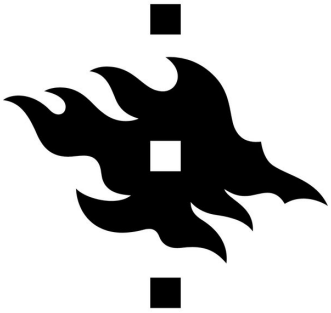

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Investigating the therapeutic efficacy of psychedelics for anorexia nervosa using animal models

Janika Pauliina Ruuska
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Supervised by Dr. Claire Foldi and Prof. Brian Oldfield

Abstract

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Abstract:

Individuals suffering from anorexia nervosa (AN) have one of the highest mortality rates of all psychiatric disorders, as a consequence of health complications that follows severe malnutrition. The impairments in cognitive flexibility, including an extreme focus on restricting food despite a rapid decline in body weight in AN, also plays an important role in the development of the disorder and has been suggested as a hallmark of AN. This cognitive inflexibility, common among many psychiatric disorders such as depression and obsessive-compulsive disorder, is linked to alterations in serotonin (5-HT) signaling in the medial prefrontal cortex (mPFC). Reduced 5-HT_{2A} receptor activity and potentially increased 5-HT_{1A} receptor activity are evident in the mPFC in individuals with AN and may be linked to impaired cognitive flexibility, however, the mechanisms through which 5-HT and inflexibility interact in AN are not fully understood. A better understanding of this link could pave the way toward more effective pharmacological treatments for AN. Psilocybin, a psychedelic compound produced by so-called "magic" mushrooms, has a high affinity for several 5-HT receptor subtypes including 5-HT_{1A} and 5-HT_{2A} receptors, and has now been empirically demonstrated to increase cognitive flexibility in individuals with major depressive disorder (MDD).

In this study, we sought to understand how the development of pathological weight loss and/or psilocybin administration influenced the expression of RNA molecules of 5-HT_{2A} and 5-HT_{1A} receptors expression in the mPFC of rats. To this end, we used the activity-based anorexia (ABA) model, the only experimental model known to elicit *voluntary* reductions in food intake and *voluntary* hyperactivity that leads to rapid body weight loss in the majority of animals exposed to ABA conditions. Outcomes were compared against an age-matched control group that were not exposed to the ABA paradigm. Animals were administered psilocybin (1.5 mg/kg) or saline (control) and 4-10 days later brain tissue was collected for processing. Receptor expression was detected using a novel multiplex RNA fluorescent in situ hybridization (FISH) technology, RNAscope®. The main aim of this study was to examine changes in the expression of RNA molecules of 5-HT_{2A} and 5-HT_{1A} receptors in the mPFC elicited by ABA conditions and determine whether these were ameliorated by the administration of psilocybin.

We found that animals exposed to ABA demonstrated a significant reduction of 5-HT_{2A} receptors' RNA levels in the mPFC, and that this was not influenced by psilocybin treatment. There have been reports from clinical trials that individuals with AN experience "less than expected" subjective effects from psilocybin, which may be explained by reduced expression of RNA molecules of 5-HT_{2A} receptors in the mPFC, and is supported by our results in rats. Taken together, these results highlight a specific serotonergic mechanism that could underly the development of pathological weight loss and offers insight into possible issues with the therapeutic application of psilocybin for AN. Future studies will need to examine the effects of psilocybin during a more acute period following treatment to define these effects. Moreover, whether or not the reduced 5-HT_{2A} receptors' RNA level expression induced by ABA is restored with body weight recovery should be determined.

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Tiivistelmä:

Anorexia nervosasta (AN) kärsivien henkilöiden kuolleisuusaste on yksi korkeimmista, kaikkien psykiatristen häiriöiden joukossa. AN:ssa kuolleisuuden aiheuttavat tekijät ovat usein vakavasta aliravitsemustilasta johtuvat terveyskomplikaatiot. Kognitiivisen joustavuuden heikkeneminen, mukaan lukien äärimmäinen keskittyminen ruoan rajoittamiseen nopeasta ruumiinpainon laskusta huolimatta, on myös tärkeä rooli AN:n kehittymisessä, ja kognitiivista joustamattomuutta onkin ehdotettu AN:n tunnusmerkiksi. Kognitiivinen joustamattomuus, joka on yleinen myös monien muiden psykiatristen häiriöiden, kuten masennuksen ja pakko-oireisen häiriön keskuudessa, liittyy mahdollisesti muutoksiin serotoniini (5-HT) signaloinnissa mediaaliprefrontaalisessa aivokuoressa (mPFC). Vähentynyt 5-HT_{2A} reseptoriaktiivisuus ja mahdollisesti lisääntynyt 5-HT_{1A} reseptoriaktiivisuus mPFC:ssa on ilmeistä AN:a kokevilla henkilöillä todennäköisesti liittyen heikentyneeseen kognitiiviseen joustavuuteen, mutta mekanismit joiden kautta 5-HT signalointi ja joustamattomuus vuorovaikuttavat AN:ssa, ei ole täysin ymmärretty. Tämän vuorovaikutuksen parempi ymmärtäminen voisi tarjota mahdollisuuksia tehokkaammille farmakologisille vaihtoehdoille AN:n hoidoissa. Niin kutsuttujen “taikasienten” tuottama psykedeelinen yhdiste, psilosybiini, omaa korkean affiniteetin useisiin 5-HT reseptorien alatyyppeihin, mukaan lukien 5-HT_{2A} ja 5-HT_{1A} reseptorit, ja psilocybiinin onkin empiiriseti osoitettu lisäävän kognitiivista joustavuutta henkilöillä joilla on vakava masennushäiriö (MDD).

Tässä tutkimuksessa pyrimme ymmärtämään, kuinka patologisen painonpudotuksen kehittyminen ja/tai psilosybiinin antaminen vaikutti 5-HT_{2A} ja 5-HT_{1A} -reseptorien RNA-molekyylien ilmentymiseen rottien mPFC:ssa. Tätä tarkoitusta varten käytimme aktiivisuuspohjaista anoreksia (ABA) -eläinmallia, ainoaa tiedettävissä olevaa kokeellista mallia, jossa ilmentyy *vapaaehtoista* ravinnon saannin vähentämistä yhdessä lisääntyneen *vapaaehtoisen* aktiivisuuden kanssa, johtaen nopeaan painonpudotukseen useimmilla ABA-olosuhteisiin altistetuilla eläimillä. Tuloksia verrattiin ikään sopivaan kontrolliryhmään, jota ei oltu altistettu ABA-olosuhteille. Eläimille annettiin psilosybiiniä (1.5 mg/kg) tai suolaliuosta kontrollina ja 4-10 päivää myöhemmin aivokudosta kerättiin prosessointia varten. Reseptorien ilmentymistä tutkittiin käyttämällä uudenlaista fluoresoivaa multipleksistä RNA *in situ* -hybridisaatiotekniikkaa (FISH), RNAscope®-tekniikkaa. Tämän tutkimuksen päätavoitteena oli tutkia ABA-olosuhteiden aiheuttamia muutoksia 5-HT_{2A} ja 5-HT_{1A} -reseptorien ilmentymisessä mPFC:ssa ja määrittää kohensiko psilosybiinin antaminen näitä muutoksia.

Havaitsimme, että eläimet jotka altistettiin ABA-olosuhteille, osoittivat merkittävää alenemista 5-HT_{2A} reseptorien RNA-tasoissa mPFC:ssa, ja että psilosybiinin antaminen ei vaikuttanut tähän. Kliinisissä tutkimuksissa on raportoitu, että AN:a sairastavat henkilöt kokevat "odotettua vähemmän" psilosybiinin aiheuttamia subjektiivisia vaikutuksia, mikä saattaa johtua vähentyneestä 5-HT_{2A} -reseptorien ilmentymisestä mPFC:ssä, mitä rotilla saamamme tulokset tukevat. Yhdessä nämä tulokset korostavat spesifistä serotonergistä mekanismia, joka voisi olla patologisen painonpudotuksen taustalla, ja tarjoaa käsityksen mahdollisista ongelmista, jotka liittyvät psilosybiinin terapeuttiseen käyttöön AN:n hoidossa. Tulevissa tutkimuksissa on selvitetävä psilosybiinin vaikutuksia akuuteimmilla hoidon jälkeisellä ajanjaksolla näiden vaikutusten määrittämiseksi. Lisäksi tulee selvittää, palautuuko ABA-olosuhteiden aiheuttama vähentynyt 5-HT_{2A} reseptorien ilmentyminen ruumiinpainon palautumisen myötä.

Table of Contents

Abbreviations

ABA	Activity-based anorexia rodent model
AcbSh	Nucleus accumbens shell
AgRP	Agouti-related protein
AN	Anorexia nervosa
BMI	Body mass index
$C_2H_6O_2$	Ethylene glycol
$C_3H_8O_3$	Glycerol
DA	Dopamine
dH_2O	Distilled water
DMN	Default mode network
DMT	N,N-dimethyl-tryptamine
DSM-5	The Diagnostic and Statistical Manual of Mental Disorders
FDA	The U.S. Food and Drug Administration
FISH	Fluorescent *in situ* hybridization
fMRI	Functional magnetic resonance imaging
IL	Infralimbic cortex
IP	Intraperitoneal injections
LSD	Lysergic acid diethylamide
MDD	Major depressive disorder
mPFC	Medial prefrontal cortex
NaCl	Sodium chloride
NPY	Neuropeptide Y
OCD	Obsessive-compulsive disorder
PB	Phosphate buffer
PFA	Paraformaldehyde
PrL	Prelimbic cortex
RNA	Ribonucleic acid
Saline	0.9% NaCl in dH_2O
SSRIs	Selective serotonin reuptake inhibitors
TRD	Treatment-resistant depression
5-HT	Serotonin

1 | INTRODUCTION

1.1 | Anorexia nervosa

In the 5th edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-5), feeding and eating disorders are characterized by continuous, disturbed eating or feeding behavior, which leads to significant impairments of physical health or psychosocial function. There are eight of these disorders, and they can affect people of all ages. One type of feeding and eating disorder is Anorexia nervosa (AN), which has one of the highest mortality rates of all psychiatric disorders with a weighted annual mortality rate of 5.39 per 1000 person-years. Physical health consequences that follow severe malnutrition are the main reasons for mortality, with around 1 out of 5 deaths among individuals with AN being a result of suicide. There are no existing pharmacological treatments approved for AN (Arcelus et al., 2011; Frank et al., 2002; Himmerich et al., 2021). According to DSM-5, AN is defined as a state in which individuals restrict energy intake lower than physiologically required, leading to significantly low body weight. This means that the individual suffering from AN has lower body weight than their peers, matched for sex, age, physical health, and trajectory of development. The severity of AN from mild to extreme, as defined by the World Health Organization, is based on body mass index (BMI). To meet the criteria of AN, the individual also needs to have an extreme fear of, or engage in behaviors to avoid weight gain or becoming obese, despite the individual already being low body weight. In addition, the person diagnosed with AN is continually lacking recognition of their extremely low body weight or has perceived flaws in their body shape or weight, either of which has the ability to affect their self-worth (Mitchell et al., 2016).

Personality and character traits, like perfectionism, harm avoidance, rigidity, obsessiveness, and overcontrol are often seen in people with AN (Frank et al., 2002; Kaye et al., 2013). Individuals with AN typically experience anhedonia, which can refer to, for example, a dislike for palatable food, or experiencing a dysphoric mood after eating (Femstrom et al., 1994; Kaye, 2008). In addition to anhedonia, extreme self-control and cognitive inflexibility are seen in individuals with AN (Tchanturia et al., 2004). Impairments in cognitive flexibility can be seen as an inability to change behavior despite changing environmental demands, which results in unsuccessful adaptation. In AN this can be seen as an excessive amount of exercise as well as an extreme focus on restricting food and the shape of the body, despite a rapid decline in body weight. Cognitive inflexibility is thought

of as independent of one's body weight as well as the duration of the disorder and may persist after individuals with AN have experienced weight recovery. This may be indicative of its role in the etiology of the AN and, as such, is an interesting target for the development of novel treatment strategies (Milton et al., 2021). Individuals with AN may have other disorders simultaneously, like depression, anxiety, or obsessive-compulsive disorder (OCD) (American Psychiatric Association, 2016). AN and OCD share similarities in genetic etiologies, and as previously mentioned, cognitive inflexibility is seen in both psychiatric conditions (Foldi et al., 2020; Milton et al., 2021). As can be seen, AN is both an extremely serious and highly complex disorder.

AN is more common among females (0.3%-1%) than males (0.1%) and the onset of the AN is typically in adolescence but can affect any individual, despite the person's sex, age, ethnicity, race, or socioeconomic status (American Psychiatric Association, 2016; Kaye et al., 2013). There are various risk factors in the development of AN, including genetic, psychosocial, and sociocultural factors (Gorwood et al., 2016). Large-scale twin studies suggest that the heritability of AN is between 0.5 and 0.8, meaning that on average 50-80 % of the differences among phenotypes are genetic (Frank et al., 2002). In recent years it has also become more clear that there are neurobiological drivers that underlie the pathogenesis of AN (Foldi et al., 2017). Human brain imaging studies have shown that individuals with AN have lower neural activity in the ventral and dorsal striatum, and higher neural activity in the medial prefrontal cortex (mPFC), especially in orbital and dorsolateral prefrontal cortices compared to healthy individuals. This imbalance is connected to extreme self-control and cognitive inflexibility seen in individuals with AN (Foldi et al., 2020). In other words, this impaired cognitive flexibility is caused, in part, by alterations in the interaction between the mPFC and ventral striatum, in the so-called frontostriatal network. More specifically, powerful inhibitory actions of the mPFC suppresses the activity of subcortical targets to produce inflexible behavior, including the projections to the ventral striatum subregion, called nucleus accumbens shell (AcbSh), that has functions in motivation and reward-related feeding behavior. It is important to keep in mind that even though these circuits are often discussed separately, their functions are also overlapping and brain areas mentioned before are playing crucial roles in both cases (Milton et al., 2021).

When looking at these neurobiological alterations at the molecular level, it is clear that although appetite-regulating neuropeptides are clearly involved in AN, dysfunctions in dopamine (DA) and

serotonin (5-HT) signaling may be more closely related to reward and cognitive deficits in individuals with AN (Kaye et al., 2013). For example, impairments in reward processing are associated with decreased endogenous DA levels in the anterior ventral striatum, which can be seen as increased DA receptor binding (Frank et al., 2005). Whereas cognitive inflexibility and changes in appetitive behavior are both linked to alterations in 5-HT signaling in the cortical regions of the brain. 5-HT receptors are G-protein coupled receptors, found in a variety of cortical and subcortical regions, which can maintain neuronal activity via a wide range of intracellular signaling pathways (Foldi et al., 2020; Rodan et al., 2021).

