreated for 1 hour with the selective $5\text{-HT}_{2A/2C}$ receptor antagonist ritanserin prior to stimulation with 5-HT. As shown in Figure 4.2, ritanserin pretreatment completely abolished 5-HT-induced Ca^{2+} responses at both 100 nM and 1 μM , confirming that the measured responses were mediated by 5-HT_{2A} receptor activation.

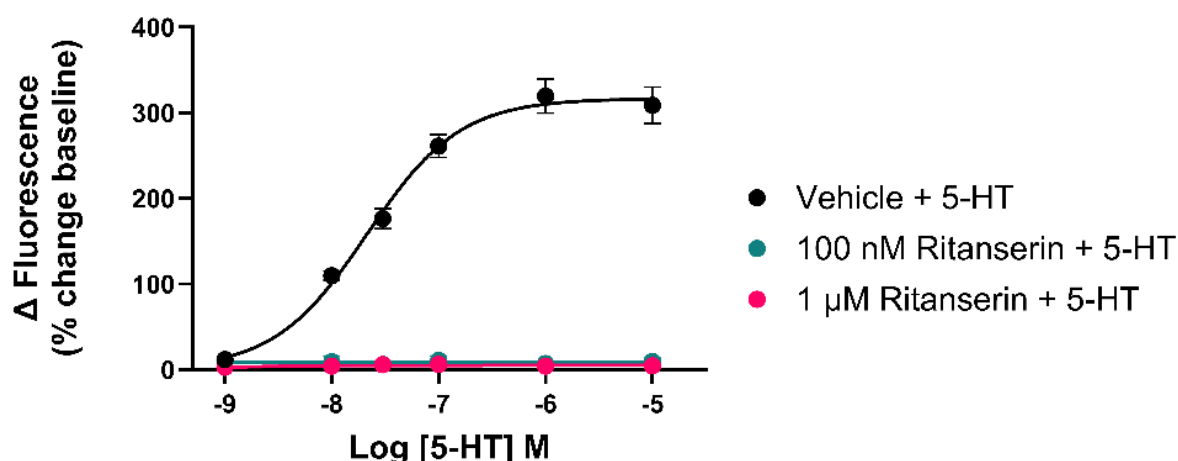


Figure 4.2. Validation of 5-HT_{2A} -mediated Ca^{2+} responses using ritanserin. HEK293 Flp-In™ T-REX™ 5-HT_{2A} cells were induced with 1000 ng/mL tetracycline and pretreated for 1 h with vehicle (HBSS + 0.01% *m/v* BSA + 0.1% *v/v* DMSO) or the $5\text{-HT}_{2A/2C}$ receptor antagonist ritanserin (100 nM or 1 μM) before stimulation with 5-HT. Data are expressed as mean Δ Fluorescence (% change from baseline) $\pm$ SEM from three independent experiments performed in duplicate.

4.1.2. Effects of CBD and (+)-CBD on 5-HT-induced Ca^{2+} responses

The effects of CBD and (+)-CBD on 5-HT_{2A} -mediated Ca^{2+} signalling were evaluated by generating CRCs for 5-HT (1 nM–10 μM), following either 5-minute or 1-hour pretreatment with CBD, (+)-CBD or vehicle (HBSS + 0.01% *m/v* BSA + 0.1% *v/v* DMSO). Cells were induced with 1000 ng/mL tetracycline to obtain high 5-HT_{2A} receptor expression and ensure robust Ca^{2+} responses. CBD was tested at 100 nM, 1 μM and 10 μM , whereas (+)-CBD was tested at 10 μM .

5-HT induced rapid concentration-dependent increases in intracellular Ca^{2+} under all conditions (Figure 4.3C–D). CRCs obtained following either 5-minute or 1-hour pretreatment with CBD or (+)-CBD largely overlapped with vehicle-pretreated controls. No clear changes in maximal response or potency were observed. As shown in Figure 4.3D, neither CBD nor (+)-CBD elicited detectable acute Ca^{2+} responses when applied alone during the pretreatment phase.

Following 5-minute pretreatment, 5-HT yielded a pEC_{50} of 7.93 ± 0.179 and an E_{max} of $288 \pm 13.6\%$ (Table 4.1). Pretreatment with CBD or (+)-CBD for 5 minutes did not significantly alter either 5-HT potency or maximal response (Table 4.1; ordinary one-way ANOVA, $n = 6$, $p > 0.05$), consistent with the overlapping CRCs shown in Figure 4.3B. Similarly, following 1-hour pretreatment, no significant differences in pEC_{50} or E_{max} were observed across pretreatment conditions (Table 4.1; ordinary one-way ANOVA, $n = 6$, $p > 0.05$).

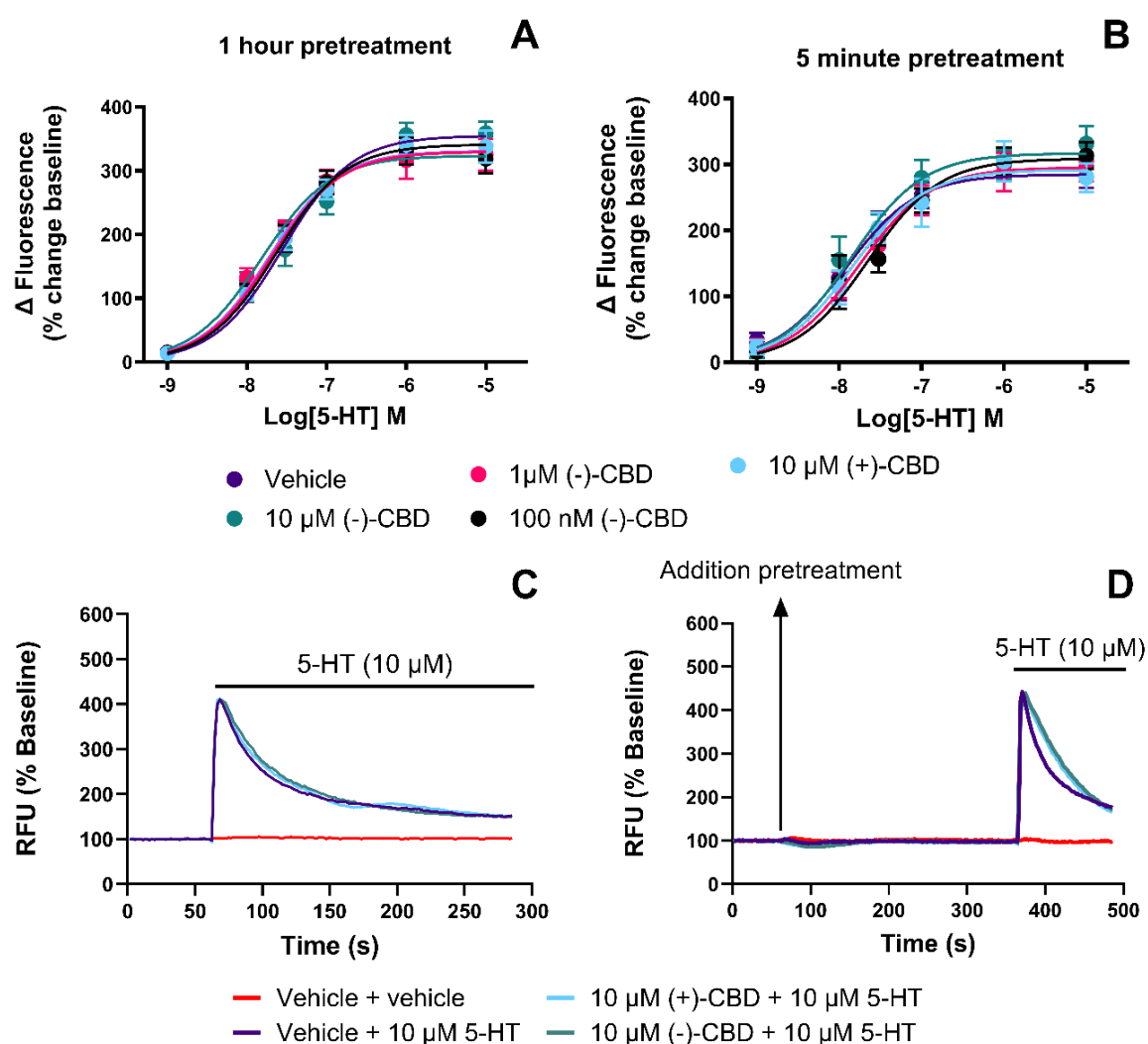


Figure 4.3. Effects of CBD and (+)-CBD on 5-HT_{2A} receptor-mediated Ca²⁺ mobilization in HEK293 Flp-In™ T-REx™ 5-HT_{2A} cells induced with 1000 ng/mL tetracycline. (A-B) CRCs for 5-HT following 1 h (A) or 5 min (B) pretreatment with vehicle (HBSS + 0.01% *m/v* BSA + 0.1% *v/v* DMSO), CBD or (+)-CBD. Data are expressed as Δ Fluorescence (% change from baseline) and represent mean ± SEM from six independent experiments performed in duplicate. (C-D) Representative fluorescence traces of 5-HT-induced Ca²⁺ responses following 1 h (C) or 5 min (D) pretreatment with vehicle, 10 μM (+)-CBD or 10 μM CBD. The vehicle-vehicle control is shown in red. The arrow indicates the time of pretreatment addition at 60 s and data are expressed as RFU (% baseline) over time.

Table 4.1. Potency (pEC_{50}) and maximal response (E_{max}) of 5-HT in the Ca^{2+} mobilization assay following 1 h or 5 min pretreatment with CBD, (+)-CBD or vehicle. Maximal responses are expressed as Δ Fluorescence (% change from baseline). Values represent mean $\pm$ SEM from six independent experiments performed in duplicate.

Pretreatment	E_{max} 1 h (% change from baseline)	pEC_{50} 1 h	E_{max} 5 min (% change from baseline)	pEC_{50} 5 min
Vehicle ^a	330 $\pm$ 22.5	7.73 $\pm$ 0.07	288 $\pm$ 13.6	7.93 $\pm$ 0.179
100 nM CBD	330 $\pm$ 21.8	7.69 $\pm$ 0.06	309 $\pm$ 19.4	7.71 $\pm$ 0.200
1 μ M CBD	324 $\pm$ 24.8	7.83 $\pm$ 0.04	291 $\pm$ 27.3	7.80 $\pm$ 0.110
10 μ M CBD	357 $\pm$ 16.6	7.54 $\pm$ 0.10	331 $\pm$ 23.7	7.79 $\pm$ 0.163
10 μ M (+)-CBD	343 $\pm$ 19.5	7.63 $\pm$ 0.07	295 $\pm$ 25.1	7.82 $\pm$ 0.131

^a Final vehicle composition in the well: HBSS + 0.01% (*m/v*) BSA + 0.1% (*v/v*) DMSO.

4.1.3. Effects of CBD metabolites on 5-HT_{2A}-mediated Ca^{2+} responses

The effects of CBD metabolites 7-COOH-CBD and 6 α -OH-CBD on 5-HT_{2A}-mediated Ca^{2+} responses were evaluated following 5-minute pretreatment. Both metabolites were tested at 100 nM, 1 μ M and 10 μ M. Cells were subsequently stimulated with submaximal concentrations of 5-HT (100 nM and 1 μ M). Across both stimulation conditions, Ca^{2+} responses were comparable between metabolite- and vehicle-pretreated cells (Figure 4.4). Neither metabolite significantly altered 5-HT-induced Ca^{2+} responses (ordinary one-way ANOVA, $n = 3$, $p > 0.05$).

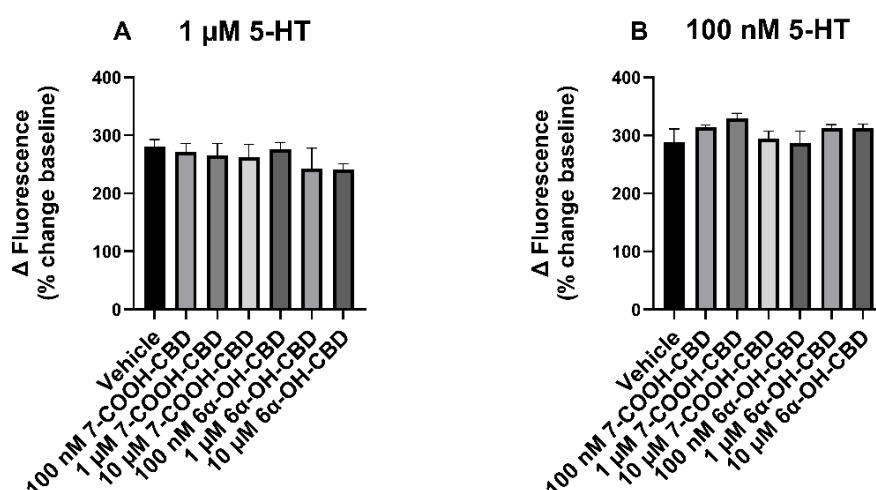


Figure 4.4. Effects of 7-COOH-CBD and 6 α -OH-CBD on 5-HT_{2A}-mediated Ca^{2+} mobilization in HEK293 Flp-In™ T-REX™ 5-HT_{2A} cells induced with 1000 ng/mL tetracycline. Cells were pretreated for 5 min with metabolites (100 nM, 1 μ M and 10 μ M) or vehicle (HBSS + 0.01% *m/v* BSA + 0.1% *v/v* DMSO) before stimulation with submaximal concentrations of 5-HT: (A) 100 nM and (B) 1 μ M. Data are expressed as Δ Fluorescence (% change from baseline) and are presented as mean $\pm$ SEM from three independent experiments performed in duplicate.

4.1.4. Effects of reported PAMs of 5-HT_{2A} on 5-HT-induced Ca²⁺ responses

The effects of the reported PAMs oleamide and its analogue JPC0323 on 5-HT_{2A}-mediated Ca²⁺ signalling were evaluated under identical experimental conditions. JPC0323 was tested at 1, 10 and 100 nM, whereas oleamide was evaluated at 10 and 100 nM. CRCs and representative RFU traces are shown in Figure 4.5.

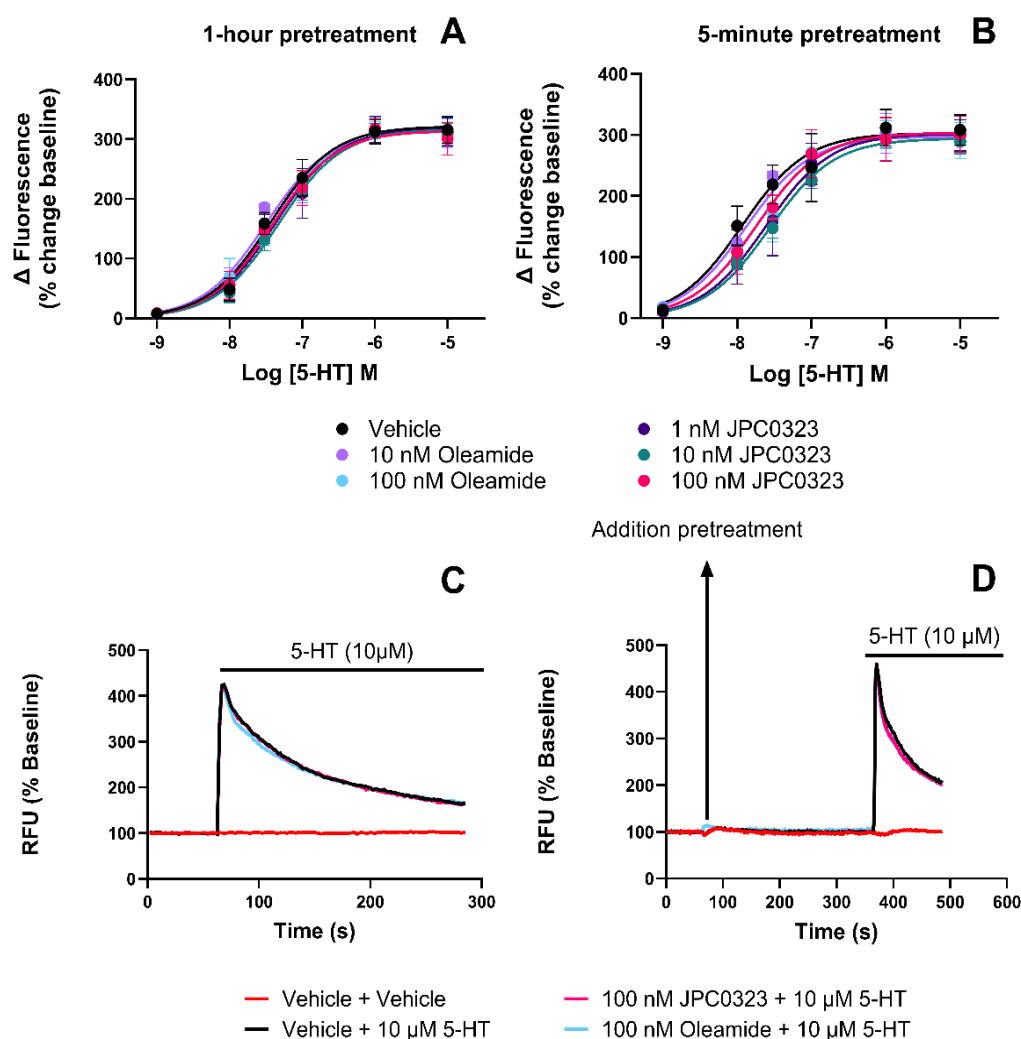


Figure 4.5. Effects of oleamide and JPC0323 on 5-HT_{2A} receptor-mediated Ca²⁺ mobilization in HEK293 Flp-In™ T-REX™ 5-HT_{2A} cells induced with 1000 ng/mL tetracycline. (A-B) CRCs for 5-HT following 1 h (A) or 5 min (B) pretreatment with vehicle (HBSS + 0.01% *m/v* BSA + 0.1% *v/v* DMSO), oleamide or JPC0323. Data are expressed as Δ Fluorescence (% change from baseline) and represent mean $\pm$ SEM from three independent experiments performed in duplicate. (C-D) Representative fluorescence traces of 5-HT-induced Ca²⁺ responses following 1 h (C) or 5 min (D) pretreatment with vehicle, 100 nM oleamide or 100 nM JPC0323. The vehicle-vehicle control is shown in red. The arrow indicates the time point (60 s) of pretreatment addition. Data are expressed as RFU (% baseline) over time.

Following 1-hour pretreatment, CRCs were largely superimposable across conditions (Figure 4.5A). In the 5-minute pretreatment condition, CRCs showed slightly greater variability between pretreatment conditions but no consistent pattern of modulation was observed (Figure 4.5B). Corresponding pEC₅₀ and E_{max} values are presented in Table 4.2.

Neither oleamide nor JPC0323 significantly altered 5-HT potency or maximal response following either 5-minute or 1-hour pretreatment (ordinary one-way ANOVA, $n=3$, $p>0.05$). In addition, neither compound elicited detectable acute Ca²⁺ responses when applied alone during the pretreatment phase (Figure 4.5D).

Table 4.2. Estimated potency (pEC₅₀) and maximal response (E_{max}) of 5-HT in the Ca²⁺ mobilization assay following 1 h or 5 min pretreatment with oleamide, JPC0323 or vehicle. Maximal responses are expressed as Δ Fluorescence (% change from baseline). Values represent mean ± SEM from three independent experiments performed in duplicate.

Pretreatment	E _{max} 1 h (% change from baseline)	pEC ₅₀ 1 h	E _{max} 5 min (% change from baseline)	pEC ₅₀ 5 min
Vehicle ^a	325 ± 22.6	7.43 ± 0.170	304 ± 33.3	7.91 ± 0.095
100 nM JPC0323	315 ± 24.5	7.42 ± 0.137	305 ± 29.9	7.72 ± 0.112
10 nM JPC0323	318 ± 19.4	7.33 ± 0.117	295 ± 16.3	7.56 ± 0.048
1 nM JPC0323	325 ± 26.2	7.35 ± 0.243	304 ± 27.7	7.58 ± 0.170
100 nM oleamide	317 ± 23.5	7.51 ± 0.148	299 ± 31.0	7.92 ± 0.155
10 nM oleamide	316 ± 20.4	7.46 ± 0.190	303 ± 25.3	7.72 ± 0.160

^a Final vehicle composition in the well: HBSS + 0.01% (w/v) BSA + 0.1% (v/v) DMSO.

4.2. EFFECTS OF CBD ON PARTIAL 5-HT_{2A} AGONIST-MEDIATED Ca²⁺ RESPONSES

The effect of CBD on partial agonist-induced 5-HT_{2A} signalling was evaluated using mescaline and psilocin. To reduce interexperimental variability, responses were normalized to the response induced by 10 μM 5-HT in vehicle-pretreated cells within the same experiment.

Following 1-hour pretreatment, 10 μM CBD significantly reduced Ca²⁺ responses induced by higher mescaline concentrations (Figure 4.6). Specifically, responses to 10 and 30 μM mescaline were reduced by 11.5 percentage points (95% confidence interval: 4.19–18.7) and 11.8 percentage points (95% confidence interval: 4.52–19.1), respectively, compared with vehicle-pretreated cells (ordinary two-way ANOVA followed by Dunnett's multiple comparisons test, $n = 3$, $p = 0.0017$ and 0.0013 , respectively). Responses at lower mescaline

concentrations (30 nM–3 μ M) were also reduced following 1-hour pretreatment with 10 μ M CBD; however, these differences did not reach statistical significance ($p > 0.05$). In contrast, pretreatment with 1 μ M CBD did not significantly alter Ca^{2+} responses at any of the tested mescaline concentrations ($p > 0.05$).

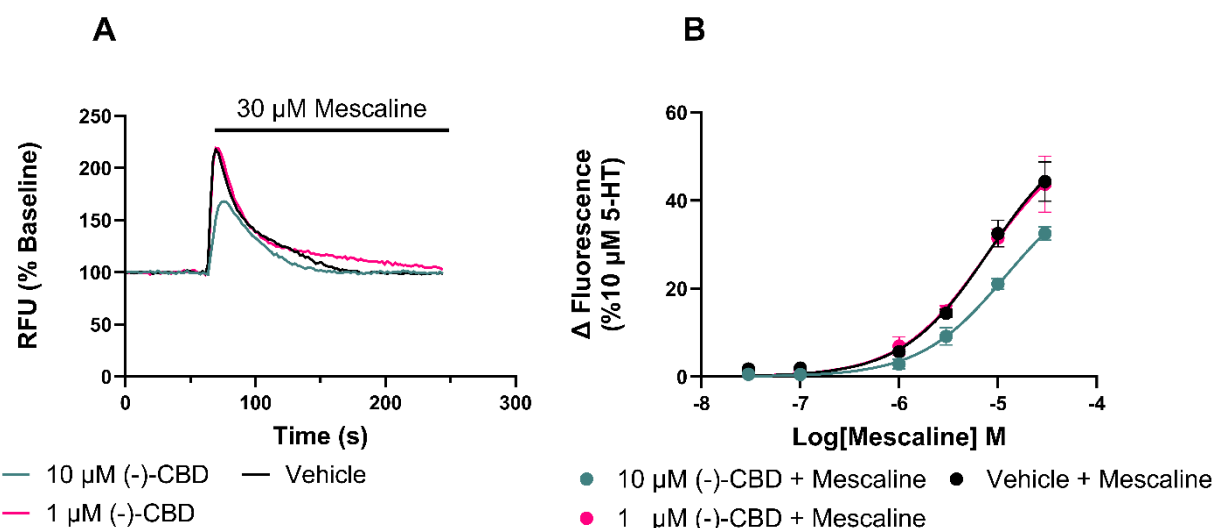


Figure 4.6. Effects of 1 h pretreatment with CBD on mescaline-induced Ca^{2+} responses in HEK293 Flp-In™ T-REx™ 5-HT_{2A} cells induced with 1000 ng/mL tetracycline. (A) Representative fluorescence traces of 30 μ M mescaline-induced Ca^{2+} responses following 1 h pretreatment with 10 μ M CBD or vehicle (HBSS + 0.01% *m/v* BSA + 0.1% *v/v* DMSO). Data are expressed as RFU (% baseline) over time. (B) CRCs for mescaline following 1 h pretreatment with vehicle or CBD (1 μ M or 10 μ M). Data are presented as mean Δ Fluorescence (% of 10 μ M 5-HT) $\pm$ SEM from three independent experiments performed in duplicate.

The effect of shorter CBD exposure was assessed by generating CRCs for mescaline following 5-minute pretreatment with 10 μ M CBD or vehicle (Figure 4.7A). Under both conditions, CRCs did not reach a clear plateau within the tested concentration range, precluding reliable estimation of potency (pEC_{50}) and maximal response (E_{max}). No significant effect of CBD was observed following 5-minute pretreatment (ordinary two-way ANOVA, $n = 6$, $p > 0.05$).

Psilocin responses were assessed under identical 5-minute pretreatment conditions. However, psilocin-induced Ca^{2+} responses were variable and not consistently reproducible across experiments. In the two experiments in which measurable responses were obtained, CRCs were broadly comparable between vehicle- and CBD-pretreated cells, although lower responses were observed at 100 nM psilocin following CBD pretreatment (Figure 4.7B). Due to the limited number of independent experiments ($n = 2$), no statistical analysis was performed and no definitive conclusions were drawn from this dataset.

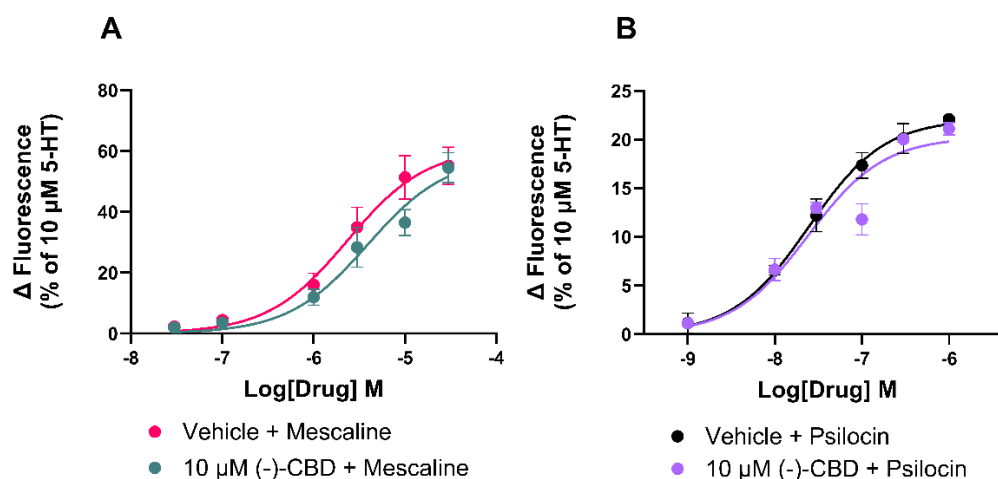


Figure 4.7. Effects of CBD pretreatment on partial agonist-induced Ca^{2+} responses in HEK293 Flp-In™ T-REx™ 5-HT_{2A} cells induced with 1000 ng/mL tetracycline. CRCs for mescaline (A) or psilocin (B) following 5 min pretreatment with vehicle (HBSS + 0.01% *m/v* BSA + 0.1% *v/v* DMSO) or CBD (10 μ M). Data are expressed as Δ Fluorescence normalized to the response induced by 10 μ M 5-HT in vehicle-pretreated cells. Note that x- and y-axis scales differ between panels. Data are presented as mean $\pm$ SEM from six (mescaline) or two (psilocin) independent experiments performed in duplicate.

4.3. INFLUENCE OF RECEPTOR EXPRESSION ON 5-HT_{2A}-MEDIATED SIGNALLING

4.3.1. Effect of tetracycline concentration on 5-HT responses

The T-REx™ inducible expression system was used to evaluate the influence of 5-HT_{2A} receptor expression levels on 5-HT-induced Ca^{2+} signalling. Cells were induced with increasing concentrations of tetracycline ranging from 1 to 1000 ng/mL or were left uninduced. CRCs for 5-HT obtained under each induction condition are shown in Figure 4.8A.

No detectable 5-HT-induced Ca^{2+} responses were observed in uninduced cells or in cells induced with 1 ng/mL tetracycline. Increasing tetracycline concentrations resulted in progressively larger 5-HT-induced Ca^{2+} responses. Cells induced with 100 and 1000 ng/mL tetracycline produced largely superimposable CRCs and comparable maximal responses with E_{max} values of $243 \pm 20.9\%$ and $231 \pm 27.2\%$, respectively (ordinary one-way ANOVA followed by Tukey's multiple comparisons test, $n = 5$, $p = 0.9828$). These responses were significantly higher than those observed in cells induced with 5 or 10 ng/mL tetracycline, where E_{max} values were $52.4 \pm 22.8\%$ and $108 \pm 21.5\%$, respectively ($p < 0.01$).

Lower tetracycline concentrations were also associated with reduced 5-HT potency. The pEC_{50} values were 7.30 ± 0.144 and 7.26 ± 0.157 at 5 and 10 ng/mL tetracycline, respectively, compared with 7.81 ± 0.093 and 7.86 ± 0.065 at 100 and 1000 ng/mL tetracycline, respectively (ordinary one-way ANOVA followed by Tukey's multiple comparisons test, $n = 5$, $p < 0.05$). However, no significant difference was observed between 5 and 10 ng/mL tetracycline or between 100 and 1000 ng/mL tetracycline ($p > 0.05$). Based on these findings, 10 ng/mL tetracycline was selected to model reduced receptor expression in subsequent experiments.

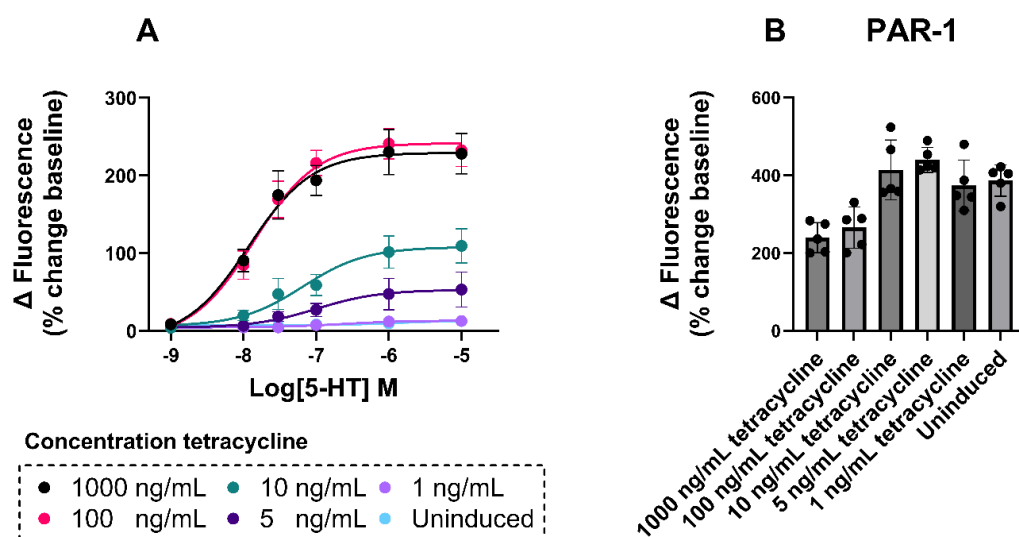


Figure 4.8. Influence of tetracycline concentration on 5-HT_{2A}-mediated and PAR-1-mediated Ca²⁺ responses in HEK293 Flp-In™ T-REx™ 5-HT_{2A} cells left uninduced or induced with 1-1000 ng/mL tetracycline. (A) CRCs for 5-HT under each induction condition. (B) Ca²⁺ responses induced by the PAR-1 agonist (100 μ M) under the same induction conditions. Data are expressed as Δ Fluorescence (% change from baseline) and represent mean $\pm$ SEM from five independent experiments performed in duplicate.

Stimulation of the endogenously expressed PAR-1 receptor showed that PAR-1-mediated Ca²⁺ responses were significantly lower at higher tetracycline concentrations (100 and 1000 ng/mL tetracycline) than at lower tetracycline concentrations (Figure 4.8B; ordinary one-way ANOVA followed by Tukey's multiple comparisons test, $n = 5$; $p < 0.05$).

4.3.2. Effects of CBD and reported PAMs under reduced receptor expression conditions

The effects of CBD, oleamide and JPC0323 on 5-HT_{2A} receptor-mediated signalling were evaluated under conditions of reduced receptor expression (cells induced with 10 ng/mL tetracycline). CRCs for 5-HT following either 5-minute or 1-hour pretreatment are shown in Figure 4.9A-B.

Following 1-hour pretreatment, 10 μ M CBD significantly reduced the maximal 5-HT response ($E_{\max}$) by approximately 26% compared with vehicle-pretreated cells (ordinary one-way ANOVA followed by Dunnett's multiple comparisons test, $n = 6$, $p = 0.0050$). A lower mean 5-HT potency (pEC_{50}) was also observed after 10 μ M CBD pretreatment, with pEC_{50} values of 6.75 ± 0.123 compared with 7.01 ± 0.141 in vehicle-pretreated cells (Table 4.3). However, this reduction in potency did not reach statistical significance. In contrast, 1-hour pretreatment with 1 μ M CBD, oleamide or JPC0323 did not significantly alter either 5-HT potency or maximal response.

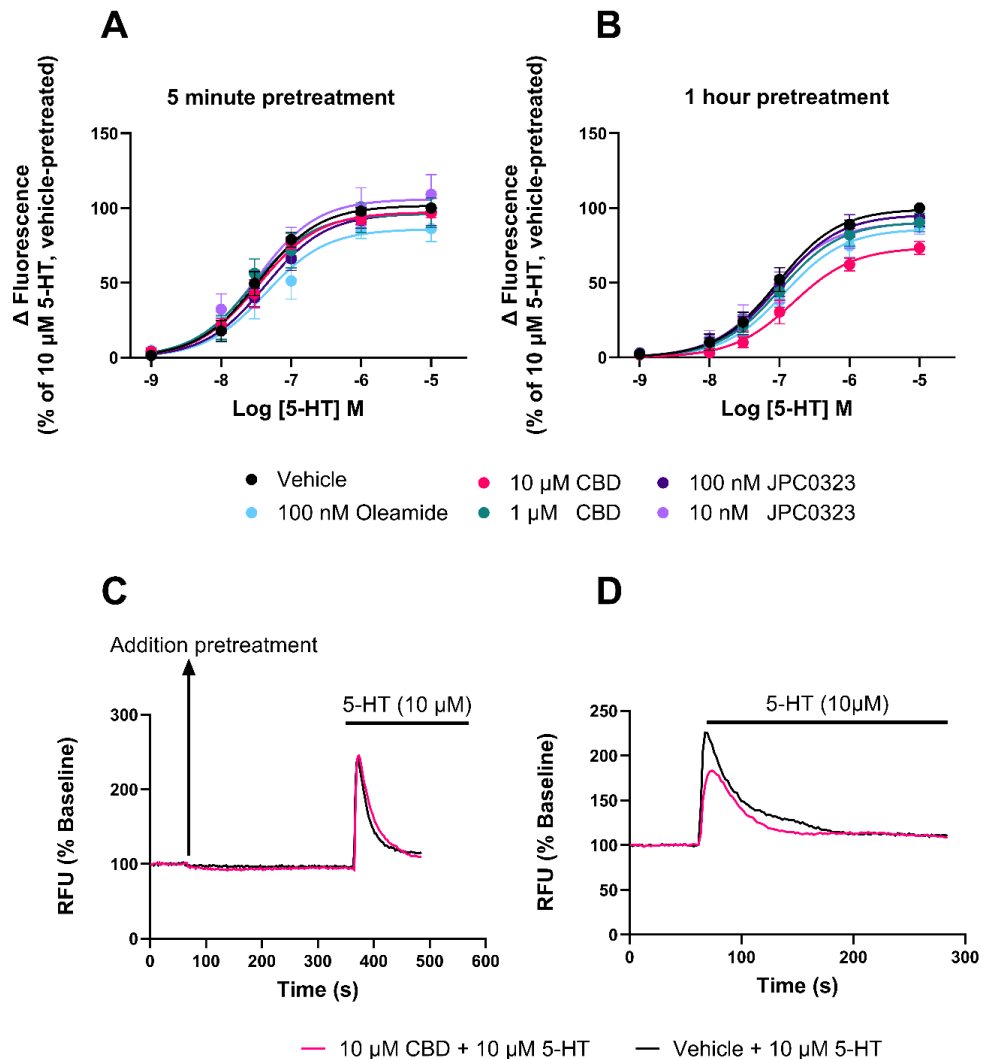


Figure 4.9. Effects of CBD, oleamide and JPC0323 on 5-HT_{2A}-mediated Ca²⁺ mobilization in HEK293 Flp-In™ T-REx™ 5-HT_{2A} cells induced with 10 ng/mL tetracycline. (A-B) CRCs for 5-HT following 5 min (A) or 1 h (B) pretreatment with vehicle (HBSS + 0.01% *m/v* BSA + 0.1% *v/v* DMSO), CBD, oleamide or JPC0323. Data are presented as Δ Fluorescence (% of 10 μ M 5-HT after vehicle pretreatment) and represent mean $\pm$ SEM from five (5 min) or six (1 h) independent experiments performed in duplicate. (C-D) Representative fluorescence traces of 5-HT-induced Ca²⁺ responses following 5 min (C) or 1 h (D) pretreatment with vehicle or 10 μ M CBD. The arrow (C) indicates the time point (60 s) of pretreatment addition. Data are expressed as RFU (% baseline) over time.

Following 5-minute pretreatment, CRCs for 5-HT were comparable across all pretreatment conditions (Figure 4.9A). Consistent with this, no significant effects on either 5-HT potency (pEC_{50}) or maximal response (E_{max}) were observed after pretreatment with CBD, oleamide or JPC0323 compared with vehicle-pretreated cells (Table 4.3; ordinary one-way ANOVA, $n = 5$, $p > 0.05$). In addition, none of the tested compounds elicited detectable acute Ca^{2+} responses when applied alone under reduced receptor expression conditions (Figure 4.9C).

Table 4.3. Estimated potency (pEC_{50}) and maximal response (E_{max}) of 5-HT in the Ca^{2+} mobilization assay under reduced receptor expression conditions following 1 h or 5 min pretreatment with CBD, oleamide, JPC0323 or vehicle. Maximal responses are expressed as Δ Fluorescence normalized to the response induced by 10 μ M 5-HT in vehicle-pretreated cells. Values are expressed as mean $\pm$ SEM from five (5 min) or six (1 h) independent experiments performed in duplicate.

Pretreatment	E_{max} 1 h (% of 10 μ M 5-HT in vehicle-pretreated cells)	pEC_{50} 1 h	E_{max} 5 min (% of 10 μ M 5-HT in vehicle-pretreated cells)	pEC_{50} 5 min
Vehicle ^a	101 $\pm$ 0.597	7.01 $\pm$ 0.141	102 $\pm$ 2.06	7.45 $\pm$ 0.133
10 μ M CBD	74.8 $\pm$ 3.90	6.75 $\pm$ 0.123	97.6 $\pm$ 3.03	7.47 $\pm$ 0.115
1 μ M CBD	92.5 $\pm$ 5.50	7.38 $\pm$ 0.161	97.2 $\pm$ 10.1	7.54 $\pm$ 0.143
100 nM JPC0323	96.6 $\pm$ 6.35	7.57 $\pm$ 0.149	98.0 $\pm$ 7.49	7.35 $\pm$ 0.124
10 nM JPC0323	92.5 $\pm$ 7.18	7.67 $\pm$ 0.198	108 $\pm$ 13.0	7.49 $\pm$ 0.203
100 nM oleamide	88.9 $\pm$ 4.38	7.47 $\pm$ 0.183	92.5 $\pm$ 6.89	7.22 $\pm$ 0.258

^a Final vehicle composition in the well: HBSS + 0.01% (m/v) BSA + 0.1% (v/v) DMSO.

4.4. EVALUATION OF PROXIMAL 5-HT_{2A} SIGNALLING USING TRUPATH BIOSENSORS

$G\alpha_{13}$ activation was assessed using a TRUPATH biosensor. None of the tested 5-HT_{2A} agonists (LSD, mescaline and 5-HT) produced a detectable change in BRET signal under the experimental conditions used (Figure 4.10A). In contrast, stimulation with S1P induced a robust decrease in BRET ratio, consistent with activation of endogenously expressed S1P receptors in HEK293 cells, which are known to couple to $G_{12/13}$ proteins.

$G\alpha_q$ activation was also evaluated using a TRUPATH biosensor under multiple experimental conditions. Several 5-HT_{2A} agonists, including LSD, mescaline and 5-HT, were tested alongside the positive controls PAR-1 agonist and S1P. However, no detectable BRET responses were observed for any of the tested compounds (Figure 4.10B). Notably, positive controls (PAR-1 and S1P) also failed to produce detectable responses under the experimental conditions used.

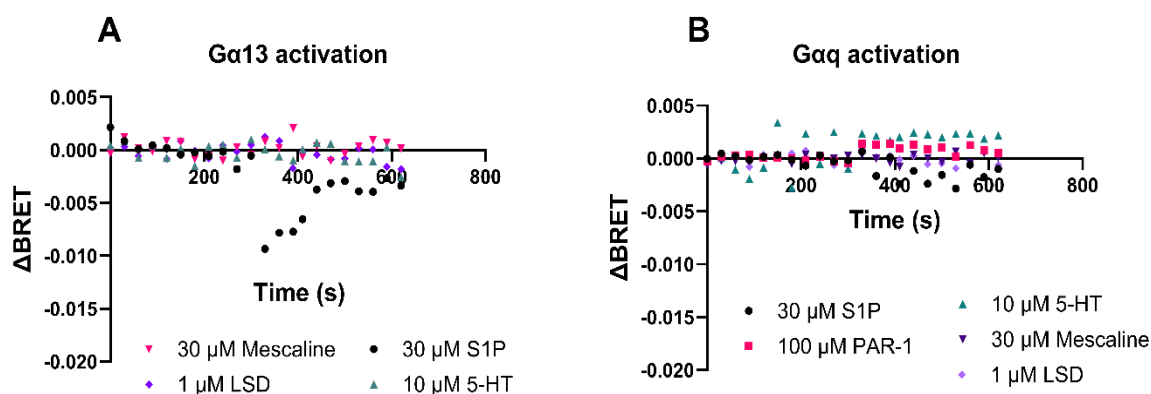


Figure 4.10. Evaluation of proximal G protein signalling using TRUPATH BRET biosensors in HEK293 Flp-In™ T-REx™ 5-HT_{2A} cells induced with 1000 ng/mL tetracycline. Compounds were added at 300 s. (A) Representative kinetic traces of Gα₁₃ BRET responses following stimulation with 5-HT_{2A} agonists or the positive control S1P. No detectable ΔBRET was observed for any of the 5-HT_{2A} agonists, whereas S1P induced a robust decrease in BRET ratio. (B) Representative kinetic traces of Gα_q BRET responses following stimulation with 5-HT_{2A} agonists and the positive controls S1P and PAR-1 agonist. Data are presented as baseline-normalized and vehicle-corrected ΔBRET over time.

4.5. VALIDATION OF CBD BIOACTIVITY USING CB1 SIGNALLING

To verify that the CBD used remained functionally intact and had not undergone degradation, its well-established NAM activity at the CB1 receptor was assessed. AtT20 Flp-In™ T-REx™ CB1 cells were pretreated for 1 hour with vehicle, 1 μM CBD or 10 μM CBD prior to stimulation with the CB1 receptor agonist CP-55,940.

CBD pretreatment attenuated CP-55,940-induced maximal responses in a concentration-dependent manner (Figure 4.11; Table 4.4). Pretreatment with 10 μM CBD significantly reduced the maximal CP-55,940-induced response compared with vehicle-pretreated cells, whereas 1 μM CBD did not significantly alter the maximal response (ordinary one-way ANOVA followed by Dunnett's multiple comparisons test versus vehicle, $n = 3$, $p = 0.0311$ and $p = 0.9059$, respectively). The maximal response was reduced from $24.8 \pm 0.394\%$ in vehicle-pretreated cells to $17.4 \pm 2.29\%$ following 10 μM CBD pretreatment.

CP-55,940 potency also appeared lower following 10 μM CBD pretreatment, with pEC₅₀ values of 7.56 ± 0.048 and 6.99 ± 0.295 for vehicle- and 10 μM CBD-pretreated cells (Table 4.4). However, this difference did not reach statistical significance (ordinary one-way ANOVA, $n = 3$, $p > 0.05$).

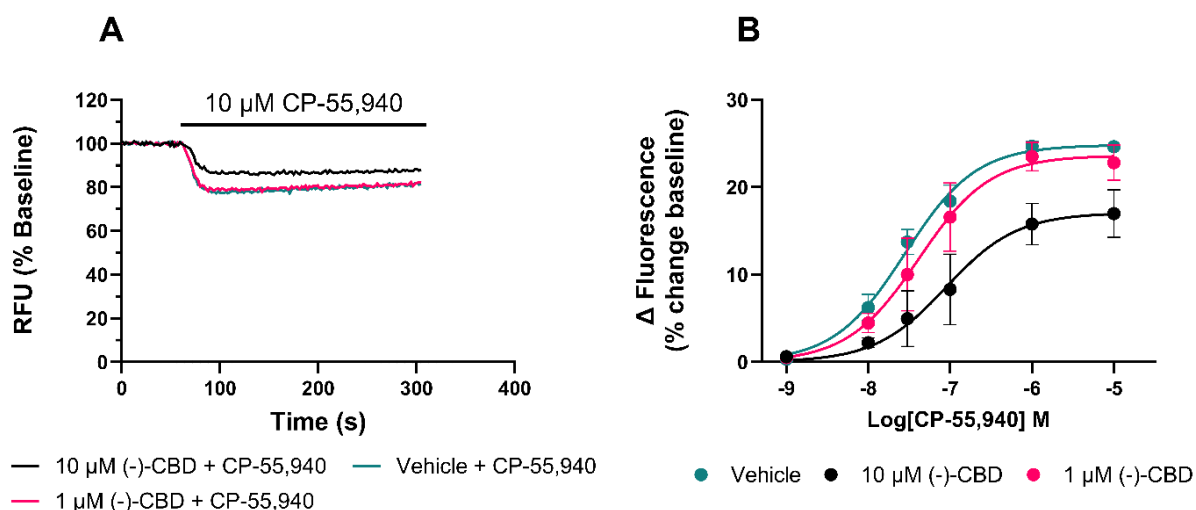


Figure 4.11. Functional validation of CBD activity at the CB1 receptor. **(A)** Representative fluorescence traces of CP-55,940-induced membrane potential responses following 1 h pretreatment with vehicle (HBSS + 0.01% *m/v* BSA + 0.1% *v/v* DMSO) or CBD (1 or 10 μ M) in AtT20 Flp-In™ T-REx™ CB1 cells induced with 1000 ng/mL tetracycline. Data are presented as RFU (% baseline) over time. **(B)** CRCs for CP-55,940 following 1 h pretreatment with vehicle or CBD (1 or 10 μ M). Data are presented as Δ Fluorescence (% change from baseline) and represent mean $\pm$ SEM from three independent experiments performed in duplicate.

Table 4.4. Estimated potency (pEC_{50}) and maximal response (E_{max}) of CP-55,940 in the MPA following 1 h pretreatment with vehicle, 1 μ M CBD or 10 μ M CBD. Values represent mean $\pm$ SEM from three independent experiments performed in duplicate.

Pretreatment	E_{max} 1 h (% change from baseline)	pEC_{50}
Vehicle ^a	24.8 $\pm$ 0.394	7.56 $\pm$ 0.048
1 μ M CBD	24.0 $\pm$ 1.58	7.32 $\pm$ 0.216
10 μ M CBD	17.4 $\pm$ 2.29	6.99 $\pm$ 0.295

^a Final vehicle composition in the well: HBSS + 0.01% (*m/v*) BSA + 0.1% (*v/v*) DMSO.

5. DISCUSSION

The present study aimed to investigate whether CBD and structurally related compounds modulate 5-HT_{2A} receptor-mediated Ca²⁺ signalling. Under high receptor expression conditions, neither CBD, (+)-CBD, nor the major CBD metabolites 7-COOH-CBD and 6 α -OH-CBD significantly altered 5-HT-induced Ca²⁺ responses. Likewise, the reported PAMs oleamide and JPC0323 did not significantly influence 5-HT_{2A}-mediated Ca²⁺ responses under these conditions. Mescaline-induced responses appeared more sensitive to CBD-mediated inhibition than 5-HT responses, although this effect was restricted to 1-hour pretreatment with 10 μ M CBD. Following reduction of receptor expression, 1-hour pretreatment with 10 μ M CBD also modestly reduced 5-HT-induced Ca²⁺ responses, whereas 1 μ M CBD and 5-minute pretreatment remained inactive. Oleamide and JPC0323 remained inactive under reduced receptor expression conditions. Collectively, these findings indicate that CBD-mediated inhibition of 5-HT_{2A} receptor-mediated Ca²⁺ signalling can be detected under specific experimental conditions, but they do not support robust modulation by CBD, CBD metabolites or the tested PAMs under most conditions investigated.

The emergence of CBD-mediated inhibition under reduced receptor expression conditions is consistent with the concept of receptor reserve and signal amplification in downstream functional assays. Ca²⁺ mobilization is an amplified downstream readout of G α_q signalling and such assays may be influenced by receptor reserve, whereby maximal responses can be maintained despite activation of only part of the receptor population [102–105]. Under high receptor expression conditions, weak negative modulation may therefore remain undetected because sufficient receptor reserve is available to maintain maximal downstream responses. Reducing receptor expression using the T-REx™ system may have reduced this reserve sufficiently to reveal subtle CBD-mediated inhibition. This interpretation is further supported by the observation that mescaline-induced responses appeared more susceptible to CBD-mediated inhibition than 5-HT-induced responses under high receptor expression conditions. As a lower-efficacy agonist, mescaline may be less able to overcome reductions in receptor signalling capacity and may therefore be more sensitive to weak negative modulation [106,107].

The present findings partially align with previous work by Billard *et al.*, who proposed CBD as a NAM of the 5-HT_{2A} receptor [41]. Using BRET-based biosensors in 5-HT_{2A}-transfected HEK293T cells, Billard *et al.* showed that CBD reduced LSD-mediated G α_q activation and DAG production, while leaving β -arrestin 2 recruitment unaffected [41]. In that study, CBD reduced LSD-induced G α_q activation and DAG production at both 1 and 10 μ M, whereas in cortical neurons significant CBD-mediated inhibition was only observed at 10 μ M CBD [41]. Additional probe-dependence experiments showed that 10 μ M CBD reduced G α_q activation induced by several agonists, including 5-HT. Compared with these findings, the present study detected a more restricted CBD effect, as

inhibition was observed only at 10 μ M CBD and only under reduced receptor expression conditions or when mescaline was used as a lower-efficacy agonist probe. This more restricted inhibition by CBD may reflect differences in the signalling readout, receptor expression level and orthosteric agonist probe. BRET-based $G\alpha_q$ activation and DAG production assays measure relatively proximal signalling events and may therefore be less susceptible to the effects of signal amplification and receptor reserve [102,104,105].

The use of different orthosteric agonists also limits direct comparison with Billard *et al.* In the present study, 5-HT and mescaline were used as the main agonist probes, whereas Billard *et al.* primarily evaluated LSD-induced signalling [41]. Robust LSD- and psilocin-induced Ca^{2+} responses could not be obtained consistently in the present study, preventing direct assessment of CBD effects on LSD-induced responses. This lack of robust responses for psilocin and LSD might be due to the transient, non-equilibrium nature of the Ca^{2+} mobilization assay or the lower efficacy of these compounds [15,105,108]. This is particularly relevant for LSD, given its slow receptor binding and dissociation kinetics [109,110]. Equilibrium-based assays, such as inositol-1-phosphate (IP1) accumulation assays, may therefore provide greater sensitivity [108,111]. In addition, the potential influence of 5-HT present in FBS was considered, as this could theoretically lead to receptor desensitization and thereby affect the observed responses [112]. However, previous experiments by our laboratory group using dialysed FBS resulted in diminished Ca^{2+} responses. Therefore, the use of non-dialysed FBS was retained.

CBD's effects in cell signalling assays can be complex and additional mechanisms should therefore be considered when interpreting the observed inhibition. CBD-mediated inhibition was concentration- and time-dependent, as it was only observed at 10 μ M following 1-hour pretreatment, whereas 5-minute pretreatment and lower concentrations were ineffective. This delayed onset may be consistent with slow membrane partitioning by CBD [113,114]. Due to its pronounced lipophilicity, CBD can partition into lipid membranes and has been reported to influence membrane properties, including membrane fluidity and microdomain organization [113,114]. Similar membrane-associated effects have been proposed to contribute to CBD pharmacology at ion channels and other GPCRs, including voltage-gated sodium channels and CB1 receptors [115–117]. Therefore, membrane-mediated effects may provide an explanation for the observed time-, context- and assay-dependent effects of CBD.

The restriction of inhibitory effects to 10 μ M CBD also warrants caution. CBD displays complex polypharmacology at micromolar concentrations and interacts with numerous molecular targets [118,119]. Moreover, CBD has been reported to form colloidal aggregates in the micromolar range, which can interfere with bioassays [120–125]. Nelson *et al.* reported a critical aggregation concentration of 12.5 μ M, whereas D'Aloia *et al.* observed aggregation at concentrations as low as 5 μ M for CBD [121–124]. Therefore, aggregation-related effects

cannot be excluded, particularly given the absence of detectable inhibition at 1 μ M CBD. This does not exclude allosteric modulation, as proposed by Billard *et al.* [41], since these mechanisms are not mutually exclusive. Future studies using appropriate controls, such as detergent-sensitivity testing or centrifugation of CBD solutions, would be necessary to clarify the possible contribution of non-specific physicochemical effects [120,121,123].

Assay functionality and specificity were evaluated using multiple controls. Robust activation of the endogenously expressed PAR-1 receptor confirmed that the Ca^{2+} signalling machinery remained intact. Importantly, these responses were not altered by CBD or (+)-CBD pretreatment, indicating that these compounds did not interfere with PAR-1 Ca^{2+} signalling or cause a general suppression of Ca^{2+} signalling. In addition, ionomycin-induced Ca^{2+} responses exceeded maximal 5-HT responses, confirming that the fluorescent dye was not saturated under the experimental conditions used. The observed Ca^{2+} responses were consistent with 5-HT_{2A} receptor activation, as pretreatment with the selective 5-HT_{2A/2C} antagonist ritanserin completely abolished 5-HT-induced Ca^{2+} responses. Likewise, the absence of detectable 5-HT responses in uninduced cells further supported the specificity of the responses for the 5-HT_{2A} receptor. Finally, the bioactivity of the CBD preparation was validated by demonstrating its established NAM activity at the CB1 receptor in the MPA. This reduces the likelihood that the absence of robust 5-HT_{2A} modulation in this study was due to inactive CBD.

Notably, PAR-1-induced Ca^{2+} responses were significantly lower under conditions of high 5-HT_{2A} receptor expression (100 and 1000 ng/mL tetracycline) than in uninduced cells or under lower-expression conditions. Since PAR-1 expression is not controlled by the T-REx™ system, this finding suggests indirect effects of increased 5-HT_{2A} receptor expression on the cellular signalling environment. One possible explanation is competition for shared signalling components such as $\text{G}\alpha_q$ proteins. Such competition for G proteins has previously been described by Tubio *et al.*, who showed that increasing expression of the histamine H2 receptor or β_2 adrenergic receptor attenuated signalling by other GPCRs in an expression-dependent manner [126]. Furthermore, signalling attenuation could be reversed by co-transfecting G protein subunits. However, alternative explanations such as altered PAR-1 expression and altered intracellular Ca^{2+} store availability cannot be excluded. Further experiments, such as quantification of PAR-1 surface expression by flow cytometry or assessment of G protein availability, would be required to clarify the mechanism underlying this observation.

The absence of detectable positive allosteric modulation by oleamide and JPC0323 indicates that PAM activity could not be reproduced under the present assay conditions. This suggests that their reported effects may be highly dependent on receptor species, assay format and experimental conditions. For oleamide, previous studies have either reported relatively modest potentiation at the 5-HT_{2A} receptor compared with that at 5-HT_{2C}

receptors or used rodent receptor systems rather than human 5-HT_{2A} receptors [89,90,127]. Importantly, one study using the human 5-HT_{2A} receptor reported no potentiation of 5-HT-induced IP accumulation [128]. Similarly, although JPC0323 has been reported to potentiate 5-HT_{2A}-mediated Ca²⁺ mobilization, its *in vivo* pharmacology appears to be preferentially selective for 5-HT_{2C} receptors [91].

A key limitation of the present study is the reliance on a single functional signalling readout. In the study of allosteric modulation, the use of multiple assay systems is necessary to fully characterize the modulatory effects [129,130]. GPCRs such as the 5-HT_{2A} receptor can engage multiple signalling pathways, including G protein activation and β -arrestin recruitment, providing the basis for possible pathway-specific effects that cannot be captured using a Ca²⁺ mobilization assay alone. Moreover, no well-established NAM control is available for the 5-HT_{2A} receptor, limiting validation of the assay and increasing the risk of false-negative results [131]. The absence of a reference NAM also makes it more difficult to determine whether the inhibitory effects of CBD reflect genuine allosteric modulation rather than non-specific actions.

An attempt was made to evaluate proximal signalling using TRUPATH BRET biosensors. Despite previous claims that the 5-HT_{2A} receptor can engage G_{12/13} signalling, this was not observed in the present system [73,74]. This finding is consistent with other reports using similar methodologies. Kim *et al.* also investigated G_{12/13} coupling in HEK293T cells and observed no coupling to G₁₂ or G₁₃ following stimulation with LSD, 5-HT and N-(2-hydroxybenzyl)-4-cyano-2,5-dimethoxyphenethylamine [132]. Detection of G_{12/13} coupling to 5-HT_{2A} may therefore depend on multiple factors, including receptor expression level, cellular background and the readout used [133]. G α_q activation was also explored using a TRUPATH biosensor. However, no detectable responses were obtained, including for the positive controls PAR-1 and S1P. Because the positive controls also failed to produce detectable responses, the G α_q biosensor could not be validated under the experimental conditions used. Consequently, further optimization would be required before this system can be employed.

From a translational perspective, the present findings do not support robust modulation of 5-HT_{2A} receptor-mediated Ca²⁺ signalling by CBD under the conditions tested. Given the widespread use of CBD, the involvement of the 5-HT_{2A} receptor in various neuropsychiatric disorders and its central role in psychedelic effects, further characterization of this interaction remains relevant [69,134]. However, the physiological relevance of the observed inhibition remains uncertain because the effect required relatively high CBD concentrations and prolonged pretreatment periods. These conditions may not reflect typical *in vivo* exposure following CBD administration. Direct extrapolation to *in vivo* exposure is difficult, as CBD is highly lipophilic, extensively bound

to plasma proteins and may distribute into tissues, including the brain. Consequently, plasma concentrations may not directly reflect free CBD concentrations at the receptor site [50,123].

Future studies should prioritize the use of complementary assay systems. Optimization of the TRUPATH BRET assay monitoring $G\alpha_q$ activation would allow more direct comparison with previous reports of CBD as a NAM at the 5-HT_{2A} receptor. Further expanding the analysis to $G\alpha_{i/o}$ signalling may be relevant given its recently proposed role in psychedelic responses [135]. In addition, equilibrium-based assays, such as IP1 accumulation assays, may provide greater sensitivity and allow assessment of CBD effects on psilocin- and LSD-induced signalling [15,129]. Given that reduced receptor expression appeared to increase sensitivity to CBD-mediated inhibition, reassessment of the major CBD metabolites and (+)-CBD under reduced receptor expression conditions would be warranted to address the study's objectives. Finally, inclusion of detergent-sensitivity controls or centrifugation of the CBD solutions could help determine whether colloidal aggregation contributes to the observed inhibitory effects [123].

In conclusion, the present study shows that CBD can modestly reduce 5-HT_{2A} receptor-mediated Ca²⁺ signalling under specific experimental conditions. This inhibition was observed after prolonged exposure to 10 μ M CBD and became apparent under conditions associated with reduced functional signalling reserve, either through lower receptor expression or through stimulation with the lower-efficacy agonist mescaline. The limited magnitude of the effect and its concentration- and time-dependent nature raise uncertainty regarding its physiological relevance, while non-specific effects cannot be excluded. Overall, these findings partially support the proposed NAM effect of CBD at the 5-HT_{2A} receptor, but indicate that this effect is strongly context-dependent and influenced by assay conditions.

6. CONCLUSION

The present study investigated whether CBD and structurally related compounds modulate 5-HT_{2A} receptor-mediated signalling. Under high receptor expression conditions, CBD did not significantly alter 5-HT-induced Ca²⁺ mobilization following either 5-minute or 1-hour pretreatment. Similarly, the enantiomer (+)-CBD and the major metabolites 7-COOH-CBD and 6 α -OH-CBD did not detectably modulate 5-HT-induced Ca²⁺ responses under these conditions. The reported 5-HT_{2A} receptor PAMs oleamide and JPC0323 also failed to modify Ca²⁺ signalling.

Modest CBD-mediated inhibition was detected under specific experimental conditions. Mescaline-induced responses appeared more sensitive to CBD-mediated inhibition than 5-HT-induced responses under high receptor expression conditions. This effect was restricted to 1-hour pretreatment with 10 μ M CBD, whereas 1 μ M CBD and 5-minute pretreatment remained inactive. In addition, reducing receptor expression revealed a modest inhibitory effect of 10 μ M CBD on 5-HT-induced responses following 1-hour pretreatment. These findings are consistent with the possibility that receptor reserve, signal amplification and agonist efficacy influence the detection of CBD-mediated inhibition in Ca²⁺ mobilization assays.

Exploratory TRUPATH BRET experiments did not detect 5-HT_{2A}-mediated G α_{13} activation, while robust responses were obtained for the positive control 5IP. Furthermore, the G α_q biosensor could not be validated because the positive controls did not produce detectable responses. Additional optimization is therefore required before this system can be used to investigate CBD-mediated modulation of proximal signalling.

Overall, the present findings are consistent with previous reports proposing CBD as a NAM of the 5-HT_{2A} receptor. However, the observed effect was modest, concentration- and time-dependent. Moreover, indirect or non-specific mechanisms, including membrane perturbation and colloidal aggregation, cannot be excluded. Consequently, the physiological relevance of this interaction remains uncertain.

Future studies should evaluate CBD using complementary and more sensitive assay systems. Optimization of the G α_q biosensor would allow more direct comparison with the previous report, while equilibrium-based assays such as IP₁ accumulation assays may improve the detection of effects on LSD- and psilocin-induced signalling. In addition, detergent-sensitivity testing or centrifugation of CBD solutions could help determine whether colloidal aggregation contributes to the observed inhibitory effects. Reassessment of the enantiomer (+)-CBD and the major metabolites under reduced receptor expression conditions would also clarify whether structurally related compounds share this context-dependent activity.

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8. APPENDICES

8.1. DRUGS, REAGENTS, MATERIALS AND EQUIPMENT

Table A8.1. Overview of reagents, drugs and substances used in this study.

Substance	Supplier/Location
(+)-CBD	Cayman Chemical Ann Arbor, MI, USA
5-HT	Sigma-Aldrich Melbourne, VIC, Australia
5-HT _{2A} gene	GenScript Piscataway, NJ, USA
6 α -OH-CBD	Cayman Chemical Ann Arbor, MI, USA
7-COOH-CBD	Cayman Chemical Ann Arbor, MI, USA
AtT20 cell line	ATCC Manassas, VA, USA
Blasticidin	InvivoGen San Diego, CA, USA
BSA	Sigma-Aldrich Melbourne, VIC, Australia
CaCl ₂	Sigma-Aldrich Melbourne, VIC, Australia
Calcium 5 kit	Molecular Devices Sunnyvale, CA, USA
CB1 receptor gene	GenScript Piscataway, NJ, USA
CBD	National Measurement Institute Sydney, NSW, Australia
CP-55,940	Cayman Chemical Ann Arbor, MI, USA
D-(+)-glucose	Sigma-Aldrich Melbourne, VIC, Australia
DMEM	Life Technologies Carlsbad, CA, USA
DMSO	Sigma-Aldrich Melbourne, VIC, Australia
FBS	Bovogen Melbourne, VIC, Australia
FLIPR membrane potential assay kit	Molecular Devices Sunnyvale, CA, USA
HEK293 Flp-In™ T-REx™	Life Technologies Mulgrave, VIC, Australia
HEPES	Sigma-Aldrich Melbourne, VIC, Australia

Table A8.1. Overview of reagents, drugs and substances used in this study [continued].

Hygromycin B gold	InvivoGen San Diego, CA, USA
JPC0323	Cayman Chemical Ann Arbor, MI, USA
KCl	Chem-Supply Gillman, SA, Australia
KH₂PO₄	Sigma-Aldrich Melbourne, VIC, Australia
Leibovitz's L-15	Life Technologies Carlsbad, CA, USA
LSD	Cayman Chemical Ann Arbor, MI, USA
Mescaline	Cayman Chemical Ann Arbor, MI, USA
MgCl₂	Sigma-Aldrich Melbourne, VIC, Australia
MgSO₄	Sigma-Aldrich Melbourne, VIC, Australia
Na₂HPO₄	Sigma-Aldrich Melbourne, VIC, Australia
NaCl	Sigma-Aldrich Melbourne, VIC, Australia
NaHCO₃	Sigma-Aldrich Melbourne, VIC, Australia
NaOH	Sigma-Aldrich Melbourne, VIC, Australia
Oleamide	Cayman Chemical Ann Arbor, MI, USA
PAR-1 agonist	AusPep Tullamarine, VIC, Australia
PBS	Life Technologies Carlsbad, CA, USA
Penicillin/streptomycin	Life Technologies Carlsbad, CA, USA
Poly-D-lysine	Sigma-Aldrich Melbourne, VIC, Australia
Polyethyleneimine	Sigma-Aldrich Melbourne, VIC, Australia
Probenecid	Biotium Fremont, CA, USA
Prolume Purple	Prolume Ltd Pinetop, AZ, USA
Psilocin	Cayman Chemical Ann Arbor, MI, USA
S1P	Cayman Chemical Ann Arbor, MI, USA

Table A8.1. Overview of reagents, drugs and substances used in this study [continued].

Tetracycline	Sigma-Aldrich Melbourne, VIC, Australia
TRUPATH plasmids	Addgene Watertown, MA, USA
Trypsin-EDTA solution 0.25%	Life Technologies Carlsbad, CA, USA

Table A8.2. Overview of materials and equipment used in this study.

Material/Equipment	Supplier/Location
-30 °C freezer	Panasonic Osaka, Japan
75 cm² cell culture flasks	Corning Inc. Kennebunk, ME, USA
-80 °C freezer	Haier Biomedical Qingdao, China
Autoclave	Memmert Schwabach, Germany
Benchtop 314 incubator (ambient CO₂)	Labline laboratory furniture Killara, NSW, Australia
Black 96-well plate	Corning Inc. Kennebunk, ME, USA
Countess II automated cell counter	Life Technologies Carlsbad, CA, USA
CLARIOstar Plus microplate reader	BMG LABTECH Ortenberg, Germany
Falcon tubes (15 and 50 mL)	Corning Inc. Kennebunk, ME, USA
FlexStation 3 multimode microplate reader	Molecular Devices Sunnyvale, CA, USA
FlexStation 3 pipet tips	Molecular Devices Sunnyvale, CA, USA
Fume hood	Thermo Fisher Scientific Waltham, MA, USA
Heidolph Reax overhead shaker (vortex)	Sigma-Aldrich Melbourne, VIC, Australia
HeraCel 150i CO₂ incubator	Thermo Fisher Scientific Waltham, MA, USA
Microcentrifuge 5430	Eppendorf Hamburg, Germany
Microscope Inverted Phase Contrast Fluorescence CKX41	Olympus Tokyo, Japan
Milli-Q® Benchtop Lab Water Purification Systems	Sigma-Aldrich Melbourne, VIC, Australia
Multichannel pipette P1200	Gilson Inc. Middleton, WI, USA

Table A8.2. Overview of materials and equipment used in this study [continued].

Pasteur pipettes	Corning Inc. Kennebunk, ME, USA
Pipette tips 10 μL, 20 μL and 1000 μL	Neptune/Interpath Services Heidelberg West, VIC, Australia
Qik spin	Edwards Narellan, NSW, Australia
Serological pipettes (5, 10, 50 mL)	Corning Inc. Kennebunk, ME, USA
Tissue culture dishes (10 cm)	Corning Inc. Kennebunk, ME, USA
V-bottomed drug plate	Greiner Bio One Heidelberg West, VIC, Australia
Water Bath NBCT2 – constant temperature (37 °C)	Labec Marrickville, NSW, Australia
Wescor 5520 Vapor Pressure Osmometer	Hyland Scientific Stanwood, WA, USA

8.2. SUPPLEMENTARY DATA EXPERIMENTAL PROCEDURES

Table A8.3. FlexStation 3 settings used for Ca²⁺ mobilization assays and membrane potential assays.

Ca²⁺ mobilization: 5-minute pretreatment (double addition)		
<i>Read mode</i>	Fluorescence bottom read	RFU
<i>wavelength</i>	Emission	525 nm
	Excitation	485 nm
<i>Sensitivity</i>	PMT ^a sensitivity	Medium
	Flashes per read	6
<i>Timing</i>	Total run time	8 minutes
	Interval	2 seconds
	Number of reads	241
<i>Compound transfer (first addition)</i>	Initial volume	180 µL
	Pipette height	170 µL
	Volume	20 µL
	Rate	2 µL/second
	Time point	60 seconds
<i>Compound transfer (second addition)</i>	Initial volume	200 µL
	Pipette height	190 µL
	Volume	22 µL
	Rate	2 µL/second
	Time point	360 seconds
<i>Triturate assay plate (first addition)</i>	Volume	20 µL
	Cycles	4
	Height	160 µL
<i>Triturate assay plate (second addition)</i>	Volume	20 µL
	Cycles	4
	Height	160 µL
<i>Auto calibrate</i>	On	
<i>Auto read</i>	Off	
Ca²⁺ mobilization: 1-hour pretreatment		
<i>Read mode</i>	Fluorescence bottom read	RFU
<i>wavelength</i>	Emission	525 nm
	Excitation	485 nm
<i>Sensitivity</i>	PMT ^a sensitivity	Medium
	Flashes per read	6
<i>Timing</i>	Total run time	4 minutes 40 seconds
	Interval	2 seconds
	Number of reads	141

Table A8.3. FlexStation 3 settings used in Ca²⁺ mobilization assays and membrane potential assays [continued].

<i>Compound transfer</i>	Initial volume	200 µL
	Pipette height	190 µL
	Volume	22 µL
	Rate	2 µL/second
	Time point	60 seconds
<i>Triturate assay plate</i>	Volume	20 µL
	Cycles	4
	Height	160 µL
<i>Auto calibrate</i>	On	
<i>Auto read</i>	Off	
MPA: 1-hour pretreatment		
<i>Read mode</i>	Fluorescence bottom read	RFU
<i>wavelength</i>	Emission	565 nm
	Excitation	530 nm
<i>Sensitivity</i>	PMT ^a sensitivity	Medium
	Flashes per read	6
<i>Timing</i>	Total run time	300 seconds
	Interval	2 seconds
	Number of reads	151
<i>Compound transfer</i>	Initial volume	200 µL
	Pipette height	190 µL
	Volume	22 µL
	Rate	2 µL/second
	Time point	60 seconds
<i>Triturate assay plate</i>	Volume	20 µL
	Cycles	4
	Height	160 µL
<i>Auto calibrate</i>	On	
<i>Auto read</i>	Off	

^a Photomultiplier tube

Table A8.4. Detailed dilution schemes for compounds used in the Ca²⁺ mobilization assays, membrane potential assays and TRUPATH BRET assays.

Compound	Stock concentration	Dilution	Concentration working solution	Final assay concentration
<i>5-HT</i>	10 mM	10 µL stock + 990 µL solvent ^b	100 µM	10 µM
		100 µL of 100 µM + 900 µL solvent ^b	10 µM	1 µM
		100 µL of 10 µM + 900 µL solvent ^b	1 µM	100 nM
		30 µL of 10 µM + 970 µL solvent ^b	300 nM	30 nM
		100 µL of 1 µM + 900 µL solvent ^b	100 nM	10 nM
		100 µL of 100 nM + 900 µL solvent ^b	10 nM	1 nM
		20 µL of 10 mM + 1980 µL solvent ^b	100 µM	10 µM
		200 µL of 100 µM + 1800 µL solvent ^c	10 µM	1 µM
		200 µL of 10 µM + 1800 µL solvent ^c	1 µM	100 nM
		200 µL of 10 µM + 1800 µL solvent ^c	1 µM	100 nM
<i>6α-OH-CBD</i>	30 mM ^a	20 µL of 10 mM + 1980 µL solvent ^b	100 µM	10 µM
		200 µL of 100 µM + 1800 µL solvent ^c	10 µM	1 µM
		200 µL of 10 µM + 1800 µL solvent ^c	1 µM	100 nM
		200 µL of 10 µM + 1800 µL solvent ^c	1 µM	100 nM

Table A8.4. Detailed dilution schemes for compounds used in the Ca²⁺ mobilization assays, membrane potential assays and TRUPATH BRET assays [continued].

<i>7-COOH-CBD</i>	30 mM ^a	20 µL of 10 mM + 1980 µL solvent ^b	100 µM	10 µM
		200 µL of 100 µM + 1800 µL solvent ^c	10 µM	1 µM
		200 µL of 10 µM + 1800 µL solvent ^c	1 µM	100 nM
<i>(+)-CBD</i>	30 mM ^a	20 µL of 10 mM + 1980 µL solvent ^b	100 µM	10 µM
<i>Ritanserine</i>	10 mM	20 µL of 10 mM + 1980 µL solvent ^b	100 µM	10 µM
		200 µL of 100 µM + 1800 µL solvent ^c	10 µM	1 µM
		200 µL of 10 µM + 1800 µL solvent ^c	1 µM	100 nM
<i>Oleamide</i>	30 mM ^a	20 µL of 10 mM + 1980 µL solvent ^b	100 µM	10 µM
		200 µL of 100 µM + 1800 µL solvent ^c	10 µM	1 µM
		200 µL of 10 µM + 1800 µL solvent ^c	1 µM	100 nM
		200 µL of 1 µM + 1800 µL solvent ^c	100 nM	10 nM
<i>SIP</i>	10 mM ^d	9 µL of 10 mM + 291 µL solvent ^b	300 µM	30 µM
		100 µL of 300 µM + 200 µL solvent ^e	100 µM	10 µM

Table A8.4. Detailed dilution schemes for compounds used in the Ca²⁺ mobilization assays, membrane potential assays and TRUPATH BRET assays [continued].

<i>JPC0323</i>	10 mM	5 µL of 10 mM + 495 µL solvent ^b	100 µM	10 µM
		50 µL of 100 µM + 450 µL solvent ^c	10 µM	1 µM
		200 µL of 10 µM + 1800 µL solvent ^c	1 µM	100 nM
		200 µL of 1 µM + 1800 µL solvent ^c	100 nM	10 nM
		200 µL of 100 nM + 1800 µL solvent ^c	10 nM	1 nM
<i>CP-55,940</i>	10 mM	5 µL of 10 mM + 495 µL solvent ^b	100 µM	10 µM
		50 µL of 100 µM + 450 µL solvent ^c	10 µM	1 µM
		50 µL of 10 µM + 450 µL solvent ^c	1 µM	100 nM
		15 µL of 10 µM + 485 µL solvent ^c	300 nM	30 nM
		50 µL of 1 µM + 450 µL solvent ^c	100 nM	10 nM
		50 µL of 100 nM + 450 µL solvent ^c	10 nM	1 nM
<i>Mescaline</i>	10 mM	15 µL of 10 mM + 485 µL solvent ^b	300 µM	30 µM
		5 µL of 10 mM + 495 µL solvent ^b	100 µM	10 µM
		50 µL of 30 µM + 450 µL solvent ^b	30 µM	3 µM

Table A8.4. Detailed dilution schemes for compounds used in the Ca²⁺ mobilization assays, membrane potential assays and TRUPATH BRET assays [continued].

<i>Mescaline</i>	10 mM	50 µL of 100 µM + 450 µL solvent ^b	10 µM	1 µM
		50 µL of 10 µM + 450 µL solvent ^b	1 µM	100 nM
		5 µL of 30 µM + 495 µL solvent ^b	300 nM	30 nM
<i>Psilocin</i>	10 mM	5 µL of 10 mM + 495 µL solvent ^b	100 µM	10 µM
		50 µL of 100 µM + 450 µL solvent ^b	10 µM	1 µM
		15 µL of 100 µM + 485 µL solvent ^b	3 µM	300 nM
		50 µL of 10 µM + 450 µL solvent ^b	1 µM	100 nM
		50 µL of 3 µM + 450 µL solvent ^b	300 nM	30 nM
		50 µL of 1 µM + 450 µL solvent ^b	100 nM	10 nM
		50 µL of 100 nM + 450 µL solvent ^b	10 nM	1 nM
<i>LSD</i>	1 mM	5 µL of 1 mM + 495 µL solvent ^b	10 µM	1 µM
		50 µL of 10 µM + 450 µL solvent ^b	1 µM	100 nM
		15 µL of 10 µM + 485 µL solvent ^b	300 nM	30 nM
		50 µL of 1 µM + 450 µL solvent ^b	100 nM	10 nM

Table A8.4. Detailed dilution schemes for compounds used in the Ca²⁺ mobilization assays, membrane potential assays and TRUPATH BRET assays [continued].

<i>LSD</i>	1 mM	50 μ L of 300 nM + 450 μ L solvent ^b	30 nM	3 nM
<i>SIP</i>	10 mM ^d	9 μ L of 10 mM + 291 μ L solvent ^b	300 μ M	30 μ M
		100 μ L of 300 μ M + 200 μ L solvent ^e	100 μ M	10 μ M

^a First 1:3 diluted in DMSO to 10 mM

^b Composition solvent: HBSS + 0.1% (m/v) BSA

^c Composition solvent: HBSS + 0.1% (m/v) BSA + 1% (v/v) DMSO (final concentration DMSO in well was 0.1% v/v)

^d NaOH (1 M)

^e Composition solvent: HBSS + 0.1% (m/v) BSA + 3 mM NaOH

Table A8.5. Calculation of the transfection mixtures used for TRUPATH BRET assays.

Calculation transfection mix G α_q -PEI		
	G α_q	PEI
Stock concentration (μ g/mL)	400	1000
Mass (μ g)	3	18
Volume (μ L)	7.5	18
V 150 mM NaCl (μ L)	492.5	482
Calculation transfection mix G α_{13} -PEI		
	G α_{13}	PEI
Stock concentration (μ g/mL)	900	1000
Mass (μ g)	3	18
Volume (μ L)	3.33	18
V 150 mM NaCl (μ L)	496.7	482

Table A8.6. CLARIOstar Plus settings used for TRUPATH BRET assays.

BRET assay CLARIOstar Plus settings	
<i>Temperature</i>	37 °C
<i>Donor emission (RLuc8)</i>	410 ± 40 nm
<i>Acceptor emission (GFP2)</i>	515 ± 10 nm
<i>Acceptor excitation (GFP2)</i>	405 ± 10 nm
<i>BRET signal</i>	515 ± 40 nm
<i>Baseline reading</i>	10 cycles (~ 5 minutes)
<i>Post-drug reading</i>	10 cycles (~ 5 minutes)

8.3. STATISTICAL ANALYSIS

Table A8.7. Statistical comparison of ionomycin-induced Ca^{2+} responses and 10 μM 5-HT-induced Ca^{2+} responses.

Data from six independent experiments were analysed using an unpaired t-test in GraphPad Prism.

Unpaired t-test	Ionomycin vs 10 μM 5-HT
<i>P value</i>	< 0.0001
<i>P value summary</i>	****
<i>Significant diff. among means ($P < 0.05$)?</i>	Yes
<i>One- or two-tailed p-value?</i>	Two-tailed
<i>t, df</i>	$t = 14.38$, $df = 10$
<i>Magnitude of effect?</i>	
▪ Difference between means	-403.4 ± 28.06
▪ 95% confidence interval	-465.9 to -340.9
▪ R squared	0.9539

Table A8.8. Statistical comparison of 5-HT potency and maximal response across the different pretreatment conditions – vehicle, CBD (100 nM, 1 μM and 10 μM) and (+)-CBD (10 μM) – in 5 min pretreatment Ca^{2+} assays.

Data from six independent experiments were analysed using an ordinary one-way ANOVA in GraphPad Prism.

ANOVA summary	5-minute pEC_{50}	5-minute E_{max}
<i>F</i>	0.1082	0.3603
<i>P value</i>	0.9785	0.8342
<i>P value summary</i>	ns	ns
<i>Significant diff. among means ($P < 0.05$)?</i>	No	No
<i>R squared</i>	0.01847	0.05896
<i>Degrees of freedom</i>		
▪ Between columns	4	4
▪ Within columns	25	25
▪ Total	29	29

Table A8.9. Statistical comparison of potency and maximal response across the different pretreatment conditions – vehicle, CBD (100 nM, 1 μM and 10 μM) and (+)-CBD (10 μM) – in 1 h pretreatment Ca^{2+} assays. Data from six

independent experiments were analysed using an ordinary one-way ANOVA in GraphPad Prism.

ANOVA summary	1-hour pEC_{50}	1-hour E_{max}
<i>F</i>	2.457	0.3559
<i>P value</i>	0.073	0.8373
<i>P value summary</i>	ns	ns
<i>Significant diff. among means ($P < 0.05$)?</i>	No	No
<i>R squared</i>	0.2905	0.0560
<i>Degrees of freedom</i>		
▪ Between columns	4	4
▪ Within columns	25	25
▪ Total	29	29

Table A8.10. Statistical comparison of PAR-1 agonist-induced responses across the different pretreatment conditions – vehicle, CBD (100 nM, 1 μ M and 10 μ M) and (+)-CBD (10 μ M) – in 1 h pretreatment Ca²⁺ assays. Data from five to six independent experiments were analysed using an ordinary one-way ANOVA in GraphPad Prism.

ANOVA summary	100 μ M PAR-1 agonist
<i>F</i>	0.1028
<i>P value</i>	0.9804
<i>P value summary</i>	ns
<i>Significant diff. among means (P < 0.05)?</i>	No
<i>R squared</i>	0.01757
<i>Degrees of freedom</i>	
▪ <i>Between columns</i>	4
▪ <i>Within columns</i>	23
▪ <i>Total</i>	27

Table A8.11. Statistical comparison of potency and maximal response across the different pretreatment conditions – vehicle, JPC0323 (1 nM, 10 nM and 100 nM) and oleamide (10 nM and 100 nM) – in 5 min pretreatment Ca²⁺ assays. Data from three independent experiments were analysed using an ordinary one-way ANOVA in GraphPad Prism.

ANOVA summary	5-minute pEC ₅₀	5-minute E _{max}
<i>F</i>	1.391	0.02192
<i>P value</i>	0.2984	0.9997
<i>P value summary</i>	ns	ns
<i>Significant diff. among means (P < 0.05)?</i>	No	No
<i>R squared</i>	0.3653	0.00905
<i>Degrees of freedom</i>		
▪ <i>Between columns</i>	5	5
▪ <i>Within columns</i>	12	12
▪ <i>Total</i>	17	17

Table A8.12. Statistical comparison of potency and maximal response across the different pretreatment conditions – vehicle, JPC0323 (1 nM, 10 nM and 100 nM) and oleamide (10 nM and 100 nM) – in 1 h pretreatment Ca²⁺ assays. Data from three independent experiments were analysed using an ordinary one-way ANOVA in GraphPad Prism.

ANOVA summary	1-hour pEC ₅₀	1-hour E _{max}
<i>F</i>	0.1480	0.03912
<i>P value</i>	0.9768	0.9989
<i>P value summary</i>	ns	ns
<i>Significant diff. among means (P < 0.05)?</i>	No	No
<i>R squared</i>	0.05807	0.01604
<i>Degrees of freedom</i>		
▪ <i>Between columns</i>	5	5
▪ <i>Within columns</i>	12	12
▪ <i>Total</i>	17	17

Table A8.13. Statistical comparison of 5-HT-induced Ca^{2+} responses across the different pretreatment conditions – vehicle, 7-COOH-CBD (100 nM, 1 μM and 10 μM) and 6 α -OH-CBD (100 nM, 1 μM and 10 μM) – in 5 min pretreatment Ca^{2+} assays. Data from three independent experiments were analysed using an ordinary one-way ANOVA in GraphPad Prism.

ANOVA summary	100 nM 5-HT	1 μM 5-HT
<i>F</i>	0.6255	1.394
<i>P value</i>	0.7077	0.2837
<i>P value summary</i>	ns	ns
<i>Significant diff. among means ($P < 0.05$)?</i>	No	No
<i>R squared</i>	0.2114	0.3741
<i>Degrees of freedom</i>		
▪ <i>Between columns</i>	6	6
▪ <i>Within columns</i>	14	14
▪ <i>Total</i>	20	20

Table A8.14. Statistical comparison of mescaline-induced Ca^{2+} responses following 5 min pretreatment with 10 μM CBD or vehicle. Data from six independent experiments were analysed using an ordinary two-way ANOVA in GraphPad Prism.

Two-way ANOVA summary	5-minute mescaline pretreatment assay
Source of variation	<i>p</i> -value
▪ <i>Mescaline concentration</i>	< 0.0001
▪ <i>Pretreatment condition</i>	0.0821
▪ <i>Interaction</i>	0.5629
Source of variation	F (DFn, DFd)
▪ <i>Mescaline concentration</i>	F (5, 60) = 47.53
▪ <i>Pretreatment condition</i>	F (1, 60) = 3.127
▪ <i>Interaction</i>	F (5, 60) = 0.7873

Table A8.15. Statistical comparison of PAR-1 responses across different tetracycline induction conditions. Data from five independent experiments were analysed using an ordinary one-way ANOVA with post hoc Tukey's multiple comparisons test in GraphPad Prism.

ANOVA summary		100 μ M PAR-1 agonist	
<i>F</i>		11.66	
<i>P value</i>		< 0.0001	
<i>P value summary</i>		****	
<i>Significant diff. among means (P < 0.05)?</i>		Yes	
<i>R squared</i>		0.7084	
<i>Degrees of freedom</i>			
▪ <i>Between columns</i>		5	
▪ <i>Within columns</i>		24	
▪ <i>Total</i>		29	

Tukey's multiple comparisons test	Mean difference	Adjusted <i>p</i> -value	Summary
1000 ng/mL vs. 100 ng/mL tetracycline	-26.12	0.9695	ns
1000 ng/mL vs. 10 ng/mL tetracycline	-174.6	0.0004	***
1000 ng/mL vs. 5 ng/mL tetracycline	-200.2	< 0.0001	****
1000 ng/mL vs. 1 ng/mL tetracycline	-134.2	0.0066	**
1000 ng/mL vs. Uninduced	-147.0	0.0027	**
100 ng/mL vs. 10 ng/mL tetracycline	-148.5	0.0014	**
100 ng/mL vs. 5 ng/mL tetracycline	-174.5	0.0004	***
100 ng/mL vs. 1 ng/mL tetracycline	-108.1	0.0397	*
100 ng/mL vs. Uninduced	-120.8	0.0169	*
10 ng/mL vs. 5 ng/mL tetracycline	-25.59	0.9721	ns
10 ng/mL vs. 1 ng/mL tetracycline	+40.37	0.8352	ns
10 ng/mL tetracycline vs. Uninduced	+27.64	0.9674	ns
5 ng/mL vs. 1 ng/mL tetracycline	+65.97	0.3974	ns
5 ng/mL vs. Uninduced	+53.24	0.6218	ns
1 ng/mL vs. Uninduced	-12.74	0.9989	ns

Table A8.16. Statistical comparison of 5-HT potency (pEC_{50}) and maximal responses (E_{max}) at different tetracycline induction conditions. Data from five independent experiments were analysed using an ordinary one-way ANOVA in GraphPad Prism followed by Tukey's multiple comparisons test. Parameter estimates for uninduced and 1 ng/mL tetracycline-induced cells were excluded because reliable curve fitting was not possible.

ANOVA summary	pEC_{50}	E_{max}
<i>F</i>	7.251	16.17
<i>P value</i>	0.0027	< 0.0001
<i>P value summary</i>	**	****
<i>Significant diff. among means ($P < 0.05$)?</i>	Yes	Yes
<i>R squared</i>	0.5762	0.7519
<i>Degrees of freedom</i>		
▪ <i>Between columns</i>	3	3
▪ <i>Within columns</i>	16	16
▪ <i>Total</i>	19	19

Tukey's multiple comparisons test	Mean difference	Adjusted <i>p</i> -value	Summary
pEC_{50}			
<i>1000 ng/mL vs. 100 ng/mL tetracycline</i>	-0.050	0.9911	ns
<i>1000 ng/mL vs. 10 ng/mL tetracycline</i>	-0.605	0.0129	*
<i>1000 ng/mL vs. 5 ng/mL tetracycline</i>	-0.566	0.0203	*
<i>100 ng/mL vs. 10 ng/mL tetracycline</i>	-0.555	0.0231	*
<i>100 ng/mL vs. 5 ng/mL tetracycline</i>	-0.514	0.0361	*
<i>10 ng/mL vs. 5 ng/mL tetracycline</i>	+0.039	0.9957	ns

Tukey's multiple comparisons test	Mean difference	Adjusted <i>p</i> -value	Summary
E_{max}			
<i>1000 ng/mL vs. 100 ng/mL tetracycline</i>	-12.0	0.9828	ns
<i>1000 ng/mL vs. 10 ng/mL tetracycline</i>	+123	0.0087	**
<i>1000 ng/mL vs. 5 ng/mL tetracycline</i>	+179	0.0003	***
<i>100 ng/mL vs. 10 ng/mL tetracycline</i>	+135	0.0041	**
<i>100 ng/mL vs. 5 ng/mL tetracycline</i>	+191	0.0001	***
<i>10 ng/mL vs. 5 ng/mL tetracycline</i>	+55.7	0.3575	ns

Table A8.17. Statistical comparison of potency and maximal response under reduced receptor expression conditions across the different pretreatment conditions – vehicle, CBD (1 and 10 μ M), JPC0323 (10 nM and 100 nM) and oleamide (100 nM) – in 5 min pretreatment Ca^{2+} assays. Data from five independent experiments were analysed using an ordinary one-way ANOVA in GraphPad Prism.

ANOVA summary	pEC ₅₀	E _{max}
<i>F</i>	0.4470	0.4103
<i>P value</i>	0.7897	0.8368
<i>P value summary</i>	ns	ns
<i>Significant diff. among means (P < 0.05)?</i>	No	No
<i>R squared</i>	0.0940	0.07875
<i>Degrees of freedom</i>		
▪ <i>Between columns</i>	5	5
▪ <i>Within columns</i>	24	24
▪ <i>Total</i>	29	29

Table A8.18. Statistical comparison of potency and maximal response under reduced receptor expression conditions across the different pretreatment conditions – vehicle, CBD (1 and 10 μ M), JPC0323 (10 nM and 100 nM) and oleamide (100 nM) – in 1 h pretreatment Ca^{2+} assays. Data from six independent experiments were analysed using an ordinary one-way ANOVA with post hoc Dunnett's multiple comparisons test against vehicle for E_{max} in GraphPad Prism.

ANOVA summary	pEC ₅₀	E _{max}	
<i>F</i>	0.4027	3.057	
<i>P value</i>	0.8430	0.0240	
<i>P value summary</i>	ns	*	
<i>Significant diff. among means (P < 0.05)?</i>	No	Yes	
<i>R squared</i>	0.06289	0.3375	
<i>Degrees of freedom</i>			
▪ <i>Between columns</i>	5	5	
▪ <i>Within columns</i>	30	30	
▪ <i>Total</i>	35	35	
Dunnett's multiple comparisons	Mean difference	Adjusted <i>p</i> -value	Summary
E _{max}			
<i>Vehicle vs 10 μM CBD</i>	26.03	0.0050	**
<i>Vehicle vs 1 μM CBD</i>	8.360	0.6689	ns
<i>Vehicle vs 100 nM JPC0323</i>	4.198	0.9665	ns
<i>Vehicle vs 10 nM JPC0323</i>	8.342	0.6707	ns
<i>Vehicle vs 100 nM oleamide</i>	11.92	0.3505	ns

Table A8.19. Statistical comparison of mescaline-induced Ca^{2+} responses following 1 h pretreatment with – Vehicle, 1 μM CBD or 10 μM CBD. Data from three independent experiments were analysed using an ordinary two-way ANOVA followed by Dunnett's multiple comparisons test against vehicle in GraphPad Prism.

Two-way ANOVA summary		1-hour mescaline pretreatment assay	
Source of variation		<i>p</i> -value	
▪ <i>Mescaline concentration</i>		< 0.0001	
▪ <i>Pretreatment condition</i>		< 0.0001	
▪ <i>Interaction</i>		0.1977	
Source of variation		F (DFn, DFd)	
▪ <i>Mescaline concentration</i>		F (5, 36) = 154.5	
▪ <i>Pretreatment condition</i>		F (2, 36) = 12.72	
▪ <i>Interaction</i>		F (10, 36) = 1.453	
Dunnett's multiple comparisons test	Mean difference	Adjusted <i>p</i> -value	Summary
<i>30 nM mescaline</i>			
• Vehicle vs 10 μM CBD	1.194	0.9017	ns
• Vehicle vs 1 μM CBD	0.1020	0.9992	ns
<i>100 nM mescaline</i>			
• Vehicle vs 10 μM CBD	1.431	0.8628	ns
• Vehicle vs 1 μM CBD	0.2491	0.9954	ns
<i>1 μM mescaline</i>			
• Vehicle vs 10 μM CBD	2.772	0.5894	ns
• Vehicle vs 1 μM CBD	-1.315	0.8825	ns
<i>3 μM mescaline</i>			
• Vehicle vs 10 μM CBD	5.265	0.1816	ns
• Vehicle vs 1 μM CBD	-0.4884	0.9826	ns
<i>10 μM mescaline</i>			
• Vehicle vs 10 μM CBD	11.46	0.0017	**
• Vehicle vs 1 μM CBD	1.005	0.9291	ns
<i>30 μM mescaline</i>			
• Vehicle vs 10 μM CBD	11.79	0.0013	**
• Vehicle vs 1 μM CBD	0.6300	0.9713	ns

Table A8.20. Statistical comparison of CP-55,940 potency and maximal response across the different pretreatment conditions in the MPA. Cells were pretreated for 1 h with vehicle or CBD (1 and 10 μ M). Data from three independent experiments were analysed using an ordinary one-way ANOVA with post hoc Dunnett's multiple comparisons test against vehicle for $E_{\max}$ in GraphPad Prism.

ANOVA summary	pEC₅₀	E_{max}	
<i>F</i>	1.794	6.287	
<i>P value</i>	0.2451	0.0337	
<i>P value summary</i>	ns	*	
<i>Significant diff. among means (P < 0.05)?</i>	No	Yes	
<i>R squared</i>	0.3472	0.6770	
<i>Degrees of freedom</i>			
▪ <i>Between columns</i>	2	2	
▪ <i>Within columns</i>	6	6	
▪ <i>Total</i>	8	8	
Dunnett's multiple comparisons	Mean difference	Adjusted <i>p</i>-value	Summary
E_{max}			
<i>Vehicle vs 10 μM CBD</i>	7.430	0.0311	*
<i>Vehicle vs 1 μM CBD</i>	0.8533	0.9059	ns

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