FACULTY OF PHARMACEUTICAL SCIENCES

Assessment of the activation of 5-HT2A, 5-HT2B and 5-HT2C receptors by PSYLO synthetic drugs

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ENGLISH SUMMARY

Depression and anxiety are common mental disorders that have significant impact on individuals' well-being. Current treatments, including medications and therapy, have limited effectiveness. Around 20% of people do not respond positively to any available treatment and even those who do often experience a relapse.

In recent years, there has been renewed interest in studying the potential therapeutic benefits of psychedelics in mental health disorders. These substances primarily interact with serotonin receptors located in the brain, important in regulating emotions, mood, cognition, appetite and various other biological and neurological processes.

This study examined the activation of 5-HT2A/B/C receptors by five synthetic drugs, that might be psychedelics. These drugs are developed by the Australian biotech company PSYLO. We compared the potency and efficacy of the drugs to serotonin. To conduct experiments, we used genetically modified HEK-293 cells that expressed the receptors. Since the 5-HT2 receptor family is coupled to a $G_{q/11}$ protein, activating these receptors results in an increase in intracellular calcium levels, measured with the FLIPR Calcium 5 Assay. A fluorescent calcium-sensitive dye emits light when calcium binds to it and the fluorescence intensity can then be measured using a FlexStation 3.

At first, serotonin was applied to the cell lines under various conditions, such as with dialyzed and non-dialyzed serum. Surprisingly, the response of the cells to non-dialyzed serum was higher than to dialyzed serum. Furthermore, the study found that all five synthetic drugs demonstrated an agonistic effect on the 5-HT2A receptor and three of them also showed an agonistic effect on the 5-HT2C receptor. None of these drugs exhibited this effect on the 5-HT2B receptor, leading to their categorization as selective agonists. However, the efficacy of these drugs was found to be lower than that of serotonin, classifying them as low-efficacy drugs. The increasing interest and ongoing research in this field suggests that psychedelics may have a meaningful impact on the future of mental health therapy. This could potentially bring new hope to individuals afflicted by treatment-resistant depression by offering them new possibilities for treatment.

DUTCH SUMMARY

Depressie en angst zijn veel voorkomende psychische stoornissen die aanzienlijke gevolgen hebben voor het welzijn van de bevolking. De huidige behandelingen, waaronder medicatie en therapie, zijn beperkt effectief. Ongeveer 20% van de mensen reageert niet positief op een beschikbare behandeling, en zelfs degenen die dat wel doen krijgen vaak een terugval.

De laatste jaren is er hernieuwde belangstelling voor onderzoek naar de potentiële therapeutische voordelen van psychedelica bij psychische aandoeningen. Deze stoffen werken voornamelijk in op serotoninereceptoren in de hersenen, die belangrijk zijn voor de regulering van emoties, stemming, cognitie, eetlust en diverse andere biologische en neurologische processen.

Deze studie onderzocht de activering van de 5-HT2A/B/C receptoren door vijf synthetische geneesmiddelen, die psychedelica zouden kunnen zijn. Zij zijn ontwikkeld door het Australische biotechnologisch bedrijf PSYLO. Wij vergeleken de werkzaamheid van deze geneesmiddelen met serotonine. Voor de experimenten gebruikten we genetisch gemodificeerde HEK-293 cellen die de receptoren tot expressie brachten. Aangezien de 5-HT2 receptorfamilie geassocieerd is met een $G_{q/11}$ eiwit, resulteert activering van deze receptoren in een verhoging van de intracellulaire calciumspiegel. Wij hebben deze veranderingen gemeten met de FLIPR Calcium 5 Assay door gebruik te maken van een fluorescerende calciumgevoelige kleurstof die in de cellen is geladen. De kleurstof zendt licht uit wanneer calcium eraan bindt en de intensiteit van de fluorescentie kan vervolgens worden gemeten met een FlexStation 3.

In eerste instantie werd serotonine op de cellijnen aangebracht onder verschillende omstandigheden, zoals met gedialyseerd of niet-gedialyseerd serum. Verrassend genoeg was de respons van de cellen op het niet-gedialyseerde serum hoger dan op het gedialyseerde serum. Voorts bleek uit het onderzoek dat alle vijf synthetische geneesmiddelen een agonistisch effect hadden op de 5-HT2A receptor en dat drie ervan ook een agonistisch effect hadden op de 5-HT2C receptor. Geen van deze geneesmiddelen vertoonde dit effect op de 5-HT2B-receptor, waardoor zij als selectieve

agonisten kunnen worden aangemerkt. De efficaciteit van deze geneesmiddelen bleek echter lager te zijn dan die van serotonine, waardoor zij als lage-efficaciteit geneesmiddelen worden geclassificeerd. De toenemende belangstelling en het lopende onderzoek in dit gebied suggereren dat psychedelica een betekenisvolle invloed kunnen hebben op de toekomst van therapie in de mentale gezondheidszorg. Dit zou mogelijks nieuwe hoop kunnen bieden aan patiënten die lijden aan behandelingsresistente depressie door hen nieuwe behandelingsmogelijkheden te bieden.

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ABBREVIATIONS

5-HT	Serotonin
CNS	Central nervous system
CRC	Concentration-response curve
DAG	Diacylglycerol
DMT	N,N-dimethyltryptamine
ER	Endoplasmic reticulum
EV	Empty vector
FBS	Foetal bovin serum
FLIPR	Fluorescent imaging plate reader
FRT	Flippase recognition target
GIRK	G-protein gated inwardly rectifying potasium channels
GOI	Gene of interest
GPCR	G-protein coupled receptor
IP3	Inositol triphosphate
LSD	Lysergic acid diethylamide
PAR-1	Protease-activated receptor 1
PBS	Phosphate buffered saline
PDL	Poly-D-lysine
PET	Positron emission tomography
PIP2	Phosphatidylinositol-4,5-biphosphate
PKC	Protein kinase C
PLC	Phospholipase C
SERT	Serotonin reuptake transporter
SSRIs	Selective serotonin reuptake inhibitors
SST	Somatostatin
SCRA	Synthetic cannabinoid receptor agonist
TAAR	Trace amine-associated receptor
TCA	Tricyclic antidepressants
TetO	Tetracycline operator
TetR	Tetracycline repressor
THC	Tetrahydrocannabinol
WT	Wild-type

1. INTRODUCTION

1.1. THE NEED FOR IMPROVED TREATMENT OF DEPRESSION

1.1.1. Depression

Depression is a common, ongoing medical condition that can have an impact on an individual's mood, thoughts and physical well-being. It affects an important portion of the population worldwide, with an estimated 3,8% of people experiencing the disease. This includes 5% of adults and 5,7% of adults older than 60, according to the world health organization (2021). It is estimated that roughly 280 million people in the world are affected by depression. The illness is defined by symptoms such as lack of energy, low mood, sadness, insomnia, and not being able to experience pleasure. (1) The disease also comes with high rates of relapse, recurrence, disfunction and mortality. (2) Clinical research has revealed that existing treatments for depression do not fully reach the desired therapeutic success for patients. (1) Approximately 30% of individuals undergoing treatment for a major depressive episode will not attain a state of remission, even after undergoing two or more treatment attempts with commonly prescribed antidepressants. These individuals are classified as having treatment-resistant depression. (3)

1.1.2. Current treatments for depression

The primary medication used to treat depression are selective serotonin reuptake inhibitors (SSRIs), which include fluoxetine, paroxetine, fluvaximen, citalopram and sertraline. They inhibit the serotonin reuptake transporter (SERT) located on the plasma membrane, thereby increasing the amount of serotonin (5-HT) in the synaptic cleft. (4) Almost all types of antidepressant treatments enhance 5-HT neurotransmission, either directly or indirectly. (5) The goal in the development of SSRIs was to achieve a specific mechanism of action while minimizing negative effects. In this way, SSRIs aimed to produce a safer and better tolerated alternative to earlier antidepressants like tricyclic antidepressants (TCAs) and monoamine oxidase inhibitors. TCAs are associated with adverse effects like gastrointestinal and urinary retention, sexual dysfunction, cardiovascular problems and weight gain. (6) Even though both TCAs and SSRIs block

SERT, TCAs have more side effects because they also interfere with sodium channels and other receptors such as alpha-adrenergic, histamine and muscarinic receptors, unlike SSRIs which don't affect them. (7)

1.1.3. Drawbacks of the current treatment

Although SSRIs are currently very widely prescribed, there are potential dangers linked to these medications, such as the emergence of suicidal tendencies and withdrawal symptoms, particularly in children and teenagers. (8,9) Other drawbacks are the requirement for ongoing administration (10,11) and an important number of patients who do not respond well to the treatment. (11) One significant disadvantage of all antidepressants is that they need to be taken for a prolonged period before achieving the maximum therapeutic effect. While some patients may exhibit partial improvement within 1-2 weeks, in most cases, it takes 3-6 weeks to reach full effectiveness. (10,12).

One theory suggests that the acute desensitization of serotonin 1A (5-HT1A) auto-receptors is insufficient to generate an antidepressant response, and that a reduction in the production of new 5-HT1A auto-receptors may be essential. This may explain the prolonged duration of the treatment. (5) One of the disputable adverse effects linked to SSRIs is the warning regarding suicidal tendencies in 18-24 years old children and adolescents. There is not enough knowledge about how SSRIs interact in the developing brain of young people with depression, adding to the complexity of this issue. It seems that adults without specific risk factors are not subject to this elevated risk of suicidal behaviour. (9) Some other depression treatments, for instance mirtazapine and venlafaxine, may show a faster response or higher efficacy compared to SSRIs in individuals with major depression. However, a significant part of them may still not experience a sufficient response to the drugs. (2)

1.1.4. Research into more effective therapies

Although antidepressant and cognitive behavioural therapy can be beneficial for certain patients, approximately 20% of individuals do not exhibit a positive response to any form of treatment, and a significant proportion of those who do respond will eventually experience a relapse. (13) This means that there is a requirement for more effective

antidepressant medications that can improve treatment responsiveness in a larger number of patients, lead to a higher level of remission in individual patients, and provide better prevention against relapse. (12) There is hope for improved treatments as research advancements are progressing rapidly. There are encouraging developments in fields such as genetics, brain function and innovative therapies that are being pursued. (11)

1.2. PSYCHEDELICS

1.2.1 Potential therapeutic effects of psychedelics

There has been an increasing curiosity in the potential of psychedelics as a remedy for various mental health conditions. The term “psychedelic” has Greek roots, where “psyche” refers to the mind or soul and “delos” means to reveal. This word was first introduced by psychiatrist Humphry Osmond in 1956, during his investigations on lysergic acid diethylamide (LSD). (14) Psychedelics, which are also called serotonergic hallucinogens, are potent psychoactive drugs that have the ability to change a person’s mood, perception and cognitive functions. (15,16) They are generally believed to be safe for the body and are not addictive. (16) Considerable research is carried out on these psychedelic drugs as potential adjuvants to psychotherapy. (17) The drugs could serve as a secure and effective substitute for typical medications used in the treatment of mood and anxiety disorders. (14)

These substances have been found to have the most potent and most effective impact on the 5-HT2A target. (15) However, it is possible that effects on other receptors may also play a role, namely 5-HT2B/C and 5-HT1A/B, but also dopamine (D2) receptors. (18) Both psylocibin and LSD, which are examples of classic psychedelics discussed in the paragraphs below, are non-selective drugs and interact with several receptors. The pharmacology of N-benzyl phenylethylamines is more restricted, as they have most potent agonist activities at 5-HT2 family receptors. (17) Uncertainty remains about which specific aspects of 5-HT2A receptor activity in the central nervous system (CNS) are responsible for the therapeutic effects. It’s also unclear to what extent these effects can be isolated by developing new chemical probes with diverse specificity and selectivity

profiles. (17) One of the reasons why people are so crazy about psychedelics, both for therapy and in the wild, is that they can provide profound insight into themselves. Another important aspect is the idea that psychedelics can make it possible for the brain to change in a positive way, which means that under influence one can alter patterns of thought and potentially change the ingrained patterns of learning or memories that drive depression or post-traumatic stress disorder. (19) While these substances are associated with relatively low physical health and mortality risks, it's important to take into account the possibility of profound psychiatric complications. In addition to the possibility of psychosis, there is also a significant risk of developing Hallucinogen Persisting Perception Disorder after just a single use of these substances. The disorder is characterized by long-term changes in perception that can negatively impact daily life and well-being. (20)

1.2.2 Classic psychedelics

1.2.2.1 Psilocybin

One example of a classic psychedelic is psilocybin. The drug has been discovered in many mushroom species across the world. (21) Psilocybin can produce hallucinogenic effects and cause temporary symptoms resembling psychosis. It has an important impact on cognition, perception and emotions. (22) Nowadays, psilocybin is among the most commonly used psychedelic in human studies as a result of its favourable safety profile, moderately long duration of action and efficient absorption when administered orally. (23) Although there is a lack of research comparing the effectiveness of psilocybin and other psychedelic drugs in treating mood and anxiety disorders, the extensive collection of evidence-based data available for psilocybin implies that it might be the most effective psychedelic drug for this purpose. Several clinical studies support the notion that psilocibin-assisted therapy has the potential to be an effective complement to psychotherapy. (24) Psilocybin undergoes rapid transformation into psilocin (4-hydroxy-N,N-dimethyltryptamine) in the human body due to alkaline phosphatase in the liver and non-specific esterase in the intestinal mucosa. Psilocybin in rodents is completely transformed into psilocin prior to entering the systemic circulation. Despite the common

belief that psilocybin is responsible for the psychotomimetic effects, it is actually psilocin, and not psilocybin, that acts as the main pharmacologically active substance. Psilocybin is considered as a prodrug to psilocin. (23)

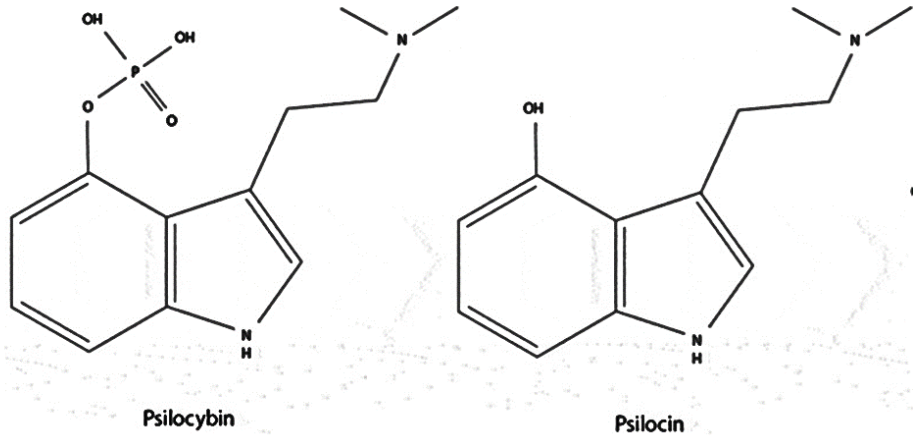


Figure 1.1: Chemical structure of psilocin and its prodrug psilocybin (25)

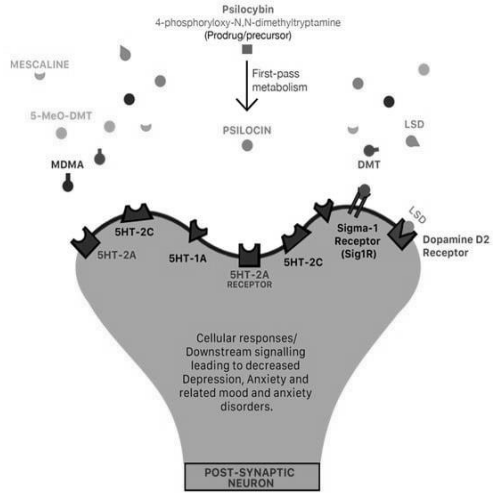
Psilocin has an agonistic effect on 5-HT2A receptors, leading to a hallucinatory experience, while its affinity for the 5-HT1A receptor is lower. The interaction between psilocin and psilocybin in producing psychotomimetic effects has been confirmed through experiments involving ketanserin, a 5-HT2A antagonist. (21) Besides interacting with 5-HT2A receptors, psilocin may also act on non-5-HT2 receptors to create its psychopharmacological effects. Psilocin and its prodrug also interact with the 5-HT1D and 5-HT2C subtypes. (21) Apart from this interaction with the serotonergic system, there is evidence to suggest that psilocybin also interacts indirectly with the mesolimbic dopaminergic pathway, which is an important component in the brain's reward system. This interaction is thought to mediate through the activation of 5-HT2A receptors by psilocybin, which results in the release of dopamine in the mesolimbic pathway. This mechanism of action is believed to be why psilocybin has low potential for addiction and abuse. Also, it has been suggested that there may be a link between depression and a deficiency of dopamine in the mesolimbic pathway. (25) It is important to note that psilocybin, unlike LSD, does not interact with the D2 receptor. This has been reported with the D2 antagonist haloperidol, which caused verry little blockade of the effects of

psilocybin. (26) Potential risks associated with psilocybin use (e.g. altered perception, hallucinations and intensified emotions (23)), can be minimized through measures such as offering preparatory counselling to ensure the patients have the right mindset, providing a medically supervised setting and offering sufficient clinical psychological and physiological support. (24)

1.2.2.2 LSD

LSD is a semisynthetic compound that originates from lysergic acid, which is found in the parasitic rye fungus C. *purpurea*. (27) The drug produces a stimulus that involves multiple components. While the primary component of the LSD stimulus is mediated through the 5-HT2A receptor, secondary components are thought to be influenced by interactions with other monoamine receptors, including 12 of the 14 human 5-HT receptors and all five dopamine receptors. LSD acts as a partial agonist at D1 and D2 receptors and as a full agonist at D4 receptors. The mixed 5-HT2A/D2 antagonist risperidone seems to be more potent than the 5-HT2A antagonist ritanserin at blocking the LSD stimulus. This indicates that both receptor interactions contribute to the sensory effects of LSD. (26) The drug is also agonist for α1 adrenergic receptors (17) and has an impact on glutamergic neurotransmission and trace amine-associated receptors (TAARs) in animal models. Additionally, LSD has a minor effect on α2 adrenergic receptors, which causes stimulation of the sympathetic nervous system. This means an increase in body temperature, tachycardia, elevated blood pressure, sweating and muscle tension. (20)

(a)



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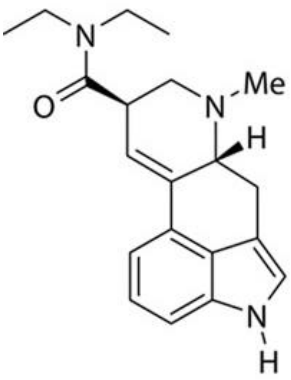


LSD

Figure 1.2: (a) Mechanism of action of classic serotonergic drugs. The 5-HT receptors are represented in high densities in brain regions that regulate mood and anxiety disorders, such as the prefrontal cortex. Classic psychedelic drugs, including LSD, psilocybin and N,N-dimethyltryptamine (DMT), all have affinity for the 5-HT receptors, which is thought to be responsible for their psychotomimetic and pharmacological effects. DMT can interact with the sigma-1 receptor and TAARs. LSD may also interact with dopamine D2 receptors and TAARs to produce the effects. (b) Chemical structure of LSD. (26)

1.3. 5-HT RECEPTORS

There are 7 different serotonin receptor groups (from 5-HT1 to 5-HT7), which in turn are divided into subgroups. For example, the 5-HT1 receptor is subdivided into 5-HT1A/B/D/E/F. This makes a total of 14 5-HT receptors. (28) The receptors belong to the G-protein coupled receptor (GPCR) family, except for the 5-HT3 family which are ion channels. These seven transmembrane receptors are found in cell membranes that act as an intermediary between molecules outside the cell, such as neurotransmitters and hormones, and proteins within the cell that signal the cell to respond, such as G-proteins and β-arrestin. (29) The focus in this study is on testing newly developed drugs on the serotonin receptors that are primarily involved in mental health diseases, which is why we only discuss the interaction with 5-HT1A/2A/2B/2C.

1.3.1. 5-HT1A receptor

The 5-HT1A receptors are linked to the Gi/o protein. Binding of a ligand to the receptor leads to the activation of the G-protein. The latter inhibits the activity of adenylate cyclase. This means that when 5-HT1A receptors are stimulated, they reduce formation of cyclic adenosine monophosphate by coupling with Gi/o, which leads to inhibition of protein kinase A-mediated protein phosphorylation. It also results in the activation of G-protein-gated inwardly rectifying potassium (GIRK) channels, by releasing G-protein $\beta\gamma$ subunits that interact with the regulatory site on GIRK channels. Activation of these channels promotes the efflux of intracellular K^+ and hyperpolarizes the cell, making it less likely to fire an action potential. The receptor also inhibits voltage gated calcium

channels, which reduces the influx of calcium in the cell. In summary, these signalling pathways result in a reduction of the activity of neurons, which is associated with the anxiolytic and antidepressant effects of drugs that interact with the 5-HT1A receptor. (30)

5-HT1A receptor activation has been shown to play an important role in moderating numerous CNS diseases. This evidence has prompted the creation of advanced 5-HT1A ligands, such as 5-HT1A agonists, with better selectivity, enhanced pharmacokinetic properties and substantial potency, and 5-HT1A ligands that have a combination of multiple pharmacological effects. This means developing or identifying drugs that in addition to acting at 5-HT1A, also act directly at other targets. (e.g. inhibition of dopamine D2 receptors, serotonin transporters and more 5-HT receptor subclasses). (31)

There are inconsistent findings regarding changes in 5-HT1A binding potential in human depression. Several studies have found reductions in post-synaptic 5-HT1A receptors in particular regions of the brain both in depression and anxiety, while other studies have found an increased level of 5-HT1A auto-receptors in depressed patients and in post-mortem raphe tissue from depressed suicide victims. These changes in 5-HT1A receptor levels in depression suggest an overall decline in 5-HT neurotransmission, as increased 5-HT1A auto-receptors reduce 5-HT neuronal activity, and less post-synaptic 5-HT1A receptors decrease the response to 5-HT. (5) As mentioned above, when stimulating a 5-HT1A auto-receptor, the release of further serotonin is inhibited because the presynaptic receptor acts as a feedback mechanism. However, when a treatment is administered over an extended period, these auto-receptors become desensitized and no longer cause a reduction in serotonin. (2) Combining SSRIs with 5-HT1A receptor agonists may ease desensitization of the auto-receptors, which can speed up the onset of the antidepressant effects. So, besides monotherapies, the agonists may be used also in combination therapy with SSRIs. (31)

1.3.2. 5-HT2A receptor

The 5-HT2 receptor subtypes, are associated with $G_{q/11}$ -related pathways. When these receptors are stimulated, they activate the Gq-protein. Thereby phospholipase C (PLC) is activated, which leads to the production of inositol triphosphate (IP3) and diacylglycerol

(DAG) from phosphatidylinositol-4,5-biphosphate (PIP2). Production of IP3 ultimately results in an increase in intracellular calcium, by releasing it from the endoplasmic reticulum (ER). (32) DAG triggers the activation of protein kinase C (PKC) and helps it move from the cytosol to the membrane. PKC adds phosphate groups to different target proteins leading to changes in their function and activity, while calcium ions are recognized for regulating the function of enzymes and ion channels. (28) While G_q - related signalling could play a role in the behavioural effects of hallucinogens in rodents, it is not the sole signalling pathway responsible for this response. Apart from $G_{q/11}$ mediated PLC-IP3 signalling, various other signalling transduction pathways associated with the 5-HT2A receptor, such as phospholipase A2, have been identified. (33) The 5-HT2A receptors have been shown to also interact with β-arrestins. These can cause receptor desensitization, internalization and signalling. The latter works via an alternative pathway that is different from the G-protein mediated pathway. Both arrestin-dependent and arrestin-independent pathways have been associated with the effects of psychedelic drugs. (34,35)

5-HT2A receptors are present in various regions of the brain including the prefrontal cortex, ventral tegmental area (VTA), thalamus and striatum, which are associated with mood regulation and cognition. (36) Along with serotonergic cell bodies, there are also dopaminergic cell bodies located in the VTA, which is a brain region that plays a role in regulating emotions, the reward process and cognitive behaviour. (24)

Some scientists have suggested that the concept of biased signalling, also known as functional selectivity, exhibited by the 5-HT2A receptor, could explain why some agonists of the 5-HT2A receptor like LSD and psilocybin cause hallucinogenic effects while others such as lisuride and ergotamine do not generate such effects. (37) The phenomenon of biased signalling has created significant excitement in the field of molecular pharmacology, as it provides the possibility to develop innovative drugs that selectively target signalling pathways associated with the intended therapeutic outcome, while minimizing undesired effects. (33)

Previous studies have observed a rise in 5-HT2A receptor binding in patients with depression, especially in those with severe negativity. These results are not yet conclusive, but indicate that individuals who have a history of recurrent major depression and have recovered may have a higher likelihood of binding potential for cortical 5-HT2A receptors. (38) There is a theory that the elevation of cortical 5-HT2A receptor binding in depression may be linked to intense negativity and develops as a coping mechanism due to prolonged serotonin deficiency. (39)

1.3.3. 5-HT2B receptor

It has been found that the 5-HT2B receptor has a strong similarity to both the 5-HT2C and 5-HT2A receptors. This similarity is not surprising given their structural related characteristics. (28) The 5-HT2B receptor is, as for the 5-HT2A receptor, also linked to $G_{q/11}$. The mechanism of action happens via activation of PLC by the G-protein, leading to conversion of PIP2 to IP3 and DAG. (40) The varied expression of the 5-HT2B receptor indicates a wide range of physiological and pathological functions. (28)

It seems that the 5-HT2B receptor enhances serotonergic activity and that it is essential for the beneficial effects of SSRIs. Therefore, the 5-HT2B receptor could be seen as a hopeful new target to tackle depression. Ex vivo studies have suggested that the 5-HT2B receptors regulate SERT in raphe neurons. Additionally, it is reported that mice without 5-HT2B receptors do not exhibit an immediate response to SSRIs in the forced swimming test, which is a well-known test used to evaluate the activity of antidepressants. (41)

1.3.4. 5-HT2C receptor

The 5-HT2C receptor also belongs to the 5-HT2 family, so it is coupled to $G_{q/11}$. Initially, doubts were raised about the significance of the 5-HT2C receptor in depression treatment, as the receptor-induced activation of phosphatidylinositol was primarily observed in the choroid plexus – an area not conventionally associated with emotions. However, it has been discovered that 5-HT2C receptors are also present in several other brain structures such as hippocampus, striatum, septum etc. These have all been identified as playing an important role in the brain mechanisms underlying emotions. (42)

At the beginning of treatment, the indirect activation of 5-HT2C receptors contributes to the anxiety-inducing impacts of SSRIs, as well as their hindrance of sleep, sexual behaviour and appetite. Conversely, as people continue to take SSRIs, the gradual onset of clinical efficacy of these drugs is accompanied by the progressive reduction of 5-HT2C receptor activity. (42,43) It is believed that the therapeutic effects of SSRIs, such as improved mood and reduced anxiety, are due to a decrease in the activity of 5-HT2C receptors. The extended antidepressant impact of these substances might be connected to a selective desensitization of the 5-HT2C receptor in specific regions in the brain. (42) So, while the initial side effects of SSRIs may be due to increased 5-HT2C receptor activity, the eventual therapeutic effects are thought to be due to reduced 5-HT2C receptor activity.

Blocking the 5-HT2C receptor with selective antagonists could be a beneficial approach for creating new medications to treat depression and anxiety. These drugs could be used alone or in combination with other antidepressants to enhance their effectiveness, as well as to alleviate negative effects caused by certain antidepressants. (44)

1.3.5. Interaction of psychedelics with 5-HT receptors

Serotonergic psychedelics share the same mechanism of action to cause significant changes in mood, thoughts and perceptions, which is agonism at cortical 5-HT1A/2A/2C receptors. Various molecular and receptor-level studies have proven that these receptors are important in the therapeutic effects of the psychedelics. The 5-HT2A receptor appears to be the most significant. The latter stimulates other signalling transduction pathways than the 5-HT1A/2C receptors. (45) Furthermore, after administration of psilocybin for example, antidepressant effects were seen in rodents. (6) For life-threatening cancer patients, controlled trials with LSD and psilocybin showed quick and long-lasting enhancements of anxiety and depression symptoms. (46)

Moreover, positron emission tomography (PET) studies were carried out to confirm that psilocin, the active form of psilocybin, functions by interacting with 5-HT2 receptors. PET is an imaging technique capable of quantifying receptor binding in vivo. Coupled with drug administration and appropriate radiotracer selection, PET-studies can provide

valuable knowledge about relationships between drug levels, drug target occupancy and associations with clinical response or side-effects. All PET scans conducted on individuals who consumed psilocybin showed a significant relationship between the amount of psilocybin taken and the level of 5-HT2A receptor occupancy. (47,48)

Nonetheless, the enthusiasm surrounding these medications needs to be balanced with concrete proof of their efficacy, tolerability and safety. Studies conducted in both experimental and clinical settings, in a controlled environment, indicate that these drugs have a favourable safety and tolerability profile. It concerns a limited number of studies with small sample sizes and brief durations, but it is worth highlighting that the effects observed were both immediate and long-lasting, and were brought about by only one or a few doses of the drugs, which is in contrast to traditional antidepressants and anxiolytics. (49,50) Although there is some promising evidence for the medical use of psychedelics, their use is still considered experimental. Therefore, there is currently insufficient data to justify routine use of psychedelics. More rigorous and extensive trials are required to compare the efficacy of psychedelic drugs with existing treatments. The current evidence should be used to support further research in this area. (19)

1.3.6. Interaction of psychedelics with receptors other than 5-HT receptors

Although the 5-HT receptors are often the most significant targets of psychedelics, these drugs can also interact with other receptors in the body and the brain. Some psychedelics, like LSD as mentioned before, have been shown to not only interact with serotonin receptors, but also with dopamine receptors. (26) Other psychedelic-like substances, such as ketamine, are known to bind with NMDA receptors, which are important for learning and memory. (51) TAAR receptors may also play a role in producing effects of psychedelic drugs, such as DMT. However, the discovery of TAARs occurred after research on DMT interaction with 5-HT2A receptors started. Consequently, there has been restricted research on the role that TAARs may play in mediating the effects of DMT. (52) The interactions between these receptors and psychedelics are complex and not fully understood yet. More research is necessary to achieve better

understanding in how these interactions contribute to the drugs' potential therapeutic advantages.

1.4. ASSAYS

We can apply various assays to determine the potency and efficacy of different compounds. This study involved assessment of the effects of novel drugs on 5-HT receptors, which can be achieved by two assays: the FLIPR Calcium 5 Assay, a high-throughput fluorescence assay that detects changes in intracellular calcium, and the FLIPR Membrane Potential Assay. To receive the new drugs that might be psychedelics, we worked in collaboration with a company called Psylo (https://psylo.bio/). This is a biotech company situated in Australia that uses natural elements as a basis to develop new therapies for mental health disorders. Our goal was to test these newly developed drugs by examining their activity on 5-HT receptors, with particular focus on their efficacy in activating the receptors.

1.4.1. FLIPR Calcium 5 assay

The FLIPR Calcium 5 assay is a widely used method for measuring changes in intracellular calcium levels in living cells, in which the abbreviation "FLIPR" stands for Fluorescent Imaging Plate Reader. These changes occur when the cells are stimulated, calcium is then mobilized from the ER into the cell cytoplasm, where a calcium-sensitive dye is present. The changes are measured by loading the cells with the calcium-sensitive fluorescent dye and exposing them to a test compound or stimulus. When the calcium ions reach the cytoplasm, they bind to the fluorescent dye that is loaded in the cells, causing the dye to emit light at a specific wavelength. The FLIPR instrument rapidly reads the fluorescence intensity of each well in a 96-well plate, allowing us to monitor changes in intracellular calcium levels over time. With the resulting data, we can determine the potency and efficacy of a compound. (53)

The FLIPR Ca^{2+} assays have several applications, among others screening for potential drugs or identifying new drug leads, and also a detailed characterizing of the pharmacology of known or novel agonists and antagonists. The assay is capable of testing large numbers of compounds every day. (54)

1.4.2. FLIPR Membrane Potential Assay

The FLIPR Membrane Potential Assay is employed as a method to measure changes in the membrane potential across living cells. This potential difference is caused by ions, such as calcium, sodium and potassium moving in and out the cell. The cell membrane can undergo a hyperpolarization, causing an increase in negativity inside the cell. On the other hand, depolarization leads to a more positively charged inside of the cell. The fluorescence intensity alters with the depolarisation or hyperpolarisation of the cellular membrane potential upon activation of a GPCR. (55)

1.4.3. The Flp-In^{TM} T-REx^{TM} system

Traditional methods for studying receptor depletion involve using an irreversible antagonist to vary the concentration of total receptors [Rt]. (56) However, this method is dependent on the availability of an appropriate pharmacological tool for the target being studied. In cases where such tools are not available, alternative methods for manipulating receptor availability can be useful. (57) In this study, we used a stably transfected Flp-In^{TM} T-REx^{TM} 293 cell line, which allowed us for tetracycline-dependent control of 5-HT receptor expression. (58) Using different levels of tetracycline, leads to different levels of expression of the GOI. By changing the number of receptors and then studying what the concentration-response curves (CRCs) are, we are allowed to determine how well an agonist activates a receptor.

Cells that have been transfected to express receptors on their surface have likely become the most commonly utilized model for conducting biochemical and pharmacological studies on GPCRs. (59) In the Flp-In^{TM} T-REx^{TM} system, a small molecule is used to induce expression of the receptors, which has several benefits over other methods. (60) In addition to transient transfection, which offers the benefit of quick expression for a short series of experiments, creating stable cell lines is the preferred method when a significant quantity of a verified and effective receptor expression is needed. (59) Firstly, there is no need for these expensive and variable transient transfections. Secondly, all stable cell lines derived from the same parental cells are integrated at the same time, ensuring they have the same genetic background, making

comparisons easier. Furthermore, stable cell lines can be grown to the required density before induction of the gene of interest (GOI), avoiding negative effects on cell growth. In addition, the level of expression can be regulated by changing the concentration of the inducing agent. Finally, since expression of the GOI is only induced when necessary, there is less chance of losing expression due to non-expressing cells outgrowing those that still express the GOI. (60)

2. OBJECTIVES

Depression is a complex and common condition that impacts a large number of individuals all over the world. It is characterized by enduring feelings of sadness or loss of interest in once-enjoyable activities, decreased focus, a negative outlook on the future and other related symptoms. Additionally, many patients have physical symptoms as a result of this condition. Currently, there are two primary treatment approaches to address depression: psychological therapy and pharmacotherapy. Due to the high possibility of recurrence, patients often undergo extended treatment. Presently, the most frequently prescribed antidepressants are SSRIs.

However, none of the currently available medications can totally eliminate depression, necessitating the development of new, more potent, more effective and safer options. Relevant studies emphasize the significance of 5-HT2A/B/C receptors in the underlying pathomechanism of depression and anxiety.

The primary objective of this research is to evaluate the activation of this 5-HT2A/B/C receptors by innovative synthetic drugs. These substances are produced by a company named PSYLO and may potentially exhibit psychedelic properties. Additionally, we will compare the efficacy and potency of these drugs to serotonin, which serves as a positive control.

The human 5-HT2A/B/C receptors were expressed in HEK-293 cells. Stable cell lines were generated, allowing us to regulate the expression of 5-HT2 receptors using tetracycline induction. This enabled us to determine the amount of receptors expressed depending on the presence or absence of tetracycline.

To ensure that the wild-type (WT) HEK-293 cells did not naturally express the 5-HT2 receptors, we conducted a test on these cells using 5-HT, somatostatin (SST) and protease-activated receptor (PAR-1). Additionally, we performed mycoplasma testing on the cell cultures, as the presence of mycoplasma would compromise the accuracy and reproducibility of our experimental outcomes. Before conducting any assays, we prepared the plates by coating them with poly-D-lysine, a substance that enhances cell adhesion to the plate.

We utilized the FLIPR Calcium 5 assay to observe alterations in intracellular calcium levels in our transfected cells. The functionality of the GPCRs, specifically the 5-HT2A/B/C receptors, was examined using a FlexStation 3. The 5-HT2 receptors belong to a diverse group of $G_{q/11}$ coupled receptors that play crucial roles in various physiological and pathophysiological processes, particularly in mental health disorders. Upon activation, these receptors trigger calcium influx from the ER. HEK-293 cells stably expressing human 5-HT2 receptors, cultivated in 96-well plates, were loaded with a calcium-sensitive fluorescent dye. The fluorescence changes in real-time were measured before and during the application of serotonin or a PSYLO synthetic drug using the FLEX mode of the FlexStation 3. The data obtained from GraphPad Prism were then used to construct concentration-response relationships for both serotonin and the PSYLO synthetic drugs.

By analysing these concentration-response curves, we obtained valuable insights into the pharmacological properties of each drug tested, specifically their potency and efficacy. We also conducted a comparative analysis of these values with those obtained for serotonin.

3. MATERIALS AND METHODS

3.1. MATERIALS

The cell culture media, antibiotics, buffers and general chemicals used in the study were sourced from Life Technologies, Sigma-Aldrich or InvivoGen (San Diego, CA, USA). The drugs were synthesized by PSYLO. Plasmids were synthesized by Genscript (Piscataway, NJ, USA).

3.2. METHODS

3.2.1. Transfection

Initially, the parental Flp-In^{TM} T-REx^{TM} HEK-293 cells were used, which already contain a Flp recognition target (FRT) site and express the tetracycline repressor protein (tetR). After transfection with pcDNA5/FRT/TO/GPCR, the GPCR DNA integrates into the FRT site, and the tetracycline repressor protein (tetR) binds to the tetracycline operator region (tetO) of the integrated pcDNA5/FRT/TO/GPCR, thereby suppressing GPCR expression. Finally tetracycline (or doxycycline) is introduced, which binds to tetR and frees tetO, allowing GPCR expression. (60) Co-transfection was done with pcDNA5/FRT/TO/GPCR and pOG44. The latter is a vector for expression of Flp recombinase, which is an enzyme that facilitates the integration of the pcDNA5/FRT/TO/GPCR vector into the genome via FRT sites. Reference sequences numbers for both the 5-HT2A, 5-HT2B and 5-HT2C receptor are given respectively: NM_000621.5, NM_000867.5 and NM_000868.4. (National Library of Medicine, NIH)

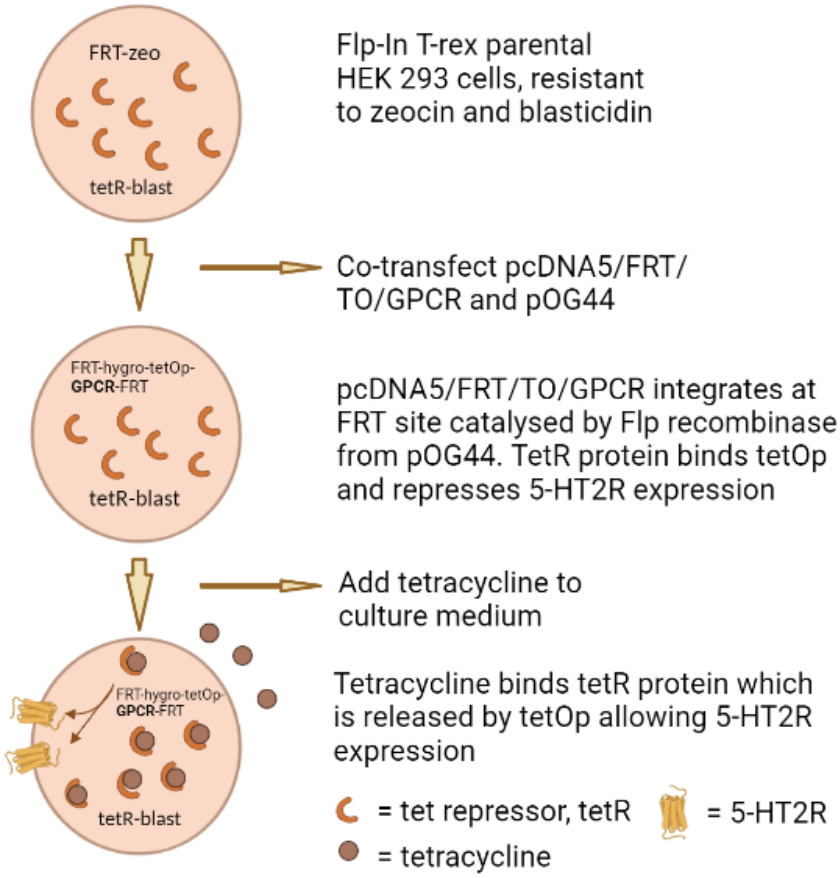


Figure 3.1: Creating Flp-In^{TM} T-REx^{TM} cell lines to express 5-HT2 receptors in an inducable and stable way. (a) Representation of Flp-In^{TM} T-REx^{TM} HEK-293 cells that already have the FRT site and tetR. The cells are resistant to zeocin and blasticdin to maintain the FRT site and tetR in the genome. (b) Transfection of pcDNA5/FRT/TO/GPCR and pOG44 into the FRT site. The integrated plasmid does contain tetO, which binds to the tetR. 5-HT2R expression is suppressed. (c) TetO region is free due to binding of the tetracycline to the tetR, which allows for 5-HT2R expression. (60) *Figure created with biorender.com*

The Flp-In^{TM} T-REx^{TM} HEK-293 cells were plated into a 6-well plate 24 hours before transfection to reach about 80% confluency the next day. This high confluency of the cells was desired as it is the optimal confluency for Fugene HD transfection. One flask was also passaged to maintain. To each of the wells, 2ml of pre-warmed maintenance medium was added and the cells were then introduced to the wells.

After 24 hours, we started the Fugene HD co-transfection of pOG44 and pcDNA5/FRT/TO/GPCR. Stock concentrations of the DNA encoding for 5-HT2A, 5-HT2B and 5-HT2C were measured with a NanoDrop One UV-Vis spectrophotometer. This Nanodrop One uses a small volume of the sample, which is placed on a measurement surface. A beam of light goes through the sample which allows the instrument to measure absorption of the light by the sample at different wavelengths. The Nanodrop One then calculates the concentration of the DNA by using Lambert-Beer's law. The vials were spun in a centrifuge for 1 minute at a temperature of 4°C and a speed of 6000 x g. Afterwards, the DNA was mixed with 40 µl of MilliQ water and 30 µl of the resulting solution was moved to a fresh tube. The concentrations measured can be found in Table 3.1.

The HEK-293 T-rex cells were genetically modified to include an FLP recognition site and then permanently transfected with plasmids encoding human 5-HT2 receptors in pDNA5 with pOG44 using FuGENE HD. The latter is a transfection reagent that forms a complex with the plasmid DNA which is then transported into the cells. The cells use endocytosis to take up this complex. The plasmids were ordered by Prof Mark Connor from Genscript. Cells were cultured in a medium called Dulbecco's modified Eagle's medium (Thermo Fisher Scientific) supplemented with 10% foetal bovin serum (FBS), 1% penicillin-streptomycin, 15 µg/ml blasticidin and 100 µg/ml hygromycin (5-HT selection antibiotics) up to passage 5 (selection phase). The transfection mix was prepared as indicated in Table 3.2. pOG44/pcDNA5 ratio of 9:1 and a µl FuGENE HD:µg total DNA ratio of 4:1 was used. The FuGENE HD transfection reagent/DNA mixture was incubated for 15 minutes at room temperature and added drop-wise to the cells. This was incubated for 48 hours at 37°C/5% CO_2. Afterwards, each well was passaged to a T75 flask (1 well per flask) and incubated without antibiotics overnight.

Table 3.1: Stock concentrations of the DNA encoding for 5-HT2A, 5-HT2B and 5-HT2C and stock concentration of pOG44

GOI	ng/µl
Human 5-HT2A receptor	167
Human 5-HT2B receptor	155
Human 5-HT2C receptor	159
pOG44 (previously propagated)	580

Table 3.2: Composition of the transfection mix for 5-HT2A, 5-HT2B and 5-HT2C

Receptor	pOG44 (µl)	GOI (µl)	Fugene HD (µl)	Diluent (µl)
5-HT2A receptor	4,7	1,80	12	82
5-HT2B receptor	4,7	1,94	12	82
5-HT2C receptor	4,7	1,89	12	82

3.2.2. Cell culture

The media of the transfected cells was changed every 3-4 days. To use the transfected cells for experiments, they had to be split, selected and expanded until they reached passage number 5. The cells expressing the 5-HT2 receptors are selected using blasticidin and hygromycin (InvivoGen, San Diego, CA, USA). Cells were passaged to a new flask at ~ 80 % confluency. The process of passaging involves several steps. The HEK-293 cells were washed with phosphate buffered saline (PBS) to rinse the cells gently and detached from the flask with trypsin for 2 minutes. The DMEM medium was then added to stop the trypsin. The cells were centrifuged for 5 minutes at 1000 rpm and the supernatant was carefully aspirated from the newly formed pellet. The cells were then resuspended in DMEM medium with antibiotics and transferred to a new 75 cm^2 flask. Finally, the flask was placed in an incubator at 37°C and 5% CO_2.

3.2.3. Wild-type HEK-293 cells

The FLIPR Calcium 5 assay was used to test whether the HEK-293 WT cells expressed the 5-HT receptors. WT cells are not transfected with the GOI, which means they may not express these receptors. Three different drugs (5-HT, SST and PAR-1) were repeatedly tested in experiments.

Cell signalling was evaluated by concurrently measuring excitation at a wavelength of 485 nm and emission at 525 nm. The measurements were captured continuously for a period of up to 300 seconds for every column of the plate. To validate that the WT cells do not express any 5-HT receptors, CRCs were constructed. These CRCs were conducted for each replicate of the experiment and are depicted in Figure 4.1. The presented data represents the average value ± SEM obtained from a minimum of three distinct experiments, with each experiment performed in duplicate.

3.2.4. Poly-D-lysine (PDL) coating

To improve the adherence of cells (especially HEK-293 cells) to surfaces, poly-D-lysine is commonly used to coat plates. Cells were plated in black wall 96-well microplates. When cells are introduced to a PDL-coated surface, the positively charged polymer interacts with the negatively charged cell membranes, promoting attachment and allowing cells to spread and multiply. Using plates coated with PDL provides a reliable and uniform surface for the attachment of cells and avoids their tendency to form clumps.

An aliquot of PDL was thawed (100 µl in 1 mg/ml) and combined with PBS. 50 µl of the mixture was added to each well and gently tapped to guarantee a uniform coating of the culture surface. After waiting for 10 minutes, the solution was removed by aspiration and the surface was thoroughly rinsed using MQ sterile water. To prevent damage to the plates and coating, the plates were left to dry in a hood rather than in an oven, which may be too hot. Once dry, the plates were sealed and stored until they were required for future experiments. Ideally, they should be used within a month.

3.2.5. Dialyzed serum

Before starting experiments on the transfected cells, it needed to be considered whether to use dialyzed L-15 instead of non-dialyzed L-15. L-15 is utilized cause it is rich in growth factors, hormones and nutrients that promote cell growth. Dialyzed serum has undergone an extra dialysis step to ensure experimental assays are not affected by unwanted substances. This includes molecules with a low molecular weight such as nucleotides, salts, amino acids and certain small proteins. Tangential flow filtration is employed for this purpose, which involves using a filter with a 10 kDa molecular weight cut-off. One such substance that is removed from the serum is serotonin, which can cause continuous activation of cells if present in the media, especially when studying receptors that are sensitive to serotonin as done in this study. However, it is necessary to test whether using dialyzed serum produces different results than using non-dialyzed L-15, as the level of serotonin may be insufficient to activate these receptors. It is also important to note that dialyzed serum is twice as expensive as non-dialyzed L-15, so the decision whether to use it or not should be carefully considered before beginning the experiments.

3.2.6. Mycoplasma testing

One important thing that had to be done before we were able to use the transfected cells for the experiments, was testing if there is mycoplasma present in the cell cultures. If so, cells would need to be discarded and the transfection process would have to be started all over again. Mycoplasma contamination can alter the behaviour of cells and impact the accuracy and reproducibility of the experimental results. Ensuring the quality of our cell cultures and obtaining accurate experimental results is crucial.

To test for mycoplasma contamination, the MycoAlert Mycoplasma Detection Kit (100 tests) from Lonza (Rockland, ME, USA) was used. This kit measures the activity of mycoplasmal enzymes, which are present in all six of the main mycoplasma cell culture contaminants and the majority of 180 mycoplasma species, but are not present in eukaryotic cells. Mycoplasma was tested at cell passage 4.

To perform the test, 500 µl of the cell culture supernatant was taken and the remaining cells were removed by spinning 5 minutes at 200 x g. This was done because the cells would increase the background signal and cause loss of sensitivity. Then, 100 µl of the samples and 100 µl of MycoAlert reagent were mixed and incubated for 5 minutes. The Mycoalert reagent contains luciferase enzyme that generates a light signal from ATP. The amount of ATP could then be measured in a first reading A.

Next, 100 µl of the substrate was added to each sample and incubated for 10 minutes. This allowed the generation of additional ATP from ADP if mycoplasma was present. In a second reading B, the amount of ATP was measured again after adding the substrate. The ratio B/A was determined and the table below shows when this result is positive, negative or borderline for mycoplasma contamination.

Table 3.3: Required results for the B/A ratio

B/A ratio		
Negative	**Positive**	**Borderline**
< 0.9	> 1.2	0.9-1.2

If the result fell between the range of 0.9-1.2, the cell culture needed to be re-tested for mycoplasma.

3.2.7. Intracellular calcium measurements

The Calcium 5 kit from Molecular Devices, together with a Flexstation 3 Microplate Reader from the same company, was utilized to measure intracellular calcium levels. HEK-293 5-HT2 cells were taken from a 75 cm^2 flask that had reached 80-90% confluence and resuspended in L-15 medium containing 1% FBS and 1% penicillin/streptomycin. The cells were then plated in 96-well Costar black-walled plates obtained from Corning in Castle Hill, Australia. 1 µg/ml tetracycline was used to induce 5-HT2 expression in the cells just before they were plated. The cells were kept in a humidified room at 37°C overnight in ambient CO_2 humidified air.

The next day, the Calcium 5 dye (Molecular Devices) was reconstituted with Hank's balanced salt solution (HBSS) and 90 µl was added to each well for an initial volume of 180 µl per well. 100 µl of probenecid per 20 ml of the reconstituted Calcium 5 dye was added. The addition of probenecid to the dye serves to block organic anion transporters (OATs), which would otherwise remove or transport the dye out of the intracellular environment. By doing so, probenecid helps to maintain a higher concentration of the Calcium 5 dye within the cells. This enables more accurate and sensitive measurements of intracellular calcium levels, which leads to stronger fluorescence signals and improved detection of any fluctuations in intracellular calcium concentrations. After, the plate was incubated at 37°C for at least 1 hour. The FlexStation 3 measured the fluorescence at intervals of 2 seconds. Unless stated otherwise, all assays were conducted at 37°C.

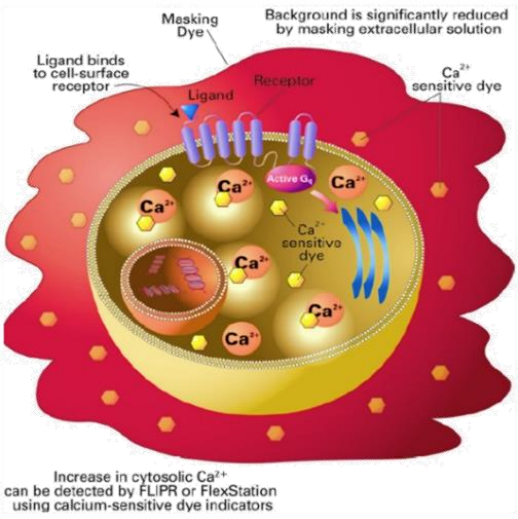


Figure 3.2: Principle of the Calcium 5 assay. When a ligand binds to the receptor located on the cell membrane, it triggers a response in which calcium levels increase from the ER. This allows calcium to bind to a calcium-sensitive fluorescent dye. To ensure that the dye remains within the cell, probenecid is introduced alongside the dye. (Diagram from Molecular Devices)

3.2.8. Serotonin experiments

The FLIPR Calcium 5 assay was used to test serotonin on the different cell lines. This was done to evaluate if the cells are actually working, which means if they express the 5-HT2 receptor and cause elevation of Calcium as a response to interaction of serotonin with the 5HT2-receptor. Tetracycline-controlled transcriptional activation was employed to regulate gene expression in the cells, so we tested the cells in the presence and in the absence of tetracycline. Furthermore, we performed the tests with dialyzed serum and with non-dialyzed serum, to make the decision whether to use it for the following experiments with the synthetic drugs or not.

Cell signalling was assessed by measuring excitation at a wavelength of 485 nm and emission at 525 nm simultaneously. The readings were taken for a duration of 360 seconds for each column of the plate. Because the responses to serotonin were achieved rapidly, we changed to 180 seconds for the experiments with the drugs. To establish a reference standard, concentration response curves (CRCs) were created for serotonin stimulation of calcium accumulation. This CRCs were performed for each experimental replicate and can be seen in Figure 4.2. Unless specified otherwise, the data presented in the study represent mean ± SEM obtained from at least three separate experiments, with each experiment conducted in duplicate.

3.2.9. PSYLO synthetic drugs experiments

We employed The FLIPR Calcium 5 Assay to evaluate the effect of five synthetic drugs developed by PSYLO on calcium levels in HEK-293 5-HT2 cells. The drugs, diluted from stock solutions in HBSS, were loaded into a 96 well source plate and introduced to the system after a 60-second initial recording to establish the baseline. The drugs were added in volumes of 20 µl with an initial volume of 100 µl per well.

We didn’t use the FLIPR Membrane Potential Assay, which was also mentioned as a possible assay in the introduction. The rationale behind this is that this research is focused on the 5-HT2 receptor family, specifically the 5-HT2A, 5-HT2B and 5-HT2C receptors, all of which are coupled to the $G_{q/11}$ protein. As described in the introduction, activating these receptors causes an increase in intracellular calcium which can be

measured using the FLIPR Calcium 5 assay. The table below shows information on the drugs, including molecular weight (MW). Drug structures can be found in the appendix.

Table 3.4. Compound list

No.	Vial code	MW (g/mol)	Amount (mg)	Form
1	P-0049-001	232.327	5	Powder
2	P-0050-002	326.663	5	Powder
3	P-1569-003	348.399	5	Powder
4	P-1816-003	304.390	5	Powder
5	P-1818-004	350.415	5	Powder

Calculations were performed to resuspend the drugs in water and can be found in Table 3.5. All of the drugs used are soluble in water, but compound 1 exhibited significantly lower solubility compared to the other drugs. As a result, the stock concentration had to be adjusted to 1 mM. This factor must be considered when preparing the different concentrations of this drug for an experiment. All the PSYLO synthetic drugs were stored in aliquots of 10 mmol L^{-1} in -30°C until needed.

Table 3.5: Calculation of the amount of water needed to resuspend the drugs

Compound	Stock concentration (mM)	MW (g/mol)	Amount (mg)	n (mmol)	Water (ml)
1	1	232.327	5	0.0215	21.5
2	10	326.663	5	0.0153	1.53
3	10	348.399	5	0.0144	1.44
4	10	304.390	5	0.0164	1.64
5	10	350.415	5	0.0143	1.43

Cell signalling was evaluated by concurrently measuring excitation at 485 nm and emission at 525 nm. The readings were recorded for 180 seconds per column on the plate. CRCs were generated to assess the drugs' impact on calcium accumulation. Each experimental replicate underwent CRC analysis, and the corresponding results are displayed in Figure 4.3. Unless explicitly mentioned, the data presented corresponds to the mean ± SEM obtained from a minimum of five distinct experiments, with each experiment conducted in duplicate

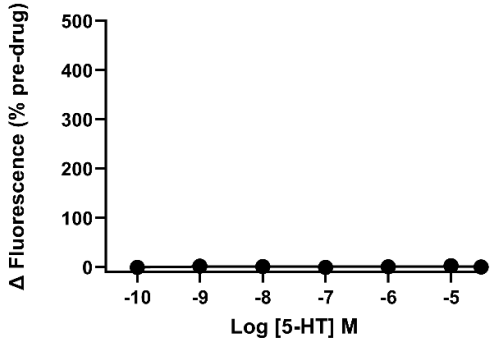
4. RESULTS

The data from all experiments was analysed using GraphPad Prism software (Version 9.5.0 (730), GraphPad Software, Inc., La Jolla, CA, USA). The effect of drugs in the FLIPR Calcium 5 assay was measured as a percentage change relative to the baseline, which was calculated by averaging the values recorded for 60 seconds right before adding the drug. Any changes caused by the solvent alone were taken into account by subtracting the corresponding blank (HBSS) values prior to normalizing the data. To determine the concentration at which the drugs had half of their maximum effect (EC50 values), the concentration-response data were fitted to the four-parameter logistic Hill equation.

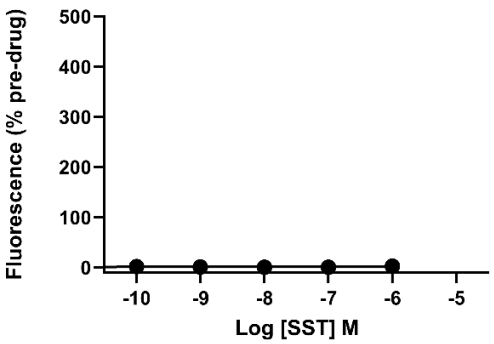
4.1. WILD-TYPE HEK-293 CELLS

Below are the concentration-response curves obtained after testing 5-HT, SST and PAR-1 peptide on WT HEK-293 cells. The curves for both 5-HT and SST are flat, indicating no significant response, whereas the curve for PAR-1 does show a response, demonstrating that the Calcium 5 assay works in these cells and experimental conditions.

(A)



(B)



(C)

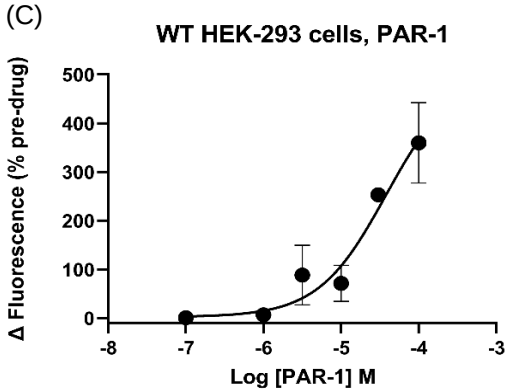


Figure 4.1: Concentration-response curves were created to examine the activation of WT HEK-293 cells by 5-HT, SST and PAR-1. Readings were recorded for a duration of up to 300 seconds, with baseline readings taken for the initial 60 seconds. The addition of increasing concentrations of 5-HT and SST to the WT HEK-293 cells resulted in no response. The addition of rising concentrations of PAR-1 has led to a concentration-dependent increase in fluorescent signal as shown. Data were normalized to the pre-drug baseline and plotted as mean ± SEM for at least three independent experiments performed in duplicate.

4.2. MYCOPLASMA TESTING

To evaluate if the different cells were contaminated with mycoplasma, readings A and B were used to calculate ratios who are presented in Table 4.1. The ratios were measured for 5-HT2A/B/C expressed HEK-293 cells, an empty vector (EV), WT HEK-293 cells and a positive control.

Table 4.1: B/A ratios

Well	Content	Raw Data A	Raw Data B	Ratio B/A
C04	HEK FlpIn T-Rex 5-HT2A P4	223	100	0.45
C05	HEK FlpIn T-Rex 5-HT2B P4	244	138	0.57
C06	HEK FlpIn T-Rex 5-HT2C P4	266	144	0.54
C07	HEK FlpIn T-Rex EV P4	261	112	0.43
C08	HEK FlpIn T-Rex WT P36	170	160	0.94
E09	Positive Control	264	18721	70.91

4.3. SEROTONIN EXPERIMENTS

Serotonin concentration-response curves were made across all the different cell lines, e.g. 5-HT2A/B/C and the EV. Experiments were performed in different conditions: with and without tetracycline and with dialyzed or with non-dialyzed serum. The curves are shown in the figures below.

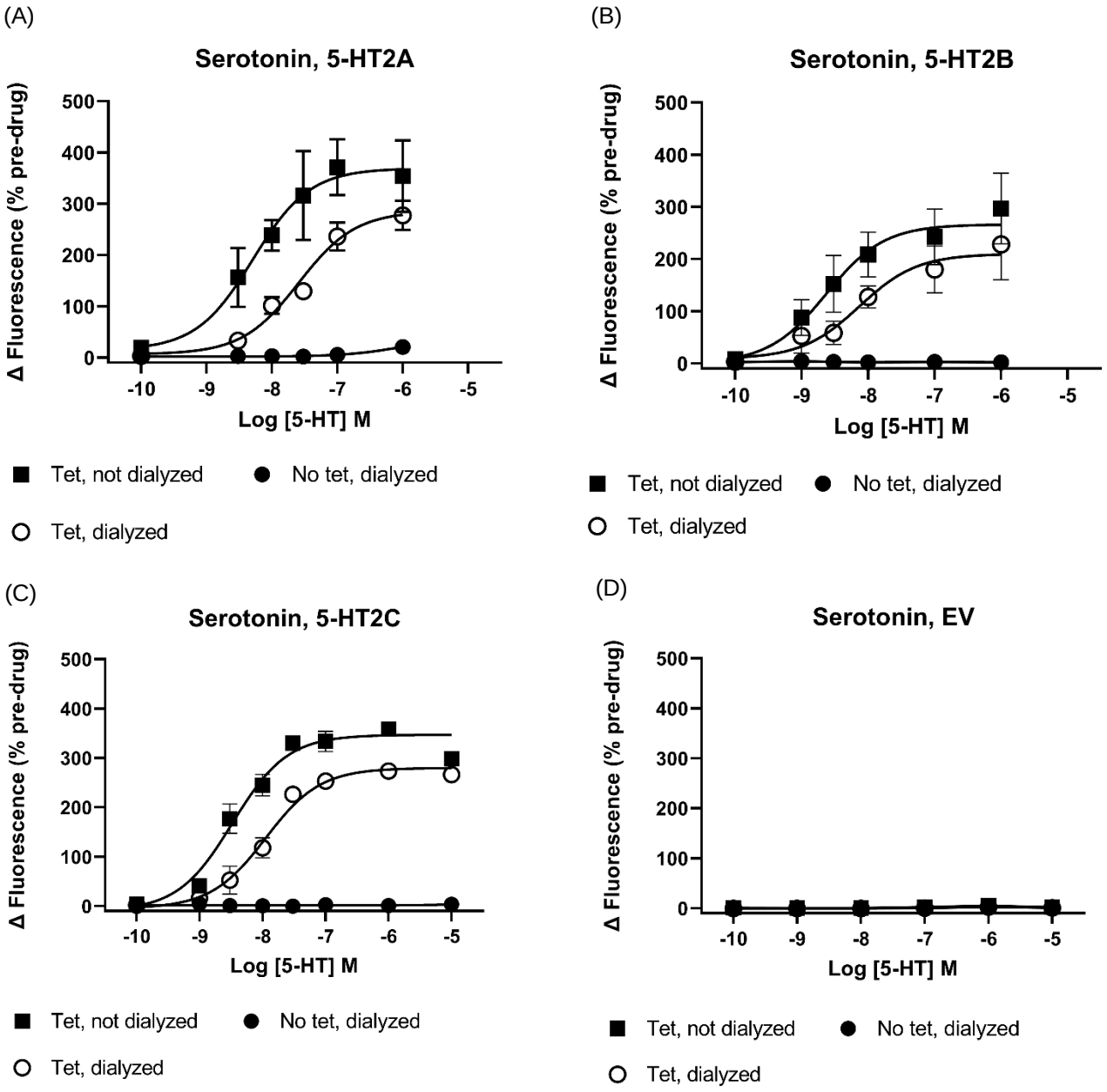


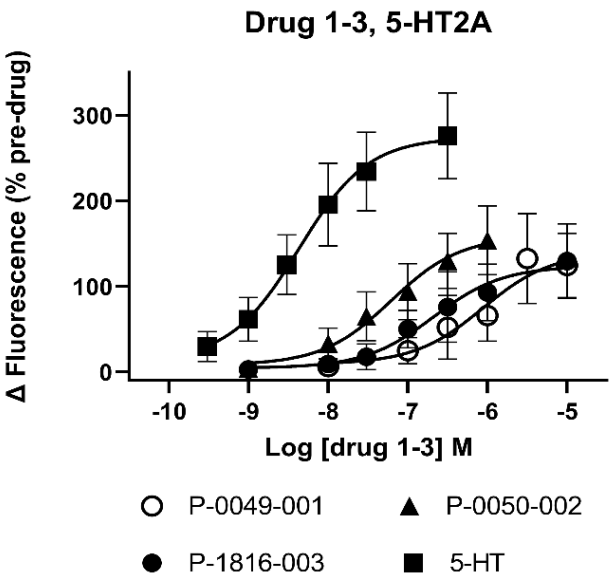
Figure 4.2: Concentration-response curves were created to examine the activation of Flp-In^{TM} T-REx^{TM} HEK-293 5-HT2 receptor cells by serotonin. These experiments were conducted both in the presence and absence of tetracycline, using both dialyzed and non-dialyzed serum. Readings were recorded for a duration of 360 seconds, with baseline readings taken for the initial 60 seconds. The addition of increasing concentrations of serotonin to the Flp-In^{TM} T-REx^{TM} HEK-293 5-HT2 receptor cells resulted in a concentration-dependent increase in

fluorescent signal as shown. Data were normalized to the pre-drug baseline and plotted as mean ± SEM for at least three independent experiments performed in duplicate. The following cell types were employed: (a) Flp-In^{TM} T-REx^{TM} HEK-293 5-HT2A receptor cells (b) Flp-In^{TM} T-REx^{TM} HEK-293 5-HT2B receptor cells (c) Flp-In^{TM} T-REx^{TM} HEK-293 5-HT2C receptor cells (d) HEK-293 EV cells.

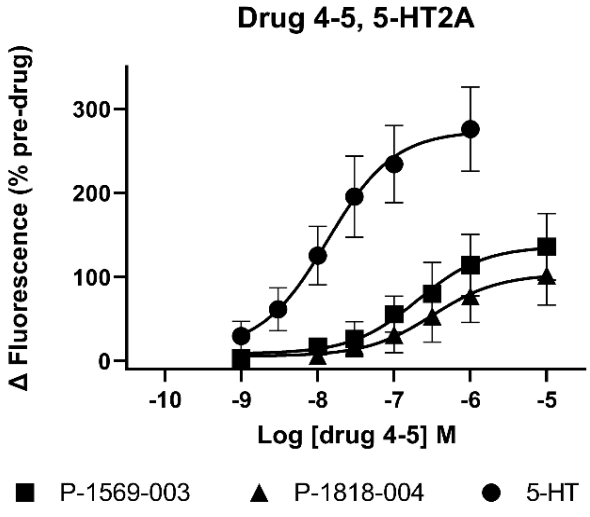
4.4. PSYLO SYNTHETIC DRUGS EXPERIMENTS

Pharmacologists and neuroscientists have been interested in understanding the mechanism of action of hallucinogens for many years. In this study, we examined the ability of five PSYLO synthetic drugs, that might have psychedelic potential, to modify 5-HT2 receptor $G_{q/11}$-mediated signalling. We immersed ourselves in the investigation of the ability of these drugs to activate 5-HT2 receptors via this pathway. CRCs of each of the five drugs, obtained with the FLIPR Calcium 5 assay, are shown below.

(A)



(B)



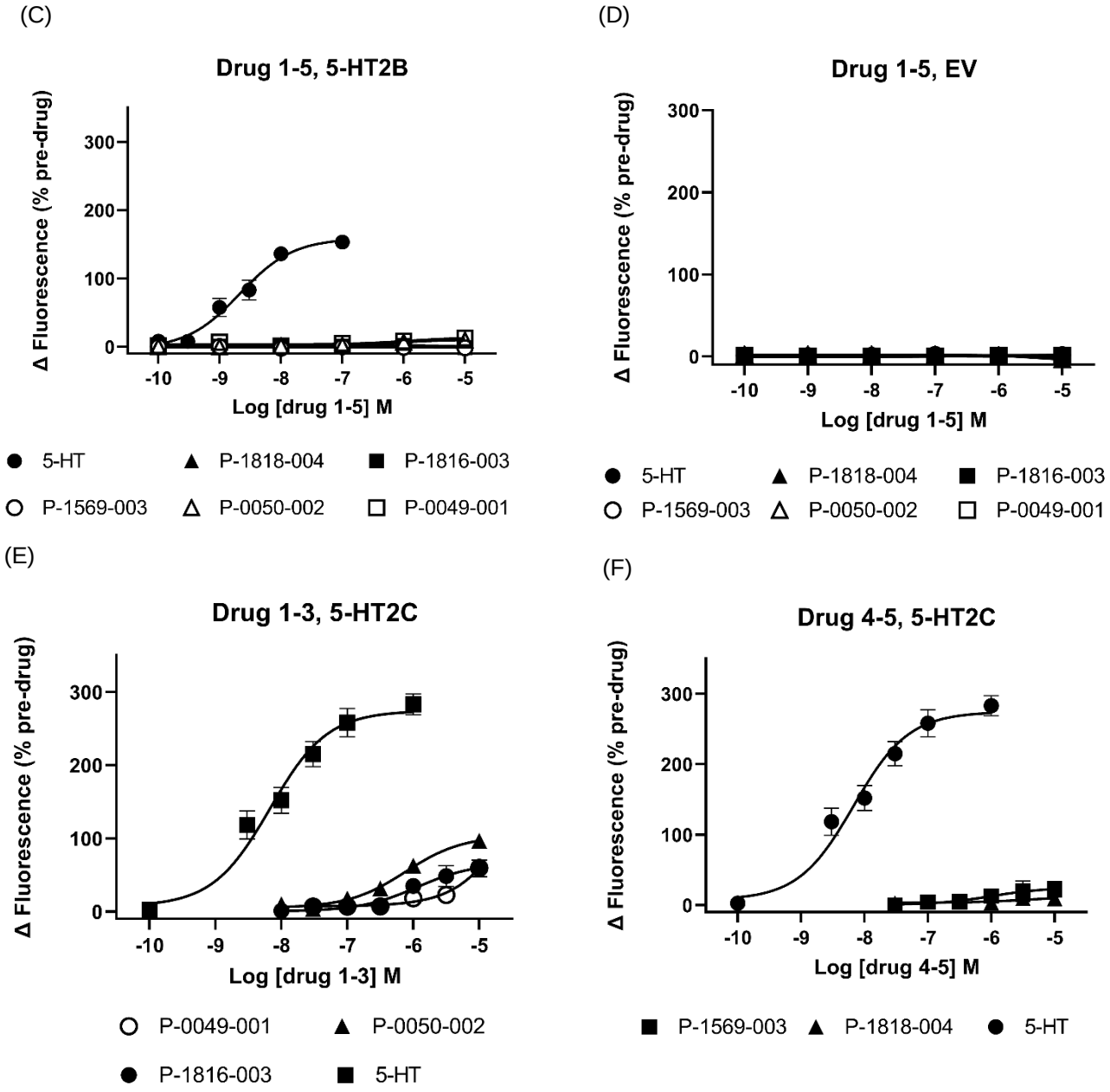


Figure 4.3: Concentration-response curves were created to examine the activation of HEK-293 5-HT2 receptor cells by the PSYLO synthetic drugs. Readings were recorded for a duration of 180 seconds, with baseline readings taken for the initial 60 seconds. Data were normalized to the pre-drug baseline and plotted as mean ± SEM for at least five independent experiments performed in duplicate. The following cell types were employed: (a, b) Flp-In^{TM} T-REx^{TM} HEK-293 5-HT2A

receptor cells (c) Flp-In^{TM} T-REx^{TM} HEK-293 5-HT2B receptor cells (d) HEK-293 EV cells (e,f) Flp-In^{TM} T-REx^{TM} HEK-293 5-HT2C receptor cells.

To provide visual evidence of the experimental data and to support our reported findings, examples of raw traces of the responses for both the 5-HT2A, 5-HT2B, 5-HT2C receptor are presented in Figure 4.4. This is a representative example of 5 experiments. As confirmed by Figure 4.4 (D), the EV cells could still respond with an elevation of calcium, but they didn't respond to any of the 5-HT agonists.

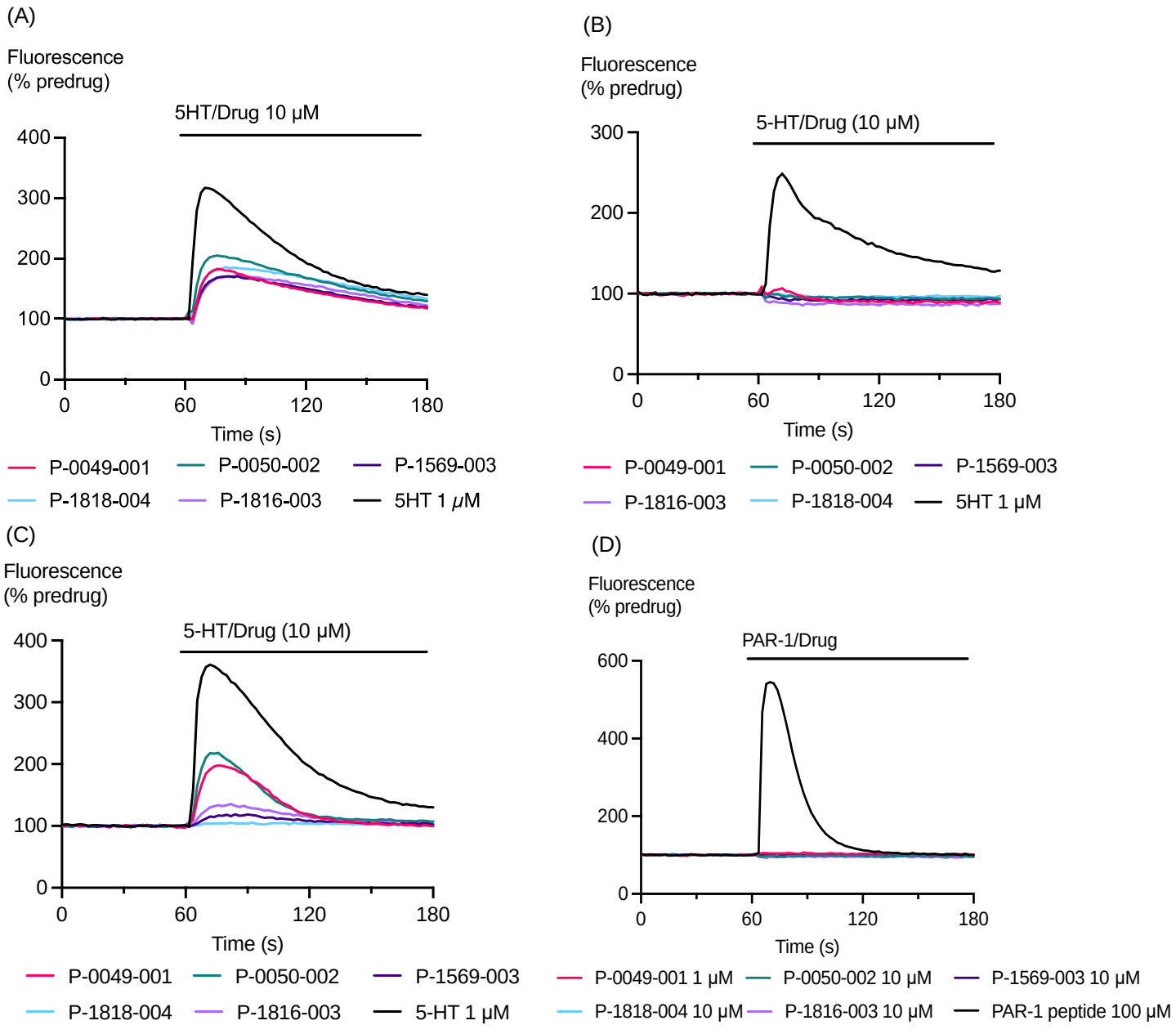


Figure 4.4: The provided data displays the changes in the fluorescent signal of the calcium 5 dye (measured in raw fluorescence units, RFU) within HEK-293 cells when a PSYLO synthetic drug is added. The drug was introduced after a duration of 60 seconds and the traces shown represent the outcomes of at least 5 experiments.

4.5. ONE-WAY ANOVA TEST

A standard one-way ANOVA test is employed to determine the log(EC50) and Emax of the five PSYLO synthetic drugs tested on the 5-HT2A/B/C receptors. We performed a one-way ANOVA for multiple comparisons between serotonin and each of the five drugs to see if any of them act different. A threshold of $p < 0.05$ was used to establish statistical significance. The obtained values for log(EC50) and Emax can be found in Table 4.2.

Table 4.2: Log(EC50) and Emax values obtained with a one-way ANOVA test for drug 1-5 tested on the 5-HT2A/B/C receptors

	5-HT2A		5-HT2B	5-HT2C	
Compound	**Log (EC50)**	**Emax (% pre-drug)**	**Emax (% pre-drug)**	**Log (EC50)**	**Emax (% pre-drug)**
P-0049-001	-5.71 **** ± 0.36	153.31 ± 37.31	13.27 **** ± 1.26	-4.86 * ± 0.23	149.74 ** ± 45.95
P-0050-002	-7.19 ± 0.19	129.78 ± 19.98	8.58 **** ± 8.89	-6.20 ± 0.18	110.08 *** ± 7.44
P-1569-003	-6.61 ** ± 0.17	106.06 * ± 25.56	-0.93 **** ± 0.91	-6.05 ± 0.033	30.55 **** ± 13.48
P-1816-003	-6.51 ** ± 0.27	102.76 * ± 25.19	0.51 **** ± 1.33	-6.16 ± 0.20	69.01 **** ± 18.59
P-1818-004	-6.28 *** ± 0.26	84.38 * ± 26.71	1.82 **** ± 1.46	-5.65 ± 1.75	15.40 **** ± 5.11
5-HT	-7.85 ± 0.12	242.98 ± 43.85	160.67 ± 3.87	-8.24 ± 0.21	273.64 **** ± 19.10

*** Significantly different to serotonin: p < 0.05**

The Emax values for P-0049-001 and P-0050-002 on the 5-HT2A receptor are not statistically different from serotonin. Similarly, P-0050-002 exhibits a log(EC50) value that is not significantly different from serotonin. However, the CRCs for all the five drugs on the 5-HT2B receptor appear to be flat, indicating a significant difference in Emax compared to serotonin. For the 5-HT2C receptor, every drug shows significantly lower p-values than 0.05 for Emax, indicating a significant difference from serotonin. The p-values calculated for their log(EC50) values, except for P-0049-001, are above 0.05.

5. DISCUSSION

5.1. WILD-TYPE HEK-293 CELLS

Both 5-HT, SST and PAR-1 were tested on WT HEK-293 cells to prove that these cells do not express the 5-HT receptor, but could be used for the Ca-mobilization assay. No response was expected for 5-HT, as the cells may not express the 5-HT receptors. SST binds only to receptors coupled to the Gi/o protein in all cells tested. Due to this association, there should be no response in the calcium assay as Gi/o proteins do not stimulate an increase in calcium levels, unlike $G_{q/11}$ proteins. The response seen for PAR-1 was used to confirm the functionality of the FLIPR Calcium 5 assay. The concentration response curves shown in Figure 4.1 confirmed our expectations.

5.2. MYCOPLASMA TESTING

While testing for mycoplasma contamination, all the B/A ratios that we observed, except for the WT HEK-293 cells, were below 0.9, which confirms the absence of mycoplasma contamination as expected. For the WT cells, a B/A ratio was obtained that was slightly higher than 0.9 but still below 1.2. This indicates a borderline result, which means the cell culture should have been re-tested. However, all the cells (5-HT2A/B/C) were derived from these original cells, so if all the other cells tested negative, it was likely that the WT cells were also negative. Therefore, there was no significant concern about the borderline result.

5.3. SEROTONIN EXPERIMENTS

The 5-HT2A/B/C cell lines responded to the different serotonin concentrations, while the empty vector did not. During these experiments, it was crucial to employ an EV. Testing serotonin on an EV enables us to confirm that the response observed in the curves of the 5-HT2A, 5-HT2B and 5-HT2C receptors is exclusively due to the presence of the GOI and not caused by any other factor. The EV serves as an important reference in order to draw conclusions regarding the function and influence of the gene that encodes for the 5-HT2 receptors.

If tetracycline-controlled transcriptional activation was employed and no tetracycline was introduced, the concentration-response curve should be flat. This is the case for the cells expressing the 5-HT2B receptor and the 5-HT2C receptor, but not for those expressing the 5-HT2A receptor. A slight response can be seen for the latter, indicating a minor leak in the system. This suggests that a tiny amount of 5-HT receptors is still expressed, even in the absence of tetracycline. As this leak is very small, we are still able to induce these cells with tetracycline. Furthermore, it can be noticed that the 5-HT2B receptor has a lower maximum response than both the 5-HT2A and the 5-HT2C receptor, which suggests there being lower expression levels of 5-HT2B receptors.

The obtained CRCs were utilized to compare the use of dialyzed and non-dialyzed serum. An intriguing observation from the graphs is that the response obtained from non-dialyzed serum is higher than from dialyzed serum, both for the 5-HT2A, 5-HT2B and 5-HT2C receptor, which is not what we expected to see. This suggests that there may be a substance present in non-dialyzed serum that potentiates calcium signalling. It is believed that a significant amount of serum may contain 5-HT, although we have not measured it in our lots. If the cells were cultured in a medium containing 5-HT, it was expected that the receptors would become desensitized, which means that there would be no response or a smaller response of 5-HT seen in the CRCs. However, based on the observed CRCs, this assumption is evidently incorrect as a significant response is observed. This suggests that the non-dialyzed serum only contains a limited amount of 5-HT and potentially other components in the serum allow the receptor to be activated more effectively or expressed at a higher level. Surprisingly, removing any 5-HT present in the medium did not potentiate the response. In fact, the response was smaller in all cell lines.

However, although the current batch of non-dialyzed serum appears to lack 5-HT, it is important to note that we will not be utilizing this particular batch indefinitely. The subsequent batch of serum could potentially contain 5-HT, leading to different outcomes in our experiments. Conversely, the dialyzed serum, being free of 5-HT, ensures consistent results as it will always be devoid of this variable. Therefore, we continued using dialyzed serum for plating during our experiments with the PSYLO synthetic drugs.

5.4. PSYLO SYNTHETIC DRUGS EXPERIMENTS

5.4.1. Efficacy and potency

The responses of the drugs on both the 5-HT2A and 5-HT2C receptors are not particularly strong, especially in the case of the 5-HT2C receptor, where some drugs such as P-1569-003 and P-1818-004 may not reach high enough efficacy to have any potential. The low responses places these drugs in the category of low efficacy drugs. However, while low efficacy drugs may not be as potent or effective as high efficacy drugs, they have their own unique advantages. These drugs offer the potential to achieve a biological effect while minimizing the risk of toxicity or other adverse effects.

An example of a low efficacy drug is tetrahydrocannabinol (THC), the main psychoactive component in cannabis. THC acts as a partial agonist of cannabinoid receptors. One notable advantage of THC is its relatively safe profile. Despite its widespread use and centuries-long medicinal use, there are no reported cases of fatal overdose solely due to cannabis consumption. Furthermore, low efficacy drugs may be better tolerated by certain patient populations who are more sensitive to the effect of the drugs. This stands in contrast to synthetic cannabinoid receptor agonists (SCRAs), which are associated with a wide range of toxic effects, including panic attacks, severe agitation, cardiovascular toxicity, renal effects such as kidney injury and even death. (61)

5.4.2. Selectivity

It’s interesting to note that some of our tested drugs, like P-1818-004 and P-1569-003, interact with the 5-HT2A receptor but do not activate the 5-HT2C or 5-HT2B receptors. On the other hand, other drugs (P-0049-001, P-0050-002 and P-1816-003) activate both the 5-HT2A and 5-HT2C receptors but also not the 5-HT2B receptor. This finding is not surprising since the 5-HT2A and 5-HT2C receptors share a high degree of amino acid sequence similarity, particularly in their seven transmembrane domains, which are critical for receptor function. Due to this structural similarity, the binding affinities of these receptors are closely correlated. (28)

Analysis of the CRCs leads us to the conclusion that the drugs tested do not have an impact up to 1 µM on the 5-HT2B receptor. We can suggest that there is no indication of these drugs exhibiting agonistic effect on this receptor, indicating their selectivity as agonists. However, this does not rule out the possibility of these drugs interacting with the receptor as antagonists. Additionally, the selectivity is in contrast to serotonin itself, which activates all subtypes of 5-HT2 receptors.

5.4.3. Therapeutic importance

5.4.2.1. Activation of 5-HT2A

Recent research highlights the significance of 5-HT2A receptor agonists in the treatment of depression, particularly in cases where patients are resistant to commonly prescribed therapies. 5-HT2A agonists have been relatively understudied but hold promise as agents for new depression treatments. It is proposed that agonists targeting the 5-HT2A receptor can yield enduring therapeutic effects in depressive diseases by promoting neuronal growth in particular brain regions like the prefrontal cortex. The antidepressant effects of 5-HT2A agonists are thought to stem from their ability to enhance neural plasticity through receptor activation. However, both agonists and antagonists of the 5-HT2A receptor are considered to own favourable properties that could beneficial in the treatment of mood disorders. (62). Moreover, the inhibition of the 5-HT2A receptor is a significant pharmacological characteristic found in numerous antipsychotic medications approved by the FDA for treating schizophrenia and bipolar disorder. (63)

5.4.2.2. No activation of 5-HT2B

As previously mentioned in the introduction, 5-HT2A receptors are found in various brain regions, which are involved in mood regulation and cognition. (36) Similarly, the 5-HT2C receptor, commonly associated with the choroid plexus, is strategically located throughout the CNS and plays a role in mediating many of the central effects of serotonin, including mood regulation. (64) On the other hand, the expression of the 5-HT2B receptor is highly diverse, indicating its involvement in a wide range of physiological and pathophysiological processes. However, because these 5-HT2B receptors are also present in organs such as heart, intestine and lung, their activation can

lead to unwanted side effects like cardiac valvular abnormalities, gastrointestinal issues and pulmonary hypertension. Selective drugs that do not activate the 5-HT2B receptor can help minimize the risk of these peripheral side effects. (28)

5.4.2.3. Activation of 5-HT2C

Some of the PSYLO synthetic drugs tested have shown agonistic effects on the 5-HT2C receptor. The use of 5-HT2C agonists has been proposed as a potential treatment for obesity, substance use disorders (SUDs) and impulse control disorders. On the other hand, antagonists of this receptor may have therapeutic value in addressing anxiety, depression and schizophrenia. Chronic substance use disorders often face difficulties with relapse, making it crucial to find medications that can enhance impulse control. This would be a ground-breaking advancement for individuals suffering from these disorders. The serotonin 5-HT2C receptor is a promising target for such medications, as it is involved in both the disinhibited behaviours associated with SUDs and those linked to obesity. By focusing on this receptor, it may be possible to address both issues and offer a fresh approach to treatment. However, our findings indicate that the PSYLO drugs are not exclusively selective for the 5-HT2C receptors. The simultaneous activation of 5-HT2A receptors can contribute to hallucinogenic effects of the drugs. The high similarity between the orthosteric sites of these 5-HT2C receptors present difficulties in selectively targeting the 5-HT2C receptor. (65)

Nonetheless, the question is if the drugs tested in this study can actually be useful in therapy. This study has some limitations, discussed in the paragraph below, which means more research will need to be done to properly answer this question. However, these drugs are not intended for regular daily use. Instead, they would typically be administered over a limited number of sessions. As a result, the therapeutic importance of a drug-receptor interaction can vary greatly between a medication taken daily for depression and a psychedelic substance administered only a few times. It’s worth noting that psychedelics will not be prescribed for feelings of sadness; rather, they would be used as a therapy for individuals with treatment-resistant depression, which is likely a distinct condition with a different underlying neurobiological basis.

5.5. LIMITATIONS

The study reveals that all five drugs examined exhibit agonistic effects on the 5-HT2A receptors. However, it's important to consider the possibility that other receptors may also play a significant role. We have already conducted tests on the 5-HT2B and 5-HT2C receptors, but there might be other receptors involved. For example, LSD affects additional receptors, such as dopamine and α-adrenergic receptors. Therefore, further investigation is required to have a better understanding about the receptor pharmacology of the drugs.

One area that remains unclear is whether these drugs must be high efficacy agonists or not. Psychedelic substances such as psilocybin have a long history of use. Generally, psilocybin is considered to have the most favourable safety profile among psychedelic drugs. The fact that psilocybin, known for its relatively low efficacy, has been used for centuries with a positive safety record suggest that achieving high efficacy may not be an absolute requirement for reaching positive effects. Nonetheless, more extensive research is needed before making conclusions regarding the therapeutic potential of these drugs. (66)

One major limitation about this study is that it only investigated one specific signalling pathway, while there are several others that should be considered in future research using these drugs. Apart from the well-known signalling pathway involving PLC and IP3, the 5-HT2 receptor can activate various other mechanisms. For instance, it has been observed that the 5-HT2A receptor can stimulate phospholipase A2, leading to an increased production of arachidonic acid derived from phospholipids. This arachidonic acid can undergo further metabolism to generate prostaglandins, leukotrienes and other lipid mediators. Additionally, the receptor can interact with the extracellular signal-regulated kinase 1 and 2 (ERK1/2) pathway. ERK1/2 translocates to the nucleus upon activation, where it phosphorylates transcription factors, enabling them to regulate the expression of specific target genes. Further research is needed to also investigate these other signalling pathways. (28)

6. CONCLUSION

Although antidepressant medications and cognitive behavioural therapy have proven beneficial for many patients, a significant proportion of individuals do not respond positively to the commonly prescribed treatments. The PSYLO drugs tested in this study could potentially serve as safe and effective substitutes for conventional medications used in the treatment of mood and anxiety disorders.

The focus in the study was to evaluate how the 5-HT2A, 5-HT2B and 5-HT2C cell lines respond to these PSYLO synthetic drugs, which are maybe psychedelic. The study confirmed that the WT HEK-293 cells do not express the 5-HT receptor. Moreover, no mycoplasma was found in the cell cultures, which means the cells were useful for our further investigations. One interesting finding was that the cell lines exhibited a higher response to serotonin when non-dialyzed L-15 was used compared to dialyzed L-15. Removing serotonin from the non-dialyzed serum leaded to a lower response, contrary to our expectations. Additionally, all of the drugs tested in the study activated the 5-HT2A receptor and three of them also activated 5-HT2C, but there was no activation of the 5-HT2B receptor. This suggests that these drugs exhibit selective agonist activation of 5-HT2A and 5-HT2C, but not of 5-HT2B. Furthermore, the drugs were found to have low efficacy compared to serotonin. However, low efficacy does not necessarily indicate that these drugs are not useful. Low-efficacy drugs are considered safe and can still have a biological effect without reaching any toxic levels.

While the examined drugs showed agonistic effects on 5-HT2A and 5-HT2C receptors, it is important to consider the possibility of other receptors playing significant roles in their mechanism of action. Further investigation is needed to explore the involvement of these additional receptors. Moreover, the study focused solely on the $G_{q/11}$ signalling pathway, while the 5-HT2 receptor can activate various other pathways that should be taken into account in future research.

The kind of drugs examined in this study are typically administered over a limited number of sessions and are not intended for daily use. Therefore, the therapeutic significance of drug-receptor interactions may differ from that of daily medications for depression. These

drugs would not be prescribed for general feelings of sadness but rather considered for individuals with treatment-resistant depression.

Overall, this study provides precious insights regarding the response of 5-HT2A/B/C cell lines to 5-HT and the PSYLO synthetic drugs. Nevertheless, additional extensive research is required to acquire a complete comprehension of the therapeutic possibilities associated with these drugs. Additional research should delve into their selectivity, efficacy, receptor pharmacology and activation of other signalling pathways. By continuing to investigate these fields, researchers can contribute to the advancement of more effective depression treatments, ultimately improving outcomes for individuals who do not respond well to existing therapies.

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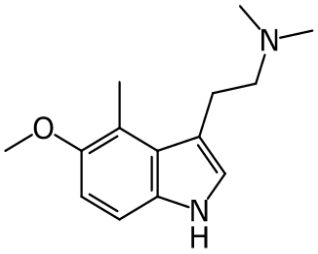
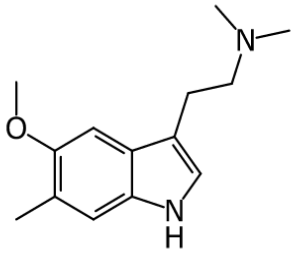
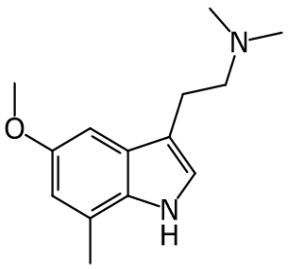
APPENDIX

1. CHEMICAL STRUCTURES OF THE DRUGS

Molecular name	Structure
P-0049-001	
P-0050-002	
P-1569-003	

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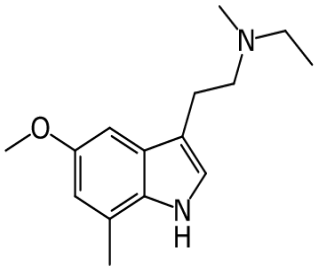

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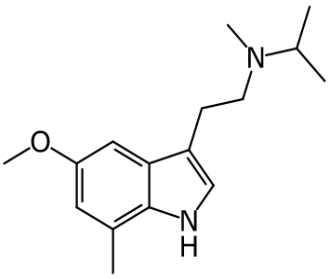

2. ANOVA TEST RESULTS

2.1. Anova results for 5-HT2A Emax

Table Analyzed	5-HT2A top
Data sets analyzed	A-F
ANOVA summary	
F	3.469
P value	0.0214
P value summary	*
Significant diff. among means (P < 0.05)?	Yes
R squared	0.4772
Brown-Forsythe test	
F (DFn, DFd)	0.5873 (5, 19)
P value	0.7097
P value summary	ns
Are SDs significantly different (P < 0.05)?	No
Bartlett's test	
Bartlett's statistic (corrected)	3.382
P value	0.6413
P value summary	ns
Are SDs significantly different (P < 0.05)?	No

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	76662	5	15332	F (5, 19) = 3.469	P=0.0214
Residual (within columns)	83985	19	4420		

Total	160647	24			
Data summary					
Number of treatments (columns)	6				
Number of values (total)	25				

2.2. Anova results for 5-HT2A EC50

Table Analyzed	5-HT2A EC50				
Data sets analyzed	A-F				
ANOVA summary					
F	10.44				
P value	<0.0001				
P value summary	****				
Significant diff. among means (P < 0.05)?	Yes				
R squared	0.6762				
Brown-Forsythe test					
F (DFn, DFd)	0.5917 (5, 25)				
P value	0.7064				
P value summary	ns				
Are SDs significantly different (P < 0.05)?	No				
Bartlett's test					
Bartlett's statistic (corrected)	5.212				
P value	0.3906				
P value summary	ns				
Are SDs significantly different (P < 0.05)?	No				
ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	15.20	5	3.039	F (5, 25) = 10.44	P<0.0001
Residual (within columns)	7.275	25	0.2910		
Total	22.47	30			
Data summary					
Number of treatments (columns)	6				
Number of values (total)	31				

2.3. Multiple comparisons for 5-HT2A Emax

Number of families	1						
Number of comparisons per family	5						
Alpha	0.05						
Šídák's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value	F-?	
5-HT vs. Drug 1	89.67	-37.51 to 216.9	No	ns	0.2613	A	Drug 1
5-HT vs. Drug 2	113.2	-13.98 to 240.4	No	ns	0.0963	B	Drug 2
5-HT vs. Drug 3	136.9	9.732 to 264.1	Yes	*	0.0311	C	Drug 3

5-HT vs. Drug 4	140.2	13.04 to 267.4	Yes	*	0.0264	D	Drug 4	
5-HT vs. Drug 5	158.6	31.42 to 285.8	Yes	*	0.0105	E	Drug 5	

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	t	DF
5-HT vs. Drug 1	243.0	153.3	89.67	44.60	5	4	2.011	19
5-HT vs. Drug 2	243.0	129.8	113.2	44.60	5	4	2.538	19
5-HT vs. Drug 3	243.0	106.1	136.9	44.60	5	4	3.070	19
5-HT vs. Drug 4	243.0	102.8	140.2	44.60	5	4	3.144	19
5-HT vs. Drug 5	243.0	84.38	158.6	44.60	5	4	3.556	19

2.4. Multiple comparisons for 5-HT2A EC50

Number of families	1
Number of comparisons per family	5
Alpha	0.05

Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value	F-?	
Column F vs. Column A	-2.149	-3.031 to -1.266	Yes	****	<0.0001	A	Column A
Column F vs. Column B	-0.6673	-1.550 to 0.2154	No	ns	0.1888	B	Column B
Column F vs. Column C	-1.251	-2.134 to -0.3682	Yes	**	0.0035	C	Column C
Column F vs. Column D	-1.343	-2.226 to -0.4606	Yes	**	0.0017	D	Column D
Column F vs. Column E	-1.572	-2.455 to -0.6892	Yes	***	0.0003	E	Column E

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	q	DF
Column F vs. Column A	-7.859	-5.710	-2.149	0.3267	6	5	6.578	25
Column F vs. Column B	-7.859	-7.191	-0.6673	0.3267	6	5	2.043	25
Column F vs. Column C	-7.859	-6.608	-1.251	0.3267	6	5	3.829	25
Column F vs. Column D	-7.859	-6.515	-1.343	0.3267	6	5	4.112	25
Column F vs. Column E	-7.859	-6.287	-1.572	0.3267	6	5	4.812	25

2.5. Anova results for 5-HT2B Emax

Table Analyzed	5-HT2B top
Data sets analyzed	A-F

ANOVA summary	
F	431.9
P value	<0.0001
P value summary	****
Significant diff. among means (P < 0.05)?	Yes
R squared	0.9954
Brown-Forsythe test	
F (DFn, DFd)	2.878 (5, 10)
P value	0.0728
P value summary	ns
Are SDs significantly different (P < 0.05)?	No
Bartlett's test	
Bartlett's statistic (corrected)	
P value	
P value summary	
Are SDs significantly different (P < 0.05)?	

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	60405	5	12081	F (5, 10) = 431.9	P<0.0001
Residual (within columns)	279.7	10	27.97		
Total	60684	15			

Data summary	
Number of treatments (columns)	6
Number of values (total)	16

2.6. Anova results for 5-HT2B EC50

Table Analyzed	5-HT2B EC50
Data sets analyzed	A-F
ANOVA summary	
F	0.8574
P value	0.5383
P value summary	ns
Significant diff. among means (P < 0.05)?	No
R squared	0.2804
Brown-Forsythe test	
F (DFn, DFd)	0.7730 (5, 11)
P value	0.5887
P value summary	ns
Are SDs significantly different (P < 0.05)?	No
Bartlett's test	
Bartlett's statistic (corrected)	
P value	
P value summary	
Are SDs significantly different (P < 0.05)?	

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	53.84	5	10.77	F (5, 11) = 0.8574	P=0.5383
Residual (within columns)	138.1	11	12.56		
Total	192.0	16			

Data summary	
Number of treatments (columns)	6

Number of values (total)	17

2.7. Multiple comparisons for 5-HT2B Emax

Number of families	1
Number of comparisons per family	5
Alpha	0.05

Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value	F-?	
5-HT vs. 1	147.4	132.9 to 161.9	Yes	****	<0.0001	A	1
5-HT vs. 2	152.1	137.6 to 166.6	Yes	****	<0.0001	B	2
5-HT vs. 3	161.6	148.6 to 174.6	Yes	****	<0.0001	C	3
5-HT vs. 4	160.2	147.2 to 173.1	Yes	****	<0.0001	D	4
5-HT vs. 5	158.8	145.8 to 171.8	Yes	****	<0.0001	E	5

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	q	DF
5-HT vs. 1	160.7	13.27	147.4	4.828	3	2	30.53	10
5-HT vs. 2	160.7	8.578	152.1	4.828	3	2	31.50	10
5-HT vs. 3	160.7	-0.9357	161.6	4.319	3	3	37.42	10
5-HT vs. 4	160.7	0.5082	160.2	4.319	3	3	37.09	10
5-HT vs. 5	160.7	1.829	158.8	4.319	3	3	36.78	10

2.8. Multiple comparisons for 5-HT2B EC50

Number of families	1
Number of comparisons per family	5
Alpha	0.05

Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value	F-?	
Column F vs. Column A	-1.671	-10.21 to 6.865	No	ns	0.9659	A	Column A
Column F vs. Column B	-3.610	-12.15 to 4.926	No	ns	0.6238	B	Column B
Column F vs. Column C	0.4155	-9.128 to 9.959	No	ns	0.9998	C	Column C
Column F vs. Column D	1.441	-7.095 to 9.977	No	ns	0.9813	D	Column D
Column F vs. Column E	1.031	-7.505 to 9.567	No	ns	0.9957	E	Column E

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	q	DF

Column F vs. Column A	-8.665	-6.994	-1.671	2.894	3	3	0.5774	11
Column F vs. Column B	-8.665	-5.055	-3.610	2.894	3	3	1.248	11
Column F vs. Column C	-8.665	-9.081	0.4155	3.235	3	2	0.1284	11
Column F vs. Column D	-8.665	-10.11	1.441	2.894	3	3	0.4981	11
Column F vs. Column E	-8.665	-9.696	1.031	2.894	3	3	0.3564	11

2.9. Anova results for 5-HT2C Emax

Table Analyzed	5-HT2C top
Data sets analyzed	A-F
ANOVA summary	
F	17.84
P value	<0.0001
P value summary	****
Significant diff. among means (P < 0.05)?	Yes
R squared	0.8321
Brown-Forsythe test	
F (DFn, DFd)	1.723 (5, 18)
P value	0.1804
P value summary	ns
Are SDs significantly different (P < 0.05)?	No
Bartlett's test	
Bartlett's statistic (corrected)	
P value	
P value summary	
Are SDs significantly different (P < 0.05)?	

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	197153	5	39431	F (5, 18) = 17.84	P<0.0001
Residual (within columns)	39789	18	2211		
Total	236942	23			

Data summary	
Number of treatments (columns)	6
Number of values (total)	24

2.10. Anova results for 5-HT2C EC50

Table Analyzed	5-HT2C EC50
Data sets analyzed	A-F
ANOVA summary	
F	2.808
P value	0.0461
P value summary	*
Significant diff. among means (P < 0.05)?	Yes
R squared	0.4249
Brown-Forsythe test	

F (DFn, DFd)	2.333 (5, 19)
P value	0.0822
P value summary	ns
Are SDs significantly different (P < 0.05)?	No
Bartlett's test	
Bartlett's statistic (corrected)	39.73
P value	<0.0001
P value summary	****
Are SDs significantly different (P < 0.05)?	Yes

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	29.11	5	5.822	F (5, 19) = 2.808	P=0.0461
Residual (within columns)	39.40	19	2.074		
Total	68.51	24			

Data summary	
Number of treatments (columns)	6
Number of values (total)	25

2.11. Multiple comparisons for 5-HT2C Emax

Number of families	1
Number of comparisons per family	5
Alpha	0.05

Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value	F-?	
5-HT vs. 1	123.9	36.06 to 211.7	Yes	**	0.0044	A	1
5-HT vs. 2	163.6	75.72 to 251.4	Yes	***	0.0003	B	2
5-HT vs. 3	243.1	155.3 to 330.9	Yes	****	<0.0001	C	3
5-HT vs. 4	204.6	116.8 to 292.5	Yes	****	<0.0001	D	4
5-HT vs. 5	258.2	162.6 to 353.9	Yes	****	<0.0001	E	5

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	q	DF
5-HT vs. 1	273.6	149.7	123.9	31.54	5	4	3.928	18
5-HT vs. 2	273.6	110.1	163.6	31.54	5	4	5.186	18
5-HT vs. 3	273.6	30.55	243.1	31.54	5	4	7.708	18
5-HT vs. 4	273.6	69.01	204.6	31.54	5	4	6.488	18
5-HT vs. 5	273.6	15.40	258.2	34.34	5	3	7.521	18

2.12. Multiple comparisons for 5-HT2C EC50

Number of families	1
Number of comparisons per family	5
Alpha	0.05

Dunnett's multiple comparisons test	Mean Diff.	95.00% CI of diff.	Below threshold?	Summary	Adjusted P Value	F-?		
5-HT vs. 1	-3.377	-6.050 to -0.7049	Yes	*	0.0105	A	1	
5-HT vs. 2	-2.045	-4.717 to 0.6279	No	ns	0.1750	B	2	
5-HT vs. 3	-2.192	-4.865 to 0.4801	No	ns	0.1325	C	3	
5-HT vs. 4	-2.075	-4.748 to 0.5971	No	ns	0.1652	D	4	
5-HT vs. 5	-2.587	-5.259 to 0.08538	No	ns	0.0598	E	5	

Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	q	DF
5-HT vs. 1	-8.245	-4.868	-3.377	0.9660	5	4	3.496	19
5-HT vs. 2	-8.245	-6.201	-2.045	0.9660	5	4	2.116	19
5-HT vs. 3	-8.245	-6.053	-2.192	0.9660	5	4	2.269	19
5-HT vs. 4	-8.245	-6.170	-2.075	0.9660	5	4	2.148	19
5-HT vs. 5	-8.245	-5.658	-2.587	0.9660	5	4	2.678	19

Master dissertation submitted to the faculty of Pharmaceutical Sciences, performed in collaboration with the Laboratory for Connor Research Group.

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