From electrophysiological recordings of neurons within rat frontal cortex, activation of different subtypes of 5-HT receptors, namely the 5-HT_{1A} and 5-HT_{2A} receptors, have contrasting effects on excitability. On the one hand, 5-HT_{1A} receptor activation is coupled to the $G_{i/o}$ protein signaling pathway, resulting in hyperpolarization of the membrane, whereas 5-HT_{2A} receptor activation is coupled to the G_q protein pathway, resulting in depolarization of the membrane. In other words, 5-HT_{1A} receptors are thought to have inhibitory (suppressive) and 5-HT_{2A} receptors to have excitatory (stimulatory) effects in neurons of the frontal cortex (Carhart-Harris & Nutt, 2017; McClure-Begley & Roth, 2022; Savalia et al., 2021). Expression of 5-HT_{1A} receptors is found in limbic and cortical regions, as well as in the midbrain, especially in serotonergic neurons of the raphe nuclei where they act mainly presynaptically as autoreceptors. The postsynaptic activity of 5-HT_{1A} receptors is focused on the cortex and limbic system, and receptors themselves are thought to be the main inhibitory receptors in the brain. In contrast, 5-HT_{2A} receptors are mainly expressed in the cortex, especially in the regions responsible for “high-level” cognitive associations. The postsynaptic activity of 5-HT_{2A} receptors may have an important role in serotonin release regulation throughout the cortex.

Complicating our understanding of receptor-specific serotonin dysfunction in AN and its effects on neuronal activity and behavior is the fact that, in the mPFC, up to 80% of pyramidal neurons express both serotonin receptor subtypes, where they have opposite effects on neuronal activity. 5-HT_{2A} receptors may play a crucial role in cognitive flexibility, whereas 5-HT_{1A} receptors may have a more crucial role in stress relief facilitation (Carhart-Harris & Nutt, 2017). Individuals with AN appear to have reduced binding in 5-HT_{2A} receptors in cortical regions, as well as alterations, in or potential increased activity of 5-HT_{1A} receptors. These changes in the 5-HT_{2A} and 5-HT_{1A} receptors continue

after weight recovery and are indicative of the pathophysiology of AN (Carhart-Harris & Nutt, 2017; Foldi et al., 2020; Bailer et al., 2006, 2007). The loss of serotonergic function in AN may be related to a polymorphism in the 5-HT_{2A} receptor promotor, an impairment that is also associated with OCD, which aligns with the putative role of 5-HT_{2A} receptors in cognitive flexibility, considering both OCD and AN are characterized by inflexible patterns of thought and behavior (Foldi et al., 2020; Frank et al., 2002).

Even though there is evidence about some neurobiological drivers of the pathophysiology of AN, the mechanism is still not clearly understood, which is one of the reasons there is no effective pharmacological treatment available for AN. Currently used pharmacotherapeutics are Selective Serotonin Reuptake Inhibitors (SSRIs) for depressive symptoms and the atypical antipsychotic medications olanzapine or haloperidol for delusional dysmorphic features of AN, meaning that current pharmacological treatments are mainly targeting co-occurring conditions (Foldi et al., 2017; Spriggs et al., 2021). Inefficacy of SSRIs in treatment for AN may be the SSRIs' ability to increase 5-HT levels in the synapse, and thus not downregulating 5-HT_{1A} receptor activity, which is shown to be exaggerated in the brain of individuals with AN (Bailer et al., 2007). A common treatment for AN is to focus on weight regain via inpatient renutrition protocols, but this is also problematic because individuals with AN are shown to experience this kind of excessive concentration on food and body weight as encouraging for symptoms rather than preventing them (Rance et al., 2017). Considering that over 50% of individuals with AN don't fully recover, chronicity of the disease is up to 20% and the illness has a high mortality rate, both understanding the development of the disease and developing new pharmacological approaches to treat AN are desperately needed (Foldi et al., 2020; Arcelus et al., 2011). It is widely shown that 5-HT plays an important role in mood regulation, and has a similarly important role in AN, but the mechanism is not fully understood (Carhart-Harris & Nutt, 2017). A study by Duriez showed that improving cognitive flexibility in AN benefits the treatment outcomes in several AN symptoms (Duriez et al., 2020). If we could pharmacologically improve cognitive flexibility, for example by targeting 5-HT signaling, this could reduce the relapse rates and be a breakthrough in AN treatment (Duriez et al., 2021).

1.2 | Psilocybin

Hallucinogens, a class of psychoactive drugs, are known to alter one's thoughts, feelings, and awareness of the surroundings. A subgroup of hallucinogens, called psychedelics, also known as ''classical'' psychedelics, or serotonergic hallucinogens (indicating their function in the serotonergic system), can be further divided into three subgroups. These subgroups are Tryptamines, such as 4-phosphoryloxy-N,N-dimethyltryptamine (psilocybin), and N,N-dimethyl-tryptamine (DMT), Phenethylamines, such as mescaline, and Ergolines, such as lysergic acid diethylamide (LSD). Naturally occurring psychedelics have been used since ancient times for various purposes and across different cultures (Jaster et al., 2021; McClure-Begley & Roth, 2022). Psychedelics are known to alter an individual's consciousness by causing changes in cognition, perception, and mood (Shao et al., 2021). In addition, the reduced feeling of social exclusion and increased emotional empathy, meaning one's ability to feel with others, are reported after psilocybin administration (Vollenweider & Preller, 2020). Although it has long been hypothesized that the therapeutic action of psychedelics relates in some way to increasing cognitive flexibility, this has now been empirically demonstrated in individuals with major depressive disorder (MDD) after psilocybin therapy. Although increased cognitive flexibility and improvements in MDD symptoms were not correlated after psilocybin administration, it has been hypothesized that psychedelic-induced cognitive flexibility may be beneficial when combined with psychotherapy due to the "opening" of a neural plasticity window (Doss et al., 2021). Cognitive flexibility is decreased (cognitive inflexibility) in several psychiatric disorders, such as depression, and anxiety as well as in anorexia nervosa, as pointed out previously. Psychedelics seem to have benefits for a whole range of psychiatric illnesses, possibly due to their ability to increase cognitive flexibility which is a feature common to many categories of psychiatric diagnoses (Davis et al., 2020). When talking about the use of psychedelics, concern has been raised about their potential addictive properties, but current research has indicated them as nonaddictive compounds. Although it is important to keep in mind, that tolerance may be increased with some of the psychedelics (Jaster et al., 2021).

More than 100 mushroom species contain psilocybin. These so-called ''magic mushrooms'' include *Psilocybe cubensis* found in South America and some parts of Mexico, as well as *Psilocybe cyanescens,* which can be found on the west coast of the United States and parts of Europe. It was

first isolated by Albert Hofmann in 1959 from Mexican mushrooms (Jaster et al., 2021; Rodan et al., 2021). In the 1960s and 1970s psychedelics research, including studies investigating the consequences of ingesting psilocybin, took a hit due to the political forces in the United States, resulting in the absence of empirical research on psychedelics for nearly half a century (Nichols, 2016). Now that research has taken its place back in the field of psychedelics, findings from several studies have suggested psilocybin especially to be suitable for various psychiatric disorders, including depression and anxiety (Jaster et al., 2021; McClure-Begley & Roth, 2022). In fact, the U.S. Food and Drug Administration (FDA) has nominated psilocybin as a ''breakthrough therapy'' for treatment-resistant depression (TRD) and MDD in 2018 and 2019 (Foldi et al., 2020; Rodan et al., 2021). In addition, short-term improvements are seen in individuals with OCD after psilocybin ingestion, who share common behavioral disturbances with individuals with AN as described above (Moreno et al., 2006).

There are more than 30 ongoing clinical studies focusing on the use of psilocybin in psychiatric disorders, including the four studies on the safety and efficacy of psilocybin in AN, from which one of the Phase I studies was completed in 2019. Phase 2 trials investigating the effects of psilocybin on eating disorder psychopathologies in AN are currently underway (*http://clinicaltrials.gov)*. So far, clinical trials investigating psilocybin for a range of mental health outcomes have suggested one to two doses of psilocybin combined with psychotherapy as producing the optimal therapeutic effects, although these data are largely in their infancy and subject to constant revision and change (McClure-Begley & Roth, 2022; Meikle et al., 2020). The onset of psychoactive effects of psilocybin after oral administration begins at 20–40 minutes, whereas the peak effects are reached after 60–90 minutes. The duration of action for psilocybin takes 4–6 h, and when compared to other serotonergic psychedelics for example LSD, which takes 8–12 h, psilocybin may be better adopted as a treatment considering the reduced time burden required for monitoring the individual (Rodan et al., 2021). Dosage is thought to be low, or rather like a placebo when the amount is 10 mg, active when the amount is 25 mg, and high, or to be precise, too strong when the dosage is 30 mg. Some studies prefer weight-dependent dosing, for example, 0.3 mg/kg, which allows more control between individuals (Meikle et al., 2020). To keep in mind, there is little consensus at the current moment regarding the most effective or appropriate dose, or even what is thought to be low or high. This is an area of high importance to understand adequately, considering that many of these dosing regimens are already being administered to individuals without this crucial understanding.

Considering the FDA designation of psilocybin as a breakthrough therapy, and that the majority of clinical trials in individuals with AN and MDD are utilizing psilocybin-assisted psychotherapy, it is of paramount importance to understand the biological effects of psilocybin over other, less widely used, psychedelic compounds.

The most consistently reported neurobiological changes resulting from psilocybin administration are changes in functional connectivity, including decreased functional connectivity and activity in the default mode network (DMN), a set of neural structures that are highly active under task-negative (or "resting") conditions. Functional connectivity is a statistical term used to define the correlation between (de)oxygenated blood levels and neuronal activity in distinct brain regions over a certain time. This data, providing *insight into indirect* neuronal activity, is typically recorded by using functional magnetic resonance imaging (fMRI) (Babaeeghazvini et al., 2021). DMN shows activation when the person concentrates on the inside world and considering that individuals with AN show higher DMN activation compared to controls, this supports the rationale for psilocybin as a potential pharmacological treatment (Foldi et al., 2020; Jaster et al., 2021). The other reasons for therapeutic outcomes followed by psilocybin administration are changes in brain network connectivity's entropy, suppression of awe/ego state, and increases in cognitive flexibility. Evidence from animal studies using a reversal-learning task indicates that cognitive flexibility is modulated by 5-HT signaling (Alsiö et al., 2021). Since one of the main characteristics of AN behavior is cognitive inflexibility, it could be possible to target these disruptions with psilocybin (Foldi et al., 2020).

Psilocybin's active metabolite 4- hydroxy-dimethyltryptamine or psilocin, is formed when psilocybin is metabolized through dephosphorylation in vivo. Psilocin has an affinity for several 5-HT receptor subtypes, and also certain dopaminergic, adrenergic, and histaminergic receptors (Savalia et al., 2021). As an example, it is shown in rats that after psilocybin administration the release of dopamine (DA) in the nucleus accumbens (NAcc) is increased, as well as the extracellular 5-HT levels in the mPFC (Sakashita et al., 2015). Here I will focus only on the serotonergic receptors for several reasons; firstly, the serotonergic system is in itself a complex system, secondly, even though the mechanisms underlying the therapeutic effects of psilocybin are not fully discovered, the importance of serotonergic activity is widely demonstrated, and lastly, 5-HT signaling is altered in several psychiatric disorders such as in MDD, OCD, and AN (Hesselgrave et al., 2021; Rodan et al., 2021).

More than 14 serotonin receptors are distributed throughout the body and the brain, to which the neurotransmitter serotonin can bind and affect various factors, for example, mood, pain, emotions, appetite, and cognition. Psilocin, the active metabolite of psilocybin, acts as an agonist and has a high affinity for at least three of these serotonin receptors, which are 5-HT_{1A}, 5-HT_{2A}, and 5-HT_{2C} (Foldi et al., 2020; Rodan et al., 2021). Here I will focus on the receptor subtypes 5-HT_{1A}, and 5-HT_{2A} because the importance of this affinity in mediating therapeutic outcomes has been indicated in several studies (Savalia et al., 2021). And even though, for example, 5-HT_{2A} receptors are expressed in both cortical and subcortical regions, several studies have shown that they are highly concentrated in apical dendrites of pyramidal neurons located in the layer V of the cortex. Some studies have also shown that individuals with AN have dysregulation of 5-HT activity in several cortical regions, especially in the prefrontal cortex, which has a key role in maintaining cognitive flexibility, as mentioned before. This all makes it reasonable to focus on this area of the brain (McClure-Begley & Roth, 2022; Rodan et al., 2021).

It is well characterized that 5-HT_{2A} receptor activation is essential for psychedelic experiences, especially for behavioral outcomes of psychedelics (McClure-Begley & Roth, 2022). Broadly speaking, the mechanisms underlying psychedelic actions depend heavily on the activation of the 5-HT_{2A} receptors which can lead for example to the activation of inositol triphosphate (IP_3) and diacylglycerol (DAG) pathways and finally to the release of neurotransmitters from the neurons. As might be expected, there are multiple other signaling pathways that can be activated due to this receptor stimulation, such as the AKT/mTOR pathway (Hibicke et al., 2020; McClure-Begley & Roth, 2022). While 5-HT_{2A} receptors are clearly important for the acute subjective effects, recent studies have questioned the role of 5-HT_{2A} receptor activation in the *therapeutic* effects of psilocybin (Hibicke et al., 2020). These concerns have been raised in several studies in both humans and mice, which have used the 5-HT_{2A} receptor antagonist, ketanserin, as a pretreatment to block its activation by psilocybin. This subsequently results in therapeutic effects without the hallucinogenic effects, measured by self-report in humans and by a head-twitch response in rodents (Hesselgrave et al., 2021; Jaster et al., 2021; McClure-Begley & Roth, 2022). Taken together, psilocybin has an affinity to 5-HT_{1A} and 5-HT_{2A} receptors and it is known that 5-HT_{2A} receptors have an important role in psychedelic experiences, but it is still unclear of the specificity of signaling through 5-HT_{2A} receptors in the therapeutic effects after psilocybin administration.

1.3 | Activity-based anorexia rodent model

The use of disease-specific animal models, when studying the effects of psychedelics from a neurobiological point of view, enables the determination of the specific effects of psilocybin without possible expectations from individuals as well as biases, which are impossible to control in human studies. Even though animals are not affected by psychedelic drugs in the exact same way as humans, the possible changes in neurobiological factors and cognitive functions altered by psychedelics can be studied with greater specificity in animal models than in humans. In addition, the stigma around psychedelics can be diminished by increasing the level of understanding of brain circuitry and neurochemistry of psychedelics from animal studies (Foldi et al., 2020).

The animal model to study these factors and the potential of psychedelic drugs as treatment for AN is the activity-based anorexia (ABA) rodent model, which was established for the first time in the late 1960s (Schalla & Stengel, 2019). ABA is the only model, where the animal chooses self-starvation over the balance of homeostasis. In this model, typically female Sprague-Dawley rats, have free access to the running wheel and *ad libitum* water access while food availability is time-limited, commonly to less than 2 hours (Schalla & Stengel, 2019). This reduced food intake results in an increase in running activity, leading to steep and rapid weight loss and when not controlled, finally to death (Foldi et al., 2020; Milton et al., 2021). The body weight typically decreases around 20% in two weeks due to scarcity of eating and starvation-induced hyperactivity (Allen et al., 2017). This behavioral hyperactivity in response to restricted availability of food, caused by the ABA paradigm, can be thought of as compulsive behavior and is often associated with inflexible adaptation to changes in the schedule of feeding (Milton et al., 2021). Note that the ABA paradigm can only happen when the two factors are paired – free access to the running wheel and time-limited access to food. If only one of these factors is introduced in isolation, the animal quickly learns compensatory mechanisms to maintain its body weight (Foldi et al., 2020; Milton et al., 2021). It is also good to keep in mind that not all animals undergoing the paradigm of ABA will develop the conditions of ABA. This is similar to the human population and therefore makes it possible to study differences in the development of the disease between susceptible and resistant individuals (Ross et al., 2016).

When the animal is considered to be in ABA conditions, there are multiple changes happening throughout the brain and the body of the animal, including alterations in neuroendocrine and neurotransmitter systems that mimic those seen in the human condition (Allen et al., 2017). As an example, there are increases in ghrelin, neuropeptide Y (NPY), and agouti-related protein (AgRP) levels, as well as decreases in leptin levels, all known to have an important role in hunger regulation as neuro-hormonal mediators. See the review from Lamanna for more detailed alterations in neuroendocrine and neurotransmitter systems in ABA (Lamanna et al., 2019).

There are several other similarities between AN and ABA, such as phenotype prevalence mainly in young females, disturbances in neuro-hormonal mediators mentioned previously, distortions in reward signaling, and cognitive inflexibility (Foldi et al., 2020). See the review from Schalla for detailed cognitive alterations in AN and ABA (Schalla & Stengel, 2019). As mentioned previously, cognitive inflexibility, possibly altered by changes in 5-HT signaling in the mPFC, is thought to be involved in the pathophysiology of AN (Foldi et al., 2020). Also in animals, serotonin has an important role in appetite inhibition, depression, anxiety, and obsessive-like behavior (Ross et al., 2016). It is shown that when depleting serotonin levels in ABA rats, results in increased running wheel activity, and decreased food intake finally leading to decreased body weight (Schalla & Stengel, 2019).

2 | AIMS

Taken together, it seems that targeting 5-HT signaling in the mPFC by using psilocybin could be a potential pharmacotherapeutic approach to reduce cognitive inflexibility in AN. But the mechanisms underlying both the altered 5-HT signaling in AN and the action of psilocybin on 5-HT signaling remain largely unknown, forming the basis of this study. The main aim of this study was to examine differences in the expression of RNA molecules of 5-HT_{1A} and 5-HT_{2A} receptors in the mPFC between animals that have (ABA) or have not (non-ABA) been exposed to the ABA paradigm and to determine whether this is influenced by psilocybin treatment.

Quantification of receptors' RNA expression levels in the mPFC was conducted by using a novel multiplex RNA fluorescent *in situ* hybridization (FISH) technology, RNAscope®. This technique

allowed the simultaneous visualization of the gene expression (RNA targets) for both receptors of interest in order to compare their expression and localization within the mPFC (Wang et al., 2012). The workflow can be seen in Figure 1, which is divided into three phases. The first phase included animal work, the second phase was for tissue collection and preparation, and in the third phase the RNAscope® was performed, receptors visualized and finally, the possible differences in RNA molecules of receptors expression and localization were analyzed.

This project was a part of a larger research program that included collaboration within and across the project - all participants are acknowledged. My main responsibilities included brain tissue processing, cutting, and sample preparation, as well as optimizing and conducting all RNAscope® steps, which was the main technique used in this project. After imaging was conducted for me, I processed the images with the Fiji and used CellProfiler to generate the data from the images. After the results tables from the data were generated for me, I conducted the statistical analysis and visualized the results by using GraphPad. In addition to these main responsibilities, I followed intensively all the steps done on animals and practiced animal care during the ABA paradigm with one cohort. This project offered valuable information to increase the understanding of the receptor-specific effects that are likely to improve the clinical application of psilocybin to individuals with AN. More broadly, these studies provide a more detailed understanding of serotonin function in the ABA model than has previously been achieved, and this molecular-level understanding will be used to inform many future studies using the model.

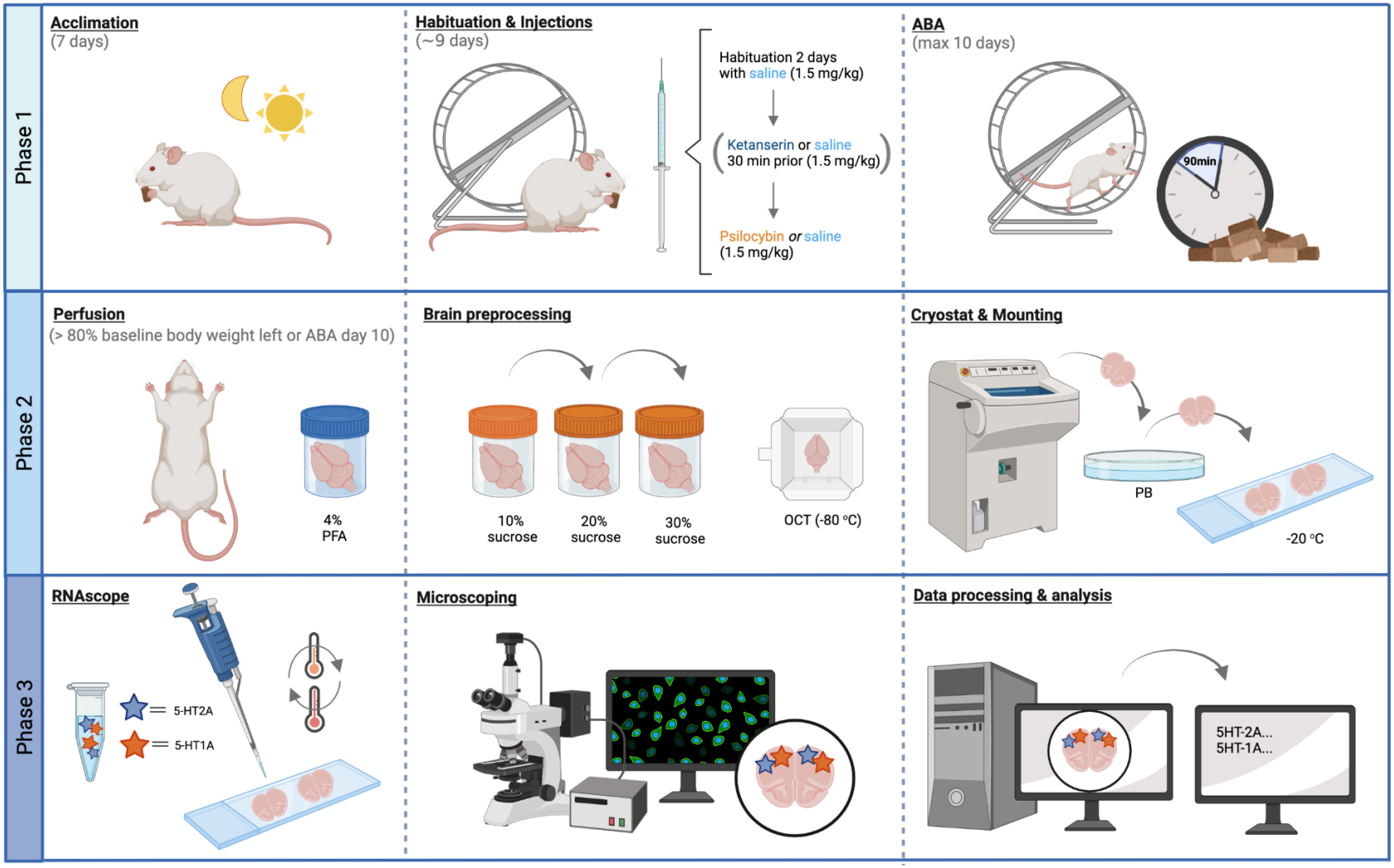

FIGURE 1 The workflow of the project. Animal work conducted is shown in phase 1, which included animals to acclimate into the environment and reversed light cycle, habituation to running wheels and injections as well as the ABA paradigm. In phase 2 the animals were euthanized, brains collected and processed, and finally sectioned for RNAscope®. RNAscope®, microscopy, data processing, and analysis were performed in phase 3.

3 | MATERIALS AND METHODS

3.1 | Animals and housing

All experiments done on animals were approved by the Monash Animal Resource Platform ethics committee (MARP-1 AEC, Project ID: 21943) and performed by following the Australian code for the care and use of animals for scientific purposes. Those animals exposed to ABA conditions had unlimited access to running wheels and water, while food accessibility was limited for 90 minutes. These animals were allowed to lose a total of 20% of their baseline body weight (susceptible individuals) or maintained under ABA conditions for a maximum of 10 experimental days (resistant individuals). Rats that remained naïve to ABA conditions (control; non-ABA) had *ad libitum* access to food and water throughout the experiments. After experiments we euthanized animals with 300 mg/kg sodium pentobarbitone (Lethabarb; Virbac, Australia), followed by transcardial perfusion by using first 200 ml 0.9% NaCl (Merck Group, Germany) in dH_2O and then 200 ml 4% paraformaldehyde (PFA; Scharlab, Spain) in 0.1 M phosphate buffer (PFA-PB).

A total number of 62 female Sprague Dawley rats were used, which were six weeks old upon arrival of (Monash Animal Research Platform, Melbourne, Australia), and weighing 125–200 g. After one week of acclimation, ABA exposed rats were housed individually in transparent activity wheel and living cages (Lafayette Instruments, model no. 80859S) and non-ABA rats in standard polypropylene rat cages (410 x 282 x 153mm) in groups. The room temperature was kept around 22.5-25°C and humidity around 60 ± 10%. Rats were under a reversed 12 h light cycle (lights off at 11:00 am) and had *ad libitum* access to food (Standard laboratory chow; Barastoc feeds) and water before the ABA animals were exposed to ABA conditions. We monitored the welfare of the rats daily. To synchronize the female rats' estrous cycle, an individually housed male rat was placed in the experimental room at acclimation and throughout the experimental procedures (known as the Whitten effect; Cora et al., 2015).

3.2 | Experimental design

We had both ABA and non-ABA animals that were divided into four cohorts. Each cohort was done at separate times. Cohorts 1, 3, and 4 had seven saline-treated and seven psilocybin-treated animals in each cohort and were exposed to the ABA paradigm. Cohort 2 included five saline-treated and five psilocybin-treated non-ABA animals (Table 1).

TABLE 1 The number of animals in each treatment group in each cohort. Cohorts 1, 3, and 4 include the animals exposed to the ABA paradigm, whereas cohort 2 includes the non-ABA animals.

Cohort	Total number of animals	Saline (number of animals)	Psilocybin (number of animals)
1	14	7	7
2	10	5	5
3	14	7	7
4	14	7	7

Following a one-week period of acclimation to reversed 12 h light cycle, we housed ABA rats individually and allowed up to nine days of running wheel training to establish a stable baseline of activity. On the first day of ABA exposure, animals' food consumption and body weight were measured daily, immediately prior to the onset of the dark phase (between 10:30 am and 11:00 am), whereas running wheel activity was recorded continuously. An example of these measures in ABA rats can be seen in Figure 2. Group housed non-ABA animals' welfare was checked daily, but since they did not have access to running wheels and the food consumption was not limited, these measurements were not conducted.

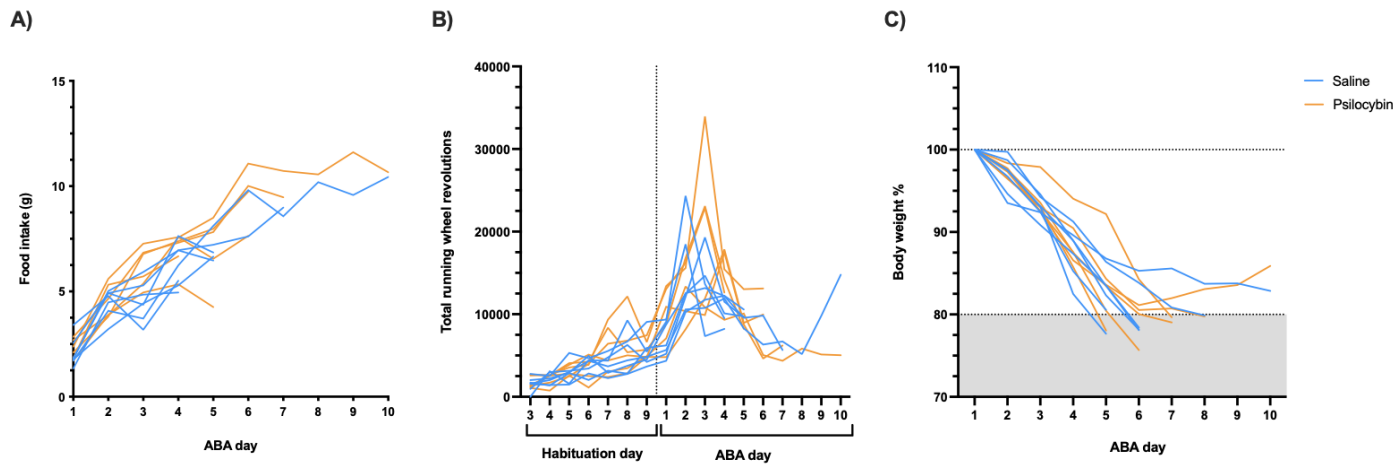

FIGURE 2 Representation of ABA paradigm. Even though **(A)** the daily food intake increases, the **(B)** increased running wheel activity causes the mismatch in homeostatic balance, leading to the **(C)** rapid bodyweight decline. The permission to use these graphs to illustrate the ABA paradigm was given by Amelia Trice whose project ran parallel to this project, focusing on the behavior of these animals.

All rats were habituated to restraint and intraperitoneal injection for two consecutive days (both times administered saline) after which the treatment of psilocybin or saline was administered on the third injection day. Cohorts 1 and 2 administered one dose of treatment, whereas cohorts 3 and 4 administered three doses of treatment either chronically (cohort 3) or on days separated (cohort 4) (Figure 3). The injections for non-ABA animals were done in an open field setting, whereas injections for ABA animals were done in their home cages. ABA exposure commenced 24 hours after the last injection, except the cohort 3 where animals received the last injection on the first day of ABA exposure (Figure 3).

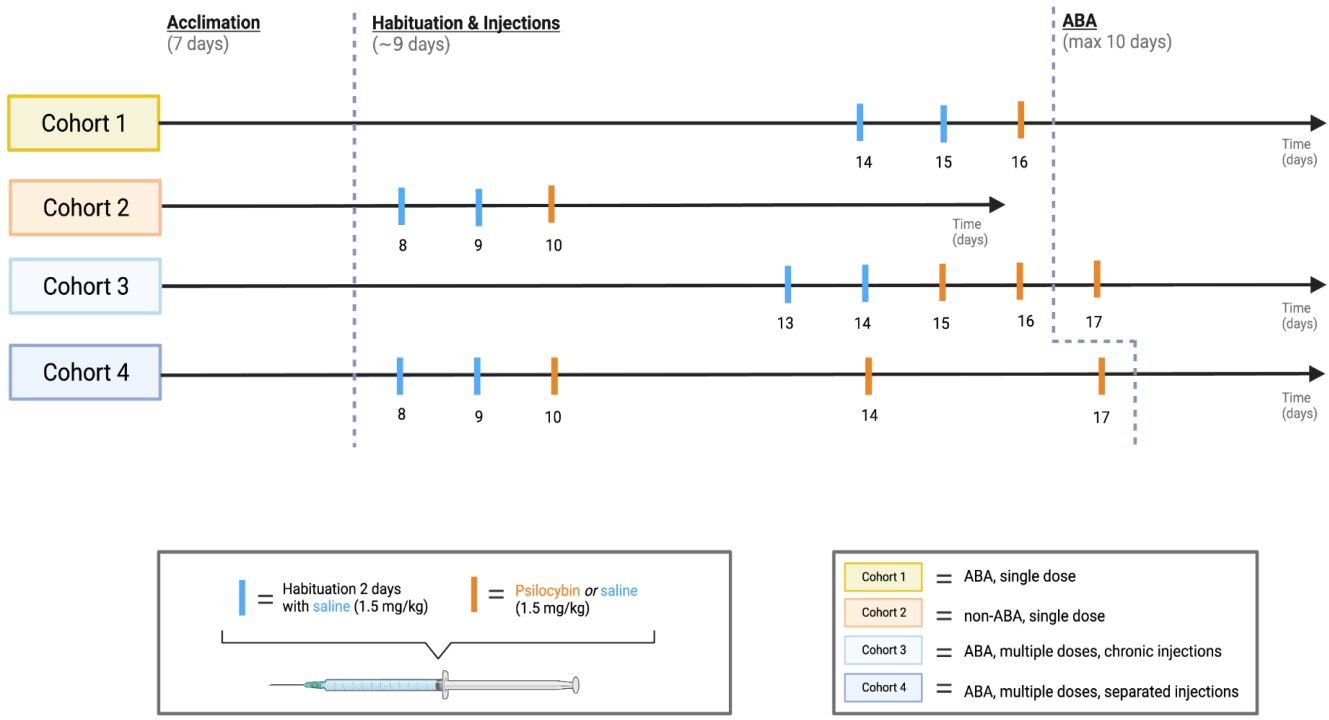

FIGURE 3 The experimental design of the project. Each cohort had a habituation period for injections with saline two days prior to the first treatment injection (saline or psilocybin). ABA animals from cohort 1 and non-ABA animals from cohort 2 administered one dose of treatment, whereas ABA animals from cohorts 3 and 4 administered three doses of treatment either chronically injected (cohort 3) or on days separated (cohort 4). The ABA paradigm started a day after treatment injections, except in cohort 3 where animals received the last injection on the first day of the ABA paradigm. ABA exposure lasted for max. 10 days or until 20 % of the baseline body weight was lost.

Animals were euthanized for brain collection either when they reached the body weight criterion in ABA (> 20 % of their baseline body weight; susceptible) or on day 10 of the ABA paradigm exposure (resistant). Any animals that gained weight during ABA exposure, likely due to food hoarding behavior, were excluded from the analyses. Non-ABA rats were euthanized for brain collection six days after drug injections, mimicking the estimated time delay between treatment and collection for susceptible ABA rats.

3.3 | Drugs and treatments

We used sterile saline solution (0.9% Sodium Chloride, Baxter, United States) to dissolve psilocybin (USONA institute, WI, USA; Lot #AMS0167, >99% purity) at a concentration of 1.5mg/ml and sterile

0.9% saline solution only as the control vehicle. Saline or psilocybin (1.5 kg/mg) was administered via intraperitoneal (IP) injections at a volume of 1 ml/kg, using a 27” gauge needle and syringe. The number of animals in each treatment group is shown in Table 1. Injections were conducted immediately prior to the onset of the dark phase, between 10:30 am and 11:00 am for ABA animals. Allocation to treatment groups was conducted by an experimenter blind to behavioral outcomes and only based on ensuring that an even spread of body weight, food intake, and baseline wheel running was present in both treatment groups. The administration route (IP) was chosen to ensure good bioavailability and solid absorption and the dose of psilocybin used (1.5 mg/kg) was chosen based on those most commonly used in the rodent literature (Hesselgrave et al., 2021; Shao et al., 2021).

3.4 | Tissue collection and preparation

Animals euthanized with 300 mg/kg sodium pentobarbitone, were transcardially perfused with 200 ml 0.9% NaCl followed by 200 ml 4% PFA-PB. After perfusion brains were post-fixed in freshly prepared 4% PFA-PB solution for 24h at 4 °C, followed by submersion in sucrose (Sigma-Aldrich, United States) series from 10% to 20% to 30% in 0.1 M PB until they sank to the bottom of the container, that took approximately four days in total. After the last 30% sucrose submersion, brains were frozen over dry ice in plastic molds using Tissue-Tek O.C.T. Compound (ProSciTech, QLD, Australia) and stored at -80 °C until sectioning. Brains were brought to -20 °C and sectioned in the coronal plane at the same temperature using a cryostat (CM1860, Leica Biosystems) Coordinates for the mPFC (interaural: 13.20-11.20 mm, bregma: 4.20-2.20 mm) were based from the “The Rat Brain in Stereotaxic Coordinates” (Figure 5; Paxinos & Watson, 1998). The areas of interest were infralimbic (IL) and prelimbic (PrL), the regions representing functionally distinct subregions of the mPFC. Sections were cut at 14 µm thickness and collected into wells of cell culture plates containing either cryoprotectant solution (30% $C_2H_6O_2$, 20% $C_3H_8O_3$, 50% 0.1 M PB) or plain 0.1 M PB and mounted onto Superfrost™ Plus slides (catalog no. SF41296SP, Eppredia).

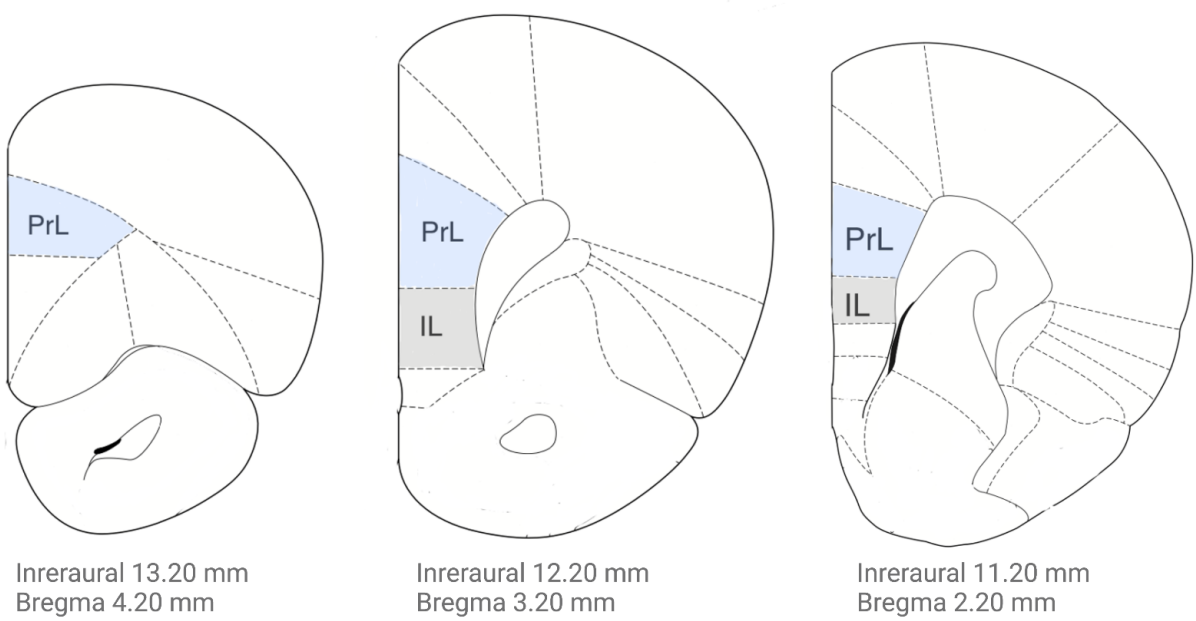

FIGURE 4 Stereotaxic coordinates of rat brain. Brains were sectioned in the coronal plane with the range of interaural: 13.20-11.20 mm, bregma: 4.20-2.20 mm (left:13.20 mm, 4.20 mm; middle: 12.20 mm, 3.20; 11.20 mm, 2.20 mm). The areas of interest were PrL and IL, the subregions of the mPFC.

Owing to time limitations, only the rat brains shown in the Table 2 that were chosen for RNAscope® were sectioned, and two sections per animal were mounted onto slides for processing. As we were interested in examining the consequences of ABA exposure on RNA molecules of specific 5-HT receptors expression compared to non-ABA conditions, all ABA brains chosen for RNAscope® were those that were susceptible to weight loss. Once slide-mounted, sections were stored with desiccants at -20 °C until the RNAscope® protocol was performed on the following day.

TABLE 2 The animals from different cohorts with different treatments chosen for RNAscope®. ABA cohorts 1, 3, and 4 had two animals from each treatment group, whereas the non-ABA animals from cohort 2 had three animals from each treatment group chosen for RNAscope® protocol.

Cohort	Animal (ID)	Treatment	The final ABA day
1	1	Saline	7
1	14	Saline	7
1	3	Psilocybin	9
1	11	Psilocybin	8
2	3	Saline	NA
2	4	Saline	NA
2	5	Saline	NA
2	13	Psilocybin	NA
2	14	Psilocybin	NA
2	15	Psilocybin	NA
3	1	Saline	5
3	11	Saline	6
3	5	Psilocybin	6
3	10	Psilocybin	6
4	1	Saline	5
4	5	Saline	5
4	4	Psilocybin	6
4	6	Psilocybin	5

3.5 | RNAscope® Protocol

The RNAscope® Multiplex Fluorescent Reagent Kit v2 (catalog no. 323100, Advanced Cell Diagnostics) was used in accordance with the protocol from the manufacturer (document no. 323100-USM) following the steps for fixed frozen tissue with slight modifications. Shortly, the steps included unmasking the target RNA and permeabilization as a pretreatment, probe hybridization to target RNA, and signal amplification, followed by the conjugation of fluorescent-labeled probes (fluorophores). These steps are visualized in Figure 5. For full protocol details, see Appendices (Appendix A). A RNAscope® Probe- Rn-Htr2a (catalog no. 424551, Advanced Cell Diagnostics) was chosen for channel 1 to detect RNA of 5-HT_{2A} receptors, and a RNAscope® Probe- Rn-Htr1a-C2 (catalog no. 404801-C2, Advanced Cell Diagnostics) for channel 2 to detect RNA of 5-HT_{1A} receptors. We prepared the probe mix following the protocol instructions by diluting the C2 probe into the C1 probe (1:50).

We used fluorophores Opal™ 620 in Dimethyl sulfoxide (DMSO) (catalog no. FP1495001KT, Akoya Biosciences) for channel 1 and Opal™ 520 in DMSO (catalog no. FP1487001KT, Akoya Biosciences) for channel 2, in this protocol, resulting in RNA molecules of 5-HT_{2A} receptors being labeled red and RNA molecules of 5-HT_{1A} receptors being labeled green. We diluted both fluorophores (Opal™ 620, 1:750; Opal™ 520, 1:500) directly before the use into RNAscope® Multiplex TSA Buffer found in the kit. DAPI solution, included in the kit, was used for nuclei detection.

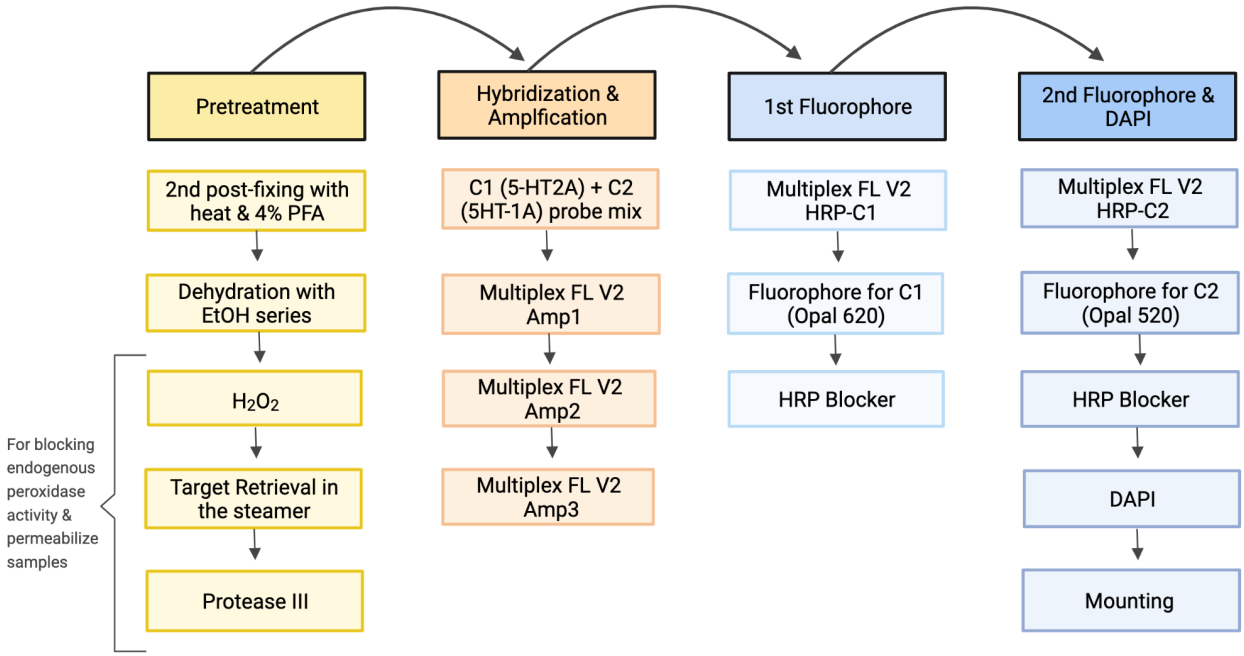

FIGURE 5 RNAscope® workflow. Pretreatment steps of RNAscope® were followed by hybridization and amplification steps and continued to the steps including the first, and the second fluorophore as well as the DAPI.

3.6 | Imaging and image analysis

We used the Leica Thunder Imager 3D Assay, a wide field fluorescent microscope for imaging the RNAscope® samples, running Leica Application Suite X (LAS X, 2021, version 3.7_5_24914) software. Micrographs were captured by Dr. Kyna Conn with a magnification of 40x oil immersion objective (NA = 1.25) and the LED light source. Wavelengths of 405 nm (DAPI to detect neuronal nuclei), 488 nm (FITC), and 532 nm (TXred) were used throughout the imaging process (see Table 3 for intensity and exposure times).

The images of mPFC subregions (IL and PrL) were captured with Z-stacks (5-15 µm) from both hemispheres (Figure 4). Micrographs were deconvoluted, images merged and saved as 16 bits lif-files. Few sections were unable to be imaged due to folding of the brain section (cohort 1: no. 3, no. 11 PrL1 and cohort 2: no. 15 IL2; Table 2) and due to technical challenges with the analysis pipeline development, time permitted imaging of only one section for each brain region per animal.

TABLE 3 Imaging settings. Detailed microscope settings for 5-HT_{1A} receptors, 5-HT_{2A} receptors, and nuclei staining.

Target	Probe	Fluorophore	Wavelength (nm)	Intensity (%)	Exposure time (ms)
5-HT_{1A}R	Rn-Htr1a-C2	Opal 520	488	30	100
5-HT_{2A}R	Rn-Htr2a	Opal 620	532	30	300
Nuclei		DAPI	405	17	300

Images were pre-processed for analysis using a macro in Fiji (version 2.3.0, 64 bit), designed to batch process all the lif-files into a maximum intensity projection (MIP) for Z-stack flattening, converting the images from 16 bits to 8 bits for reducing the volume of the file, splitting the three channels and finally saving the resulting images as a tiff format. These images were then analyzed with a custom-built pipeline in the CellProfiler (version 4.2.1) that was modified from Carpenter et al. (2006). The pipeline was designed to recognize primary objects, including nuclei and the dots representing either RNA molecules of 5-HT_{1A}, or 5-HT_{2A} receptors (“dots”). The pipeline also created an artificial cell membrane around the nuclei, and by doing so related the other primary objects for each cell.

The number of both dots was the main factor evaluated by the pipeline since each dot represented one RNA molecule and was the interest of this study. It is important to note that the size nor intensity of the dots does not describe the number of transcripts, but instead the number of probes bound to the target RNA and thus was not taken to the statistical evaluation. Both the macro and pipeline were designed and modified to suit this project by Dr. Jihane Homman-Ludiye. An overview of the pipeline can be seen in Appendices (Appendix B; Figure 14).

3.7 | Statistical analysis

The CellProfiler pipeline generated absolute numbers of cells and dots associated with these cells for every section (Figure 6). These outputs were used to calculate a multitude of outcome variables for each section (or region by combining sections) for each animal using a custom Python script designed by Dr. Laura Milton. The script generated results tables for each treatment group ready for input into GraphPad for graphing and statistical analysis for every outcome of interest.

GraphPad Prism (version 9.4.1 (458)) was used both for statistical analysis and graphics. One - and two-way analysis of variance (one- and two-way ANOVA) were used to examine differences elicited by ABA exposure (ABA vs non-ABA) and treatment (saline vs psilocybin) followed by post hoc Tukey's multiple comparisons when applicable. Unpaired t-tests were used when evaluating the mean number of cells between ABA and non-ABA, regardless of treatment. All data were represented with the mean ± SEM and were considered statistically significant when $p < .05$ ($*p < .05$, $**p < .01$, $***p < .001$, $****p < .0001$ and not significant, ns $p > .05$).

4 | RESULTS

In order to study how the development of pathological weight loss and/or psilocybin administration influenced the expression of RNA molecules of 5-HT_{1A} and 5-HT_{2A} receptors expression in rats' mPFC (Figure 4), we analyzed the results from the CellProfiler (Figure 6). This was done by looking at the outcomes such as the cells expressing either RNA molecules of receptor subtype and the number of dots representing the RNA molecules of either receptor subtype. From now on "the RNA molecule of 5-HT_{1A} and 5-HT_{2A} receptors" is replaced by "5-HT_{1A} and 5-HT_{2A} receptors" to aid readability. Cells expressing at least three dots of one receptor subtype, regardless if they had less than three dots of the other receptor subtype, were counted as cells that express the receptor of interest (here called cells exclusively expressing the receptor of interest). This outcome was chosen for analysis by assuming it means those cells will affect the action of the cell with greater probability. Whereas the number of dots evaluating the extent of expression of RNA was chosen for analysis, because the processing power and actions in the mPFC may depend on the intensity of these molecules.

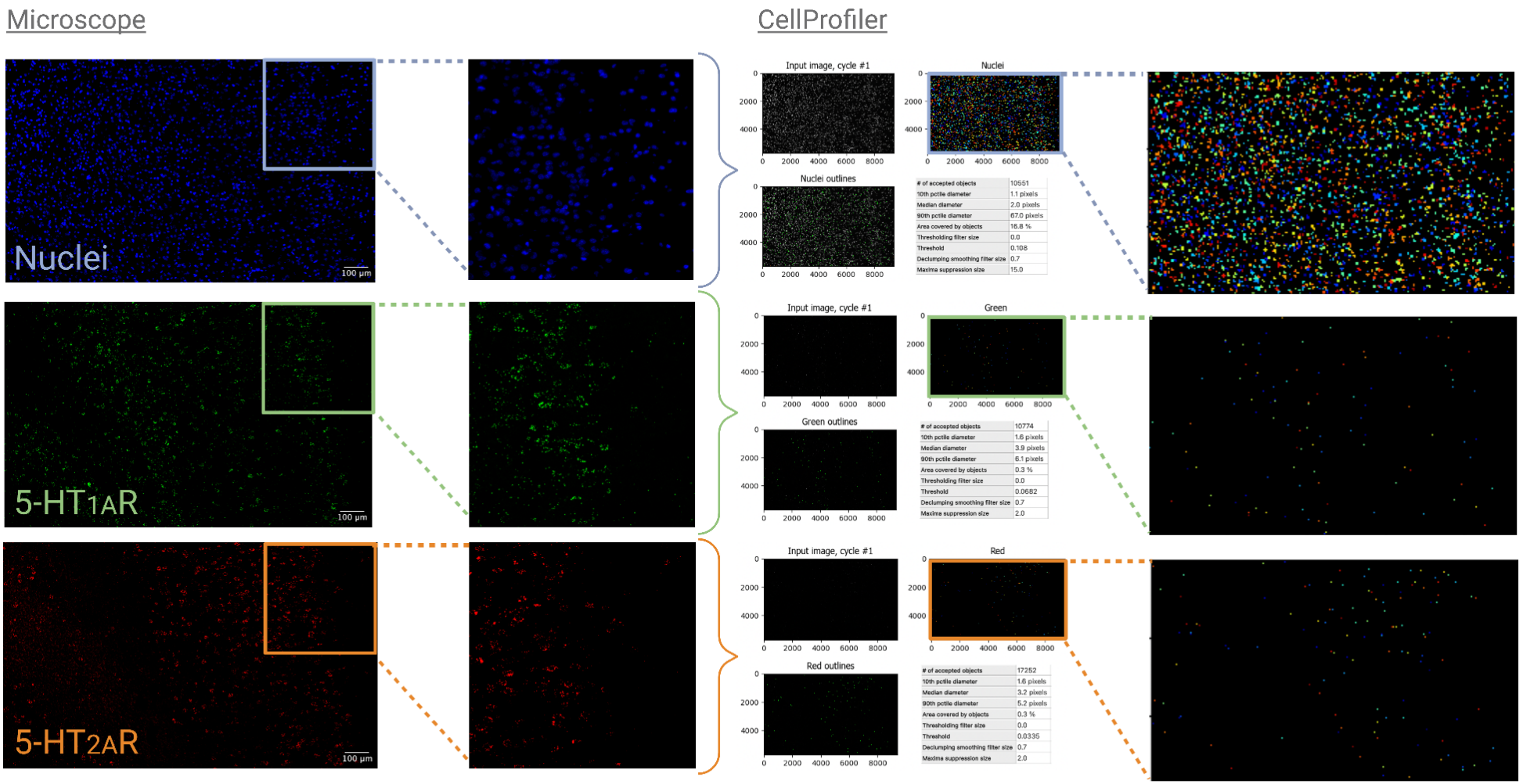

FIGURE 6 Illustration of CellProfiler outcome measurements from micrographs. Images from micrographs representing nuclei (blue), 5-HT_{1A}- (green), and 5-HT_{2A} (red) receptors followed by the CellProfiler outcome measurements of identifying these primary objects. Results representing IL section 1, from animal number 1, from cohort 1, (Table 2).

4.1 | Volume of cells

In order to ensure that any differences in expression of receptor subtypes (5-HT_{1A} and 5-HT_{2A}) were not simply due to variation in total neuron density in the microscopy images, we first determined the total number of neurons (cells) in each image. At first, we did the total cell count comparison for all saline and psilocybin-treated ABA and non-ABA animals separately to see if there were any differences in the number of cells in the different subregions of the mPFC (IL and PrL). There were no significant differences in total cell count between the different mPFC subregions within the ABA nor in the non-ABA group. However, there were some outlying measures for IL and PrL sections within the ABA and non-ABA cohorts possibly due to variance in section thickness within the sections (ABA, $p = .515$; non-ABA, $p = .581$; Figure 7A). Next, we compared the mean number of cells per section in the mPFC and found that there was a significant difference between cohorts ($p = .025$; Figure 7B). This difference can be explained by the variance both in the rostral-caudal axis (ABA from more caudal vs non-ABA from the more rostral side) and in sections' thicknesses (5 - 15 µm) caused by the unreliability of cryostat.

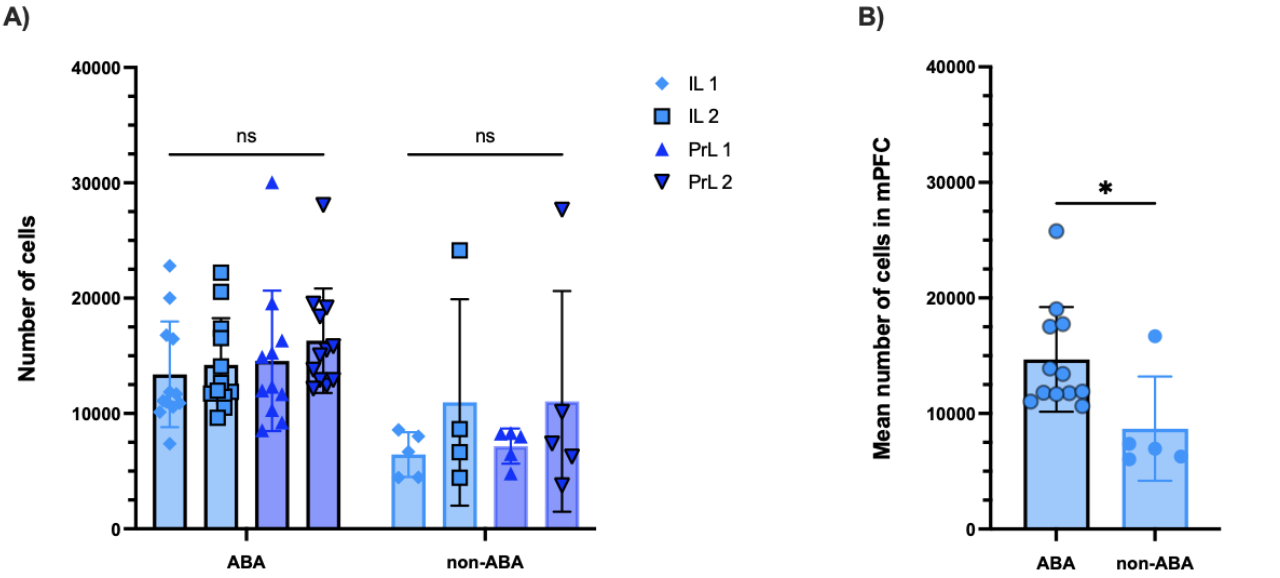

FIGURE 7 The total number of cells in subregions of the mPFC and the mean number of cells in the mPFC. One-way ANOVA showed no significant differences in **(A)** total cell count between the regions of the mPFC within cohorts (ABA, *F(3, 43) = .774, p = .515*; non-ABA, *F(3, 15) = .675, p = .581*), but the unpaired t-test done for **(B)** mean number of cells in the mPFC showed significant difference between cohorts (*t(15) = 2.497, p = .025*). All data is represented with the mean ± SEM. *n* (ABA, total) = 12, *n* (non-ABA, total) = 5.

Based on these results we decided to continue the comparison between cohorts by looking at the proportion of the cells that express RNA molecules of specific receptors together with the average number of these RNA molecules across all cells to ensure a reliable quantitative analysis of the results.

4.2 | ABA alters the expression of 5-HT$_{2A}$, but not 5-HT$_{1A}$ receptors in cells exclusively expressing the receptors

In order to determine whether the proportion of cells expressing specific receptors differed between subregions of the mPFC within ABA and non-ABA cohorts, we compared the results between these regions. We pooled ABA cohorts 1,3 and 4 (Table 2) with the same treatment together, regardless of the dosing paradigm, because there were no significant differences in the expression of receptor subtypes between the cohorts, treated with psilocybin (Appendix C; Figure 15). No significant differences in cells exclusively expressing 5-HT$_{1A}$ or 5-HT$_{2A}$ receptors were shown between the subregions of mPFC within cohorts (all *p's* > .05; Figure 8A-B), therefore the results that will follow, will be presented for the total mPFC including both IL and PrL subregions.

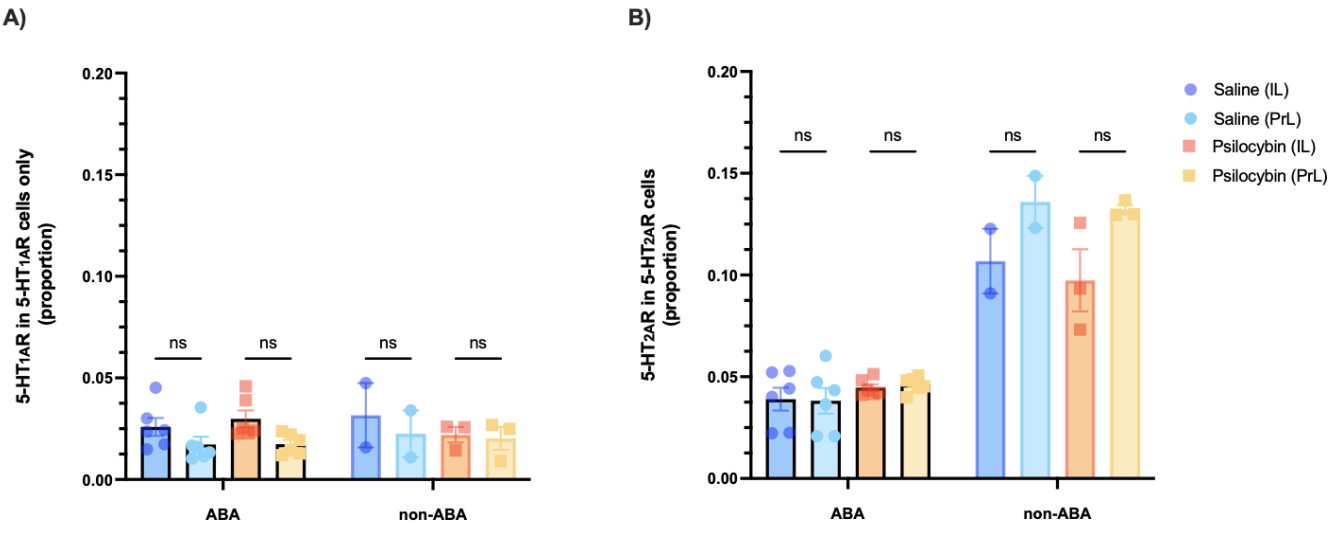

FIGURE 8 The proportion of cells exclusively expressing 5-HT$_{1A}$ or 5-HT$_{2A}$ receptors in the subregions of mPFC within cohorts and treatment groups. Two-way ANOVA showed no significant differences in **(A)** cells exclusively expressing 5-HT$_{1A}$ receptors nor in **(B)** cells exclusively expressing 5-HT$_{2A}$ receptors between the regions of the mPFC within the treatments of each cohort (all *p's > .05*). All data is represented with the mean ± SEM. *n* (ABA, total saline) = 6, *n* (non-ABA, total saline) = 2, *n* (ABA, total psilocybin) = 6, *n* (non-ABA, total psilocybin) = 3.

In order to examine the effects of psilocybin treatment and ABA exposure on 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors expression, we compared the cells expressing exclusively these receptor subtypes between treatment groups (saline and psilocybin) within cohorts (ABA and non-ABA), and between cohorts within different treatment groups seen in Figure 9. The exposure to the ABA paradigm did not cause significant differences in the proportion of cells expressing 5-HT$_{1A}$ receptors within the treatment group (p = .813; Figure 9A). Whereas when looking at the proportion of cells expressing 5-HT$_{2A}$ receptors, they were significantly lower within the treatment group caused by ABA exposure (both p's < .0001; Figure 9B). Treatment with psilocybin did not cause significant differences in the proportion of cells expressing 5-HT$_{1A}$ or 5-HT$_{2A}$ receptors (both p's > .05; Figure 9A-B). The subregional differences considering these results were not statistically significant and are shown in the Appendices (Appendix D; Figure 16A-B).

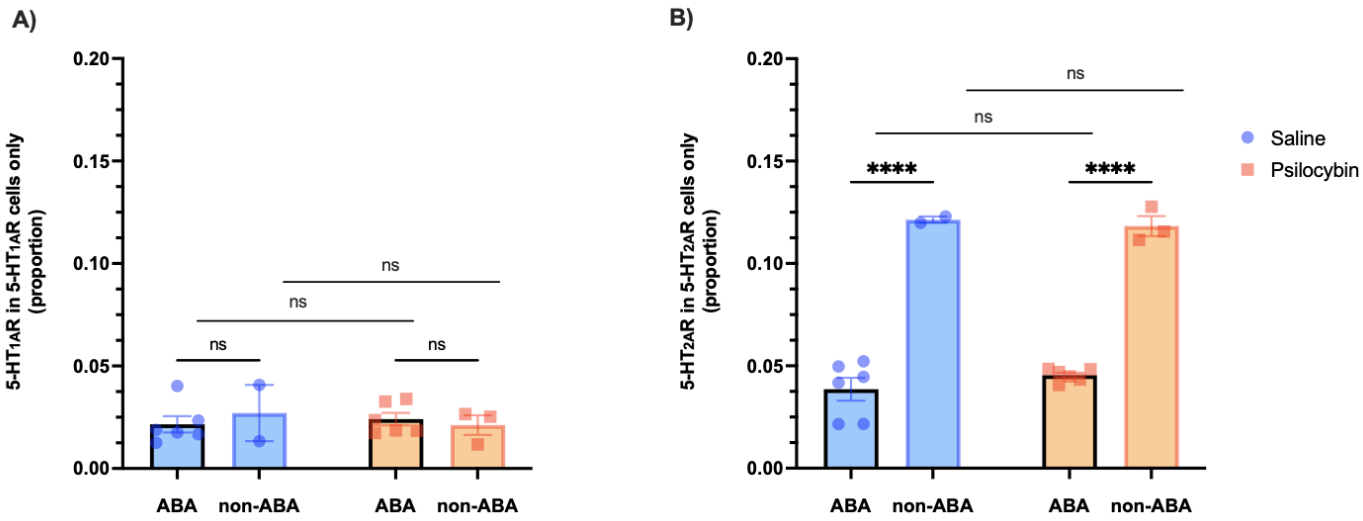

FIGURE 9 The proportion of cells exclusively expressing 5-HT$_{1A}$ or 5-HT$_{2A}$ receptors in the mPFC within and between cohorts and treatment groups. Two-way ANOVA showed no significant differences in **(A)** the proportion of cells exclusively expressing 5-HT$_{1A}$ receptors neither between cohorts or treatments (Cohort, *F(1, 13) = .058, p = .813*; Treatment, *F(1, 13) = .098, p = .759*) in the mPFC. Whereas two-way ANOVA performed for **(B)** the proportion of cells exclusively expressing 5-HT$_{2A}$ receptors showed significant differences between cohorts within treatments (*F(1, 13) = 239.1, p < .0001*) followed by post hoc Tukey's Multiple comparisons (both *p's < .0001*), but no significant differences between treatments within cohorts (*F(1, 13) = .140, p = .714*). All data is represented with the mean ± SEM. *n* (ABA, saline) = 6, *n* (non-ABA, saline) = 2, *n* (ABA, psilocybin) = 6, *n* (non-ABA, psilocybin) = 3.

Based on these results we can say that only exposure to the ABA paradigm caused significant differences in the proportion of cells exclusively expressing 5-HT$_{2A}$ receptors by lowering the levels. To see if the extent of RNA expression of 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors would have differences in results, we continued examining the average number of 5-HT$_{1A}$ or 5-HT$_{2A}$ receptors across all cells.

4.3 | ABA alters the expression of 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors across all cells

We repeated the same comparison for the average number of 5-HT$_{1A}$ or 5-HT$_{2A}$ receptors across all cells, with significant differences observed in 5-HT$_{1A}$ receptors across cells within saline-treated, but not in psilocybin-treated -animals due to ABA exposure (saline, p = .001; psilocybin p = .344; Figure 10A). But due to the low n in saline-treated non-ABA animals and high variance among these individuals, especially the results showing cells expressing 5-HT$_{1A}$ receptors should be treated with caution (Figure 10A). ABA exposure caused statistically significant differences in 5-HT$_{2A}$ receptors expression across all cells within treatment groups (saline, p < .0001 ; psilocybin, p = .0001 ; Figure 10B). Treatment with psilocybin did not cause significant differences in average number of either receptor subtypes within cohorts (5-HT$_{1A}$R, p = .376; 5-HT$_{2A}$R, p = .279; Figure 10A-B).

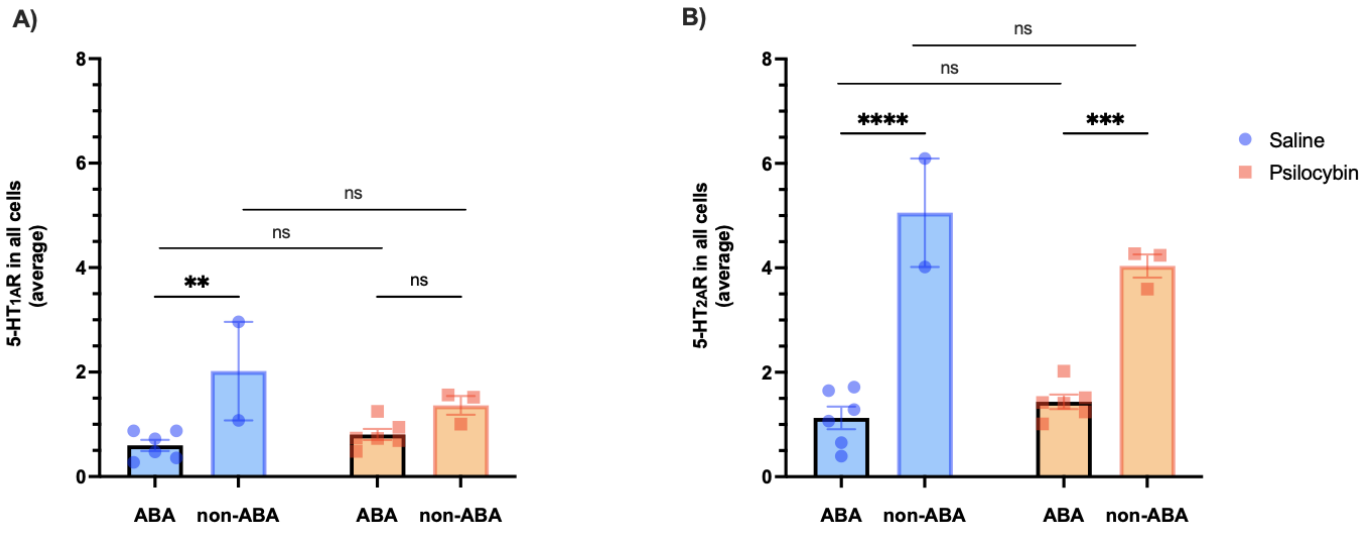

FIGURE 10 The average number of 5-HT$_{1A}$ or 5-HT$_{2A}$ receptors across all cells in the mPFC within and between cohorts and treatment groups. Two-way ANOVA was performed for **(A)** average number 5-HT$_{1A}$ receptors across all cells and showed significant differences between saline-treated cohorts (*F(1, 13) = 16.380, p = .001*) followed by post hoc Tukey's Multiple comparisons (*p = .001*), but no significant differences between psilocybin-treated cohorts nor between treatments within cohorts in the mPFC (Psilocybin cohort, *p = .344*); Treatment, *F(1, 13) = .841, p = .376*). Whereas two-way ANOVA performed for **(B)** average number of 5-HT$_{2A}$ receptors showed significant differences between cohorts within treatments (*F(1, 13) = 107.3, p < .0001*) followed by post hoc Tukey's Multiple comparisons (saline, *p < .0001*; psilocybin, *p = .0001*), but no significant differences between treatments within cohorts (*F(1, 13) = 1.277, p = .279*). All data is represented with the mean ± SEM. *n* (ABA, saline) = 6, *n* (non-ABA, saline) = 2, *n* (ABA, psilocybin) = 6, *n* (non-ABA, psilocybin) = 3.

4.4 | ABA alters the expression of 5-HT$_{1A}$ and/or 5-HT$_{2A}$ receptors regardless of the psilocybin treatment

Based on the previous finding we wanted to see if these alterations caused by ABA exposure, would happen regardless of drug treatment. For this comparison, all saline- and psilocybin-treated animals were pooled together within cohorts. ABA exposure did not cause statistically significant differences in the proportion of cells exclusively expressing 5-HT$_{1A}$ receptors, but it caused significantly lower levels of cells expressing 5-HT$_{2A}$ receptors, regardless of the psilocybin treatment (5-HT$_{1A}$R, p = 0.999; 5-HT$_{2A}$R, p < .0001, Figure 11A). Whereas ABA exposure caused significantly lower levels of 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors across all cells, regardless of the psilocybin treatment (both *p's* < .0001; Figure 11B).

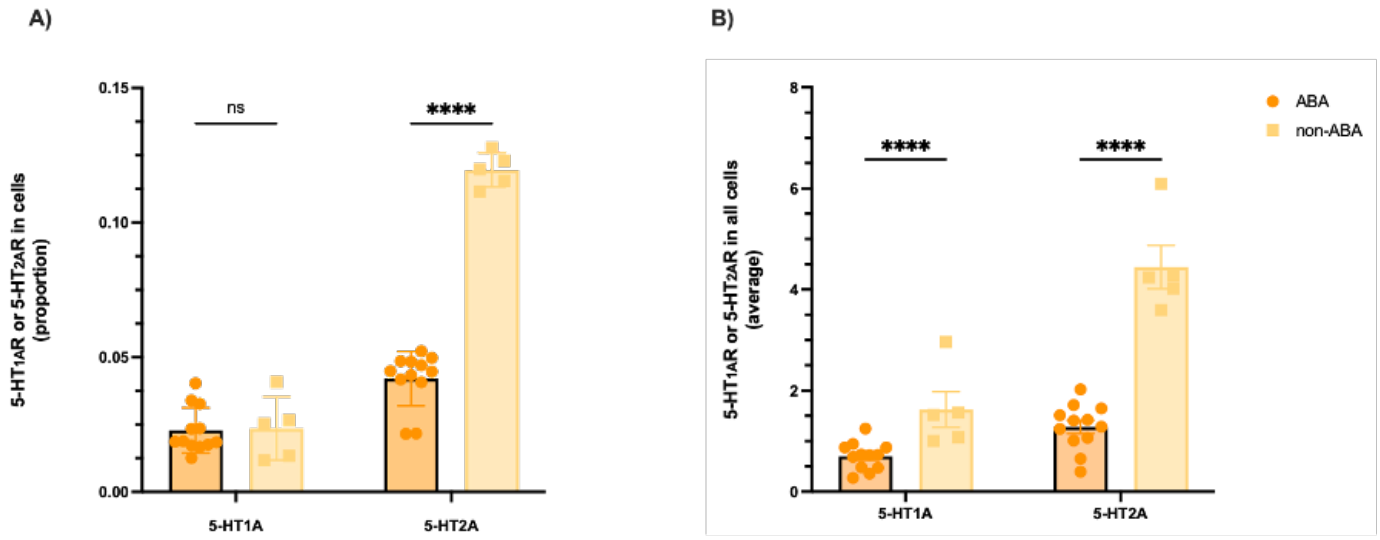

FIGURE 11 The proportion of cells exclusively expressing 5-HT$_{1A}$ or 5-HT$_{2A}$ receptors and the average number of 5-HT$_{1A}$ or 5-HT$_{2A}$ receptors across all cells in the mPFC between cohorts regardless of the psilocybin treatment. Two-way ANOVA was performed for **(A)** the proportion of cells exclusively expressing 5-HT$_{2A}$ and 5-HT$_{1A}$ receptors (*F(1, 30) = 123.8, p < .0001*), followed by post hoc Tukey's Multiple comparisons indicating no statistically significant differences in cells expressing 5-HT$_{1A}$, but significant differences in 5-HT$_{2A}$ receptors (5-HT$_{1A}$R, *p = .999*; 5-HT$_{2A}$R, *p < .0001*) between cohorts in the mPFC. There were also significant differences between the 5-HT$_{2A}$ and 5-HT$_{1A}$ receptors expression alone, (*F(1, 30) = 268.6, p < .0001*). Two-way ANOVA showed **(B)** statistically significant differences in the average number of 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors across all cells between cohorts (*F(1, 31) = 50.420, p < .0001*), followed by post hoc Tukey's Multiple comparisons (both *p's < .0001*). There were also significant differences between the 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors' expression alone (*F(1, 31) = 22.320,* both *p's < .0001*). All data is represented with the mean ± SEM. *n* (ABA) = 12, *n* (non-ABA) = 5.

These results together show that ABA exposure caused significantly lowered levels of the proportion of cells exclusively expressing 5-HT_{2A} receptors as well as reduced levels of 5-HT_{1A} and 5-HT_{2A} receptors across all cells, regardless of psilocybin treatment (Figure 11A-B). However, many neurons in the mPFC express both 5-HT receptor subtypes, and thus it was important to assess the effects of ABA exposure and psilocybin treatment on the full complement of 5-HT expressing neurons.

4.5 | ABA alters both the proportion of *all* cells expressing 5-HT_{1A} or 5-HT_{2A} receptors, and cells co-localizing these receptor subtypes

In order to examine the effects of psilocybin treatment and ABA exposure in the proportion of all cells expressing 5-HT_{1A} or 5-HT_{2A} receptors, these expression levels were analyzed. ABA exposure caused a statistically significant difference in cells expressing 5-HT_{1A} receptors in saline-treated animals, but not in psilocybin-treated animals (saline, p = .012; psilocybin, p = .192; Figure 12A). Whereas cells expressing 5-HT_{2A} receptors were significantly lower in both treatment groups caused by ABA exposure (saline, p < .0001; psilocybin, p = .0002; Figure 12B). Due to the low n in saline-treated non-ABA animals and high variance among these individuals, especially the results showing cells expressing 5-HT_{1A} receptors should be treated with caution (Figure 12A). The psilocybin treatment did not cause significant difference in cells expressing either receptor subtypes withing cohorts (5-HT_{1A}R, p = .608; 5-HT_{2A}R, p = 0.716; Figure 12A-B). These results show that ABA exposure causes significantly lowered levels of the proportion of all cells expressing 5-HT_{2A} receptors in the mPFC.

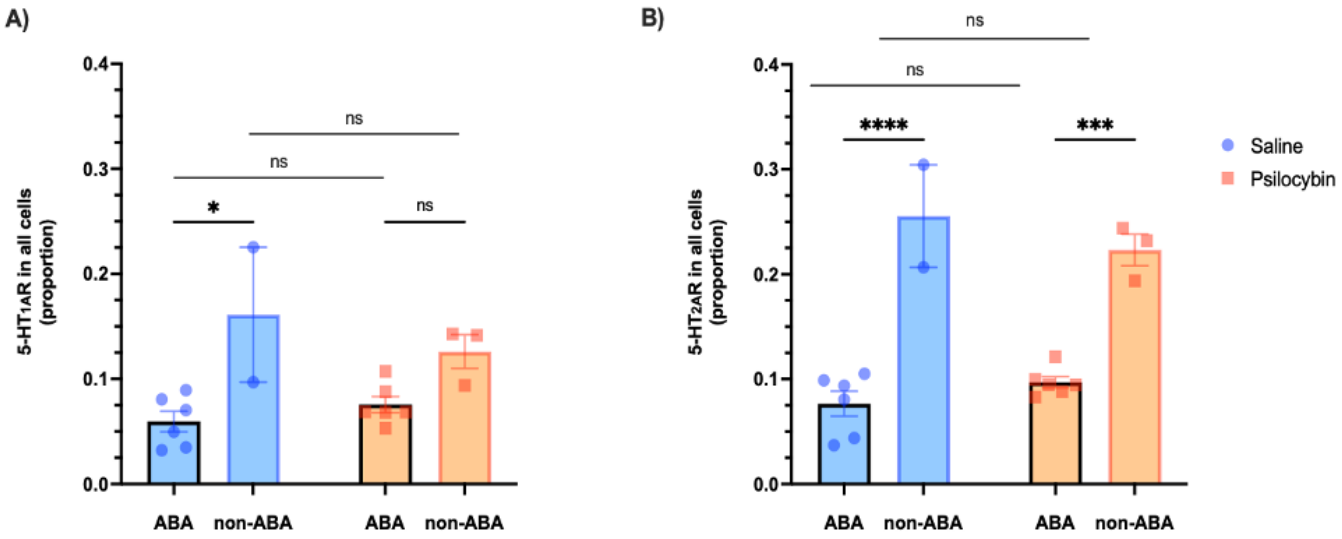

FIGURE 12 The proportion of all cells expressing 5-HT$_{1A}$ or 5-HT$_{2A}$ receptors in the mPFC within and between cohorts and treatment groups. Two-way ANOVA was performed for **(A)** the proportion of all cells expressing 5-HT$_{1A}$ receptors and showed significant differences within saline-treated animals between cohorts, but no significant differences within psilocybin-treated animals (*F (1, 13) = 17.79, p = .001*), followed by post hoc Tukey's Multiple comparisons (saline, *p = .012;* psilocybin, *p = 0.192*). Treatments did not have significant differences within cohorts (F (1, 13) = .276, p = .608). Two-way ANOVA performed for **(B)** the proportion of all cells expressing 5-HT$_{2A}$ receptors showed significant differences between cohorts within the treatment (*F (1, 13) = 91.77 ,p < .0001*), followed by post hoc Tukey's Multiple comparisons (saline, *p < .0001* ; psilocybin, *p = .0002*), but no significant differences between treatments within cohorts (*F (1, 13) = 0.139, p = 0.716*). All data is represented with the mean ± SEM. *n* (ABA, saline) = 6, *n* (non-ABA, saline) = 2, *n* (ABA, psilocybin) = 6, *n* (non-ABA, psilocybin) = 3.

Next, we examined the effect of psilocybin treatment and ABA exposure on the expression of co-localized 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors in the proportion of cells expressing both receptor subtypes. ABA exposure showed a statistically significant difference in cells expressing both 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors within treatment groups (saline, *p* = .002; psilocybin, *p* = 0.045; Figure 13A). But due to the low *n* in saline-treated non-ABA animals and high variance between these individuals, the results considering this data should be treated with suspicion. Psilocybin treatment did not significantly affect cells expressing both 5-HT$_{1A}$ and 5-HT$_{2A}$ receptors (*p* = .576; Figure 13B). The subregional differences considering both results were not statistically significant and are shown in Appendix D (Figure 16C).

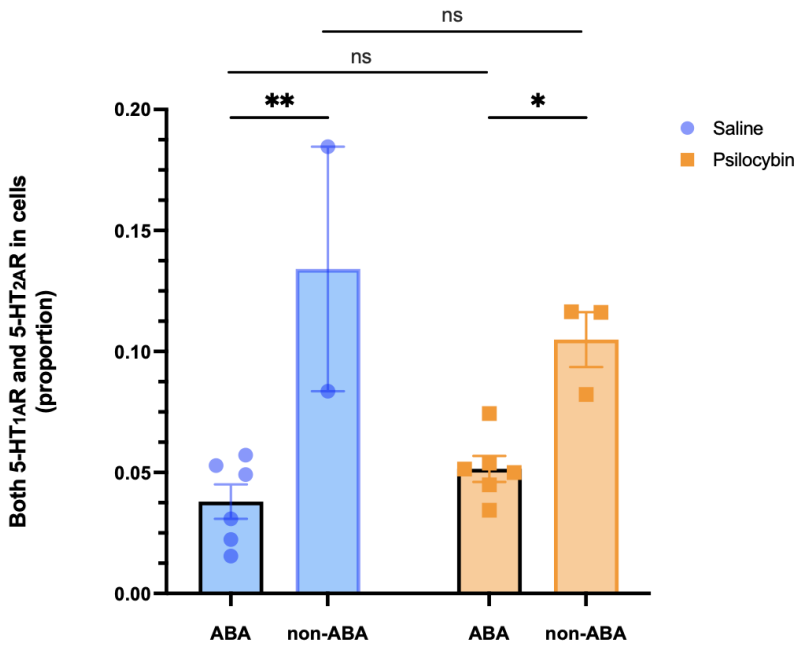

FIGURE 13 The proportion of co-localized 5-HT_{1A} and 5-HT_{2A} receptors in cells expressing both receptor subtypes in the mPFC within and between cohorts and treatment groups. Two-way ANOVA showed statistically significant differences in co-localized 5-HT_{1A} and 5-HT_{2A} receptors in cells expressing both receptor subtypes between cohorts within treatment groups (*F (1, 13) = 30.18, p = .0001*), followed by post hoc Tukey's Multiple comparisons (saline, *p = .002*; psilocybin, *p = .045*), but no significant differences between treatments within cohorts (*F (1, 13) = .329, p = .576*) in the mPFC. All data is represented with the mean ± SEM. *n* (ABA, saline) = 6, *n* (non-ABA, saline) = 2, *n* (ABA, psilocybin) = 6, *n* (non-ABA, psilocybin) = 3.

The results from the proportion of all cells expressing 5-HT_{1A} or 5-HT_{2A} receptors showed significantly lowered levels of 5-HT_{2A}, and possibly 5-HT_{1A} receptors, caused by ABA exposure (Figure 12). Also, co-localized receptors in the proportion of cells expressing both receptor subtypes were significantly lowered in ABA-exposed animals (Figure 13).

5 | DISCUSSION

Individuals with AN demonstrate reduced binding to 5-HT_{2A} receptors and increased binding to 5-HT_{1A} receptors in the prefrontal cortex, with some evidence that this persists the following weight regain (Bailer et al., 2006, 2007; Frank et al., 2002; Kaye et al., 2013). The results presented here support the idea that 5-HT_{2A} receptor expression is central to the neurobiological consequences of pathological weight loss and shows this for the first time at the molecular level in ABA rats. Specifically, we show that neurons exclusively expressing RNA molecules of 5-HT_{2A} receptors, as well as the levels of these molecules across all cells in the mPFC, were significantly lowered in ABA rats compared to controls (non-ABA). There were no significant changes in the cells exclusively expressing RNA molecules of 5-HT_{1A} receptors between ABA and non-ABA, whereas the extent of RNA levels of these receptors were significantly lowered in the ABA animals compared to non-ABA animals. These changes were seen regardless of the treatment (saline or psilocybin). In terms of how these observed changes influence the overall function of the mPFC after exposure to ABA conditions, a reduction in cells exclusively expressing RNA molecules of these receptors is likely to have a net "receptor-specific" functional outcome, with less overall 5-HT_{2A} receptor-mediated signaling. In addition, the extent of expression of both 5-HT receptors was reduced after ABA indicating that a dampened serotonergic tone that is not specific to 5-HT_{2A} receptors occurs following pathological weight loss in ABA rats. This makes it important to view both results.

Since expression of these specific 5-HT receptor subtypes seemed to mirror the changes in AN, these data support the continued use of the ABA animal model as a tool for gaining a deeper understanding of serotonergic dysfunctions leading to pathological weight loss in AN as well as offering important insight into possible issues regarding the use of psilocybin as a pharmacotherapeutic approach for treating AN (Alsiö et al., 2021; Duriez et al., 2021; Schalla & Stengel, 2019). However, it is still unknown whether changes in altered RNA levels of the 5-HT_{2A} receptor expression are restored with body weight recovery in ABA rats, which is a question that will be resolved with future studies. Moreover, reduced levels of 5-HT_{2A} receptors need to be considered in the refinement of clinical trials conducted in individuals with AN. These results align with binding studies in individuals with AN described previously, and, if it is the case that the therapeutic action of psilocybin is mediated by the 5-HT_{2A} receptors in the mPFC, the standard dose

of psilocybin may have reduced effects in this patient population and could warrant a larger dose, or a multiple dose treatment strategy.

In terms of how these results are likely to cause changes in the function of the mPFC in ABA rats, it is important to note that stimulation of 5-HT_{1A} and 5-HT_{2A} receptors in pyramidal neurons have opposing effects on neuronal activity downstream signaling. At the molecular level, 5-HT_{1A} receptor activation engages the $G_{i/o}$ protein signaling pathway and leads to the hyperpolarization of the cellular membrane due to potassium conductance, resulting in inhibitory effects in neurons of the mPFC (Celada et al., 2004). In contrast, stimulation of the 5-HT_{2A} receptors engages the G_q protein signaling pathway and leads to the depolarization of the membrane, resulting in excitatory effects in neurons and the release of 5-HT in the mPFC. However, there is also evidence of neuronal inhibition via 5-HT_{2A} receptors located in GABAergic interneurons (Celada et al., 2004). There are many other complexities that contribute to differential signaling between these two 5-HT receptors, including their anatomical location (5-HT_{1A} receptors are densely expressed at the soma, whereas 5-HT_{2A} receptors are expressed on dendrites), that receptor activation can have ligand-specific effects, and that intracellular signaling cascades can be influenced by receptor internalization that occurs as a result of prolonged receptor occupation and recycling of the receptor, aspects that are also dependent on the type of ligand bound (Raote et al., 2007). Indeed there are many unanswered questions regarding what exactly happens after receptor activation, but so far we know that typically 5-HT_{1A} receptors suppress and 5-HT_{2A} receptors stimulate serotonin release in the mPFC (Carhart-Harris & Nutt, 2017; McClure-Begley & Roth, 2022; Savalia et al., 2021). These receptors are often expressed within the same pyramidal neurons in the mPFC, which begs the question of how individual neuron activity changes as a result of increased or decreased receptor expression (Carhart-Harris & Nutt, 2017).

With this in mind, the major finding from this study - that ABA induces an overall reduction in the level of 5-HT_{2A} receptor transcripts, as well as reduced co-localization of the 5-HT_{1A} and 5-HT_{2A} receptor transcripts is likely to result in a less active G_q signaling pathway and an overall suppression of serotonin release in the mPFC of ABA rats. Considering that 5-HT_{2A} receptors may play a role in cognitive flexibility it is plausible that the molecular changes described here could underlie the cognitive inflexibility seen among other things as an inability to maintain body weight in ABA rats (Carhart-Harris & Nutt, 2017). In order to examine this further, the ABA paradigm could be

combined with tests of cognitive flexibility, such as a reversal-learning task, in which changes in 5-HT receptor expression could be correlated with flexible behavior. Moreover, as there are multiple behavioral aspects of the ABA paradigm that contribute to pathological weight loss, it will be important in future studies to determine whether the individual components of food restriction and wheel running are associated with changes in 5-HT receptor expression. These studies will determine the *specificity* of ABA in causing functional changes to the serotonin system. In addition, as noted above, whether or not the changes described here are restricted to the "acute" phase of ABA or whether they persist after body weight recovery will be important to ascertain.

Contrary to our hypotheses, this study did not reveal any differences in RNA levels of 5-HT_{1A} and 5-HT_{2A} receptors elicited by psilocybin treatment. However, our protocol included a delay between the psilocybin administration and brain tissue collection, which was necessary to incorporate ABA exposure, which may have prevented effects from being observed due to the time course of receptor internalization and recycling described above. Expanding on this idea, it could be the case that the acute effects of psilocybin binding to the 5-HT_{2A} receptors led to the internalization of these receptors followed by increased receptor transcription, but when the receptors have recycled to the cell surface, the increased transcription of these receptors was not further needed. Depending on the time scale of these events, and considering that the acute action of psilocybin lasts for only several hours, this level of receptor cycling would not have been apparent after several days. Support for this idea comes from a study in Male Wistar rats on the effects of psilocin (the active metabolite of psilocybin) at 10 mg/kg on 5-HT levels in the mPFC using microdialysis, which showed the levels to return back to the baseline within 80 minutes (Sakashita et al., 2015). This may be followed by the transcription of the receptors, and the transcription of G protein-coupled receptors generally takes hours (Tréfier et al., 2018).

To understand the time-dependent changes in the RNA levels of 5-HT_{1A} and 5-HT_{2A} receptors after psilocybin administration, future studies should examine whether psilocybin causes changes in this more acute period (between 2-24 hours post-administration). The absence of differences in RNA levels of these receptors' expression between treatments could be driven by other things too, including the psilocybin dose. While the dose used in our studies was chosen between the doses used in two reliable studies (Hesselgrave et al., 2021; Shao et al., 2021), the dose-response in 5-HT signaling or 5-HT receptor occupancy have not been published in female rats, and although studies

using male rats have indicated that this dose of psilocybin elicits transcriptional changes in genes related to plasticity, there are possible sex-dependent differences in the actions of psilocybin in rodents (Jaster et al., 2022; Jefsen et al., 2020). Another possibility is that because the ABA exposure itself was carried out after psilocybin administration, which caused a significant reduction in RNA levels of 5-HT_{2A} receptors, this may have masked any effects caused by psilocybin due to delay in brain collection. The acute effects of psilocybin in ABA rats could be studied by administering psilocybin after body weight loss caused by ABA exposure and collecting the tissue right after the administration. This kind of study setup would better mimic the situation in individuals with AN who would be intended to be treated with psilocybin.

ABA is currently the only existing animal model, where animals voluntarily choose self-starvation over homeostasis, and as discussed previously it shares similarities with AN including the mirroring of specific 5-HT receptor subtypes, making it a suitable model to study AN (Alsiö et al., 2021; Duriez et al., 2021; Schalla & Stengel, 2019). It is worth noticing that ABA does not, and cannot model the psychosocial aspects of AN. And that the animal models in general are unlikely to experience the possible outcomes of the same subjective effects of psychedelics as humans. But they do give us detailed information about underlying neurobiological effects of pathological weight loss and psilocybin actions, as well as a better understanding of behavioral outcomes relevant to both the causes and treatment of AN.

The novel fluorescent in situ hybridization (FISH) technology, RNAscope®, is a beneficial method to answer questions considering the detection of gene expression and spatial information of the gene. Currently, this is the only way to detect differences in the level of 5-HT_{1A} and 5-HT_{2A} receptors, because, for example, the antibodies for immunohistochemistry detecting the protein levels of these receptors are notoriously unreliable. In any case, immunohistochemistry could be used together with RNAscope® for spatial analysis of specific cell types, such as glutamatergic and GABAergic neurons. This would give valuable information about the excitatory/inhibitory properties of neurons in relation to the ratio of 5-HT_{1A} and 5-HT_{2A} receptors, as it is known that both of these receptor subtypes are also present in GABAergic interneurons in addition to pyramidal (excitatory) neurons (Celada et al., 2004). To increase the understanding of the functional consequences of 5-HT receptors' actions across time, 5-HT levels could be studied by using microdialysis (Raote et al., 2007). These results could be combined with the studies focusing on the transcription of the

receptors at different time points. This would increase the understanding of the interaction between the serotonin and serotonin receptors and possibly guide to the right direction on what should be studied about signaling cascades happening after receptor activation (Raote et al., 2007).

Although AN occurs relatively more in females than males (0.3% - 1% females vs 0.1 % males), it would be important to study these same questions in male rats (American Psychiatric Association, 2016). When conducting the studies in both sexes, an additional and informative question to study could focus on the role of estrogen levels and its influence on 5-HT signaling, since it is known to have a role in depression - possibly via 5-HT signaling (Rybaczyk et al., 2005; Schorr & Miller, 2017). This question is also interesting when thinking about the psilocybin actions in 5-HT_{1A} and 5-HT_{2A} receptors, which are also influenced by the most active form of estrogen in mammals, 17-β estradiol (E2). It is known that either naturally or pharmacologically induced E2 levels increase the levels of E2β receptors, further increasing the 5-HT_{2A} receptor density and binding while decreasing the ERα receptors resulting decrease in 5-HT_{1A} receptor activity and by doing so upregulating the activity of 5-HT (Rybaczyk et al., 2005). If the therapeutic efficacy of psilocybin is dependent on these factors, as our study suggests, the question is whether the pretreatment of hormonal replacement therapy could help the action of psilocybin in the treatment of AN by increasing the density and action of 5-HT_{2A} receptors together with decreasing 5-HT_{1A} receptor activity. It is still important to keep in mind that since the action of psilocybin via 5-HT_{1A} receptors is not fully understood, together with the findings from animal studies that have used ketanserin as a pretreatment to block 5-HT_{2A} receptors and retained some possible therapeutic efficacy, the role of 5-HT_{1A} receptors in possible therapeutic outcomes should not be ignored (Hesselgrave et al., 2021; Jaster et al., 2021; McClure-Begley & Roth, 2022). And when thinking of psilocybin as a treatment for AN, our results showed significantly lowered levels of RNA molecules of 5-HT_{1A} receptors across all cells in ABA compared to non-ABA, which signals both the importance of these receptors in body weight maintenance and a possible therapeutic target of psilocybin.

In conclusion, this study showed that ABA rats have significantly lowered levels of cells exclusively expressing RNA molecules of 5-HT_{2A}-, but not 5-HT_{1A} receptors in the mPFC, compared to non-ABA rats. These results were consistent regardless of psilocybin treatment and are the first known molecular findings implicating these receptor systems in the ABA models. Across all cells in the mPFC, exposure to ABA conditions also elicited a significant reduction in the extent of 5-HT_{1A}

receptors, which again was not altered by psilocybin treatment. Taken together, our results indicate that at least the RNA levels of 5-HT_{2A} receptors are influenced by ABA exposure, and should be considered when thinking about the pharmacological treatment for AN. The role of these receptors in mediating the therapeutic effects of psilocybin administration should be studied further, for example by focusing on more acute time-dependent changes.

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RNAscope® Multiplex Fluorescent Reagent Kit v2 Assay for Fixed-frozen tissue, steps: pretreatment, hybridization & amplification, 1st fluorophore, and 2nd fluorophore & DAPI (only for channels 1 and 2)

PRETREATMENT

TURN THE WATER BATH ON (60°C) AND PUT THE CONTAINER WITH A LID IN TO WARM UP. MELT FROZEN 4% PFA.

1. Bake the slides in a 60°C water bath, inside the warmed container with the lid, for 30 MIN. Turn the water bath to 40°C after.

2. Post-fix the slides by immersing them in 4% PFA in 1X PB at 4°C for 15 MIN.

TAKE HYDROGEN PEROXIDASE AND PROTEASE ||| TO RT. PREPARE 50%, 70% AND 100% EtOH.

3. Remove the slides from 4% PFA, and immerse them in 50% EtOH at RT for 5 MIN.

4. Remove the slides from 50% EtOH, and immerse them in 70% EtOH at RT for 5 MIN.

5. Remove the slides from 70% EtOH, and immerse them in 100% EtOH at RT for 5 MIN.

6. Remove the slides from 100% EtOH, and let them air dry at RT for 5 MIN.

PREPARE 200 mL OF 1x TARGET RETRIEVAL REAGENTS BY ADDING 180 mL DISTILLED WATER TO 20 mL 10x TARGET RETRIEVAL REAGENTS (1:10). MIX WELL (always prepare fresh).

PREPARE THE STEAMER BY USING DISTILLED WATER, PLACE TWO CHAMBERS INTO THE STEAMER - ONE WITH DISTILLED WATER, AND ONE WITH 1x TARGET RETRIEVAL REAGENTS.

FILL A CHAMBER (with rack) WITH DISTILLED WATER AT RT.

7. Take the dehydrated slides and lay them on the bench, and add ~5–8 drops of Hydrogen Peroxide to cover the entire section. Incubate the slides at RT for 10 MIN.

TURN THE STEAMER ON AND ALLOW THE TEMPERATURE TO RISE TO AT LEAST 99°C (measure from 1x Target Retrieval Reagents).

8. Remove the Hydrogen Peroxide solution from one slide at a time by tapping and/or flicking the slide on absorbent paper. Immediately insert the slide into a chamber with distilled water (with a rack, at RT).

9. Wash the slides 3–5 times by moving the rack up and down in the chamber with distilled water. Repeat with fresh distilled water.

FILL ONE CHAMBER WITH DISTILLED WATER, AND ONE WITH 100% EtOH at RT (both 200 mL). PREPARE A CONTAINER WITH WET PAPER AND A LID, PUT IT INTO THE 40 °C WATERBATH TO WARM UP

10. Add the slides into a steamer to the chamber containing distilled water for 10 SEC to acclimate the slides.

11. Remove the slides and move them to the chamber containing 1x Target Retrieval Reagent. Cover the steamer with the lid and let them be there for 5 MIN (for mild and standard conditions).

12. Remove the slides from the steamer and transfer them to the chamber with 200 mL of distilled water at RT for 15 SEC.

13. Transfer the slides to the chamber with 200 mL 100% EtOH at RT for 3 MIN.

14. Dry the slides at RT for 5 MIN.

15. Use the ImmEdgeTM hydrophobic barrier pen to draw a barrier around each section and let it dry at RT ~5 MIN.

16. Add ~5 drops of Protease III to each section. Use enough solution to completely cover the sections.

17. Incubate the samples in a humid container (with wet paper and a lid) at 40°C for 30 MIN.

WARM 50X WASH BUFFER UP TO 40°C FOR 10–20 MIN BEFORE PREPARATION. PREPARE 2 L of 1x WASH BUFFER BY ADDING 1960 mL DISTILLED WATER TO 40 mL 50x WASH BUFFER. MIX WELL (store at RT for up to one month).

PREPARE THE PROBES (C1 = 5-HT$_{2A}$ & C2 = 5-HT$_{1A}$) BY WARMING THEM UP FOR 10 MIN AT 40°C IN A WATER BATH, THEN COOL THEM TO ROOM TEMPERATURE. SPIN C2 BRIEFLY. MIX PROBES BY DILUTING C2 IN C1 1:50 (25 uL/sample is needed). MIX BY INVERTING THE TUBE.

PLACE AMP1, AMP2, AMP3, HRP-C1, HRP-C2, HRP-C3, AND HRP BLOCKERS AT RT.

18. Wash twice with fresh distilled water at RT, move gently while washing.

HYBRIDIZATION & AMPLIFICATION

19. Remove excess liquid from the slides, but don't let them dry. Add probe mix to each sample and incubate in a humid container (with wet paper and a lid) at 40°C for 2 HRS.

20. Wash twice with fresh 1x Wash Buffer at RT for 2 MIN.

21. Remove excess liquid from the slides, but don't let them dry. Add AMP1 to each sample and incubate in a humid container (with wet paper and a lid) at 40°C for 30 MIN.

22. Wash twice with fresh 1x Wash Buffer at RT for 2 MIN.

23. Remove excess liquid from the slides, but don't let them dry. Add AMP2 to each sample and incubate in a humid container (with wet paper and a lid) at 40°C for 30 MIN

24. Wash twice with fresh 1x Wash Buffer at RT for 2 MIN.

25. Remove excess liquid from the slides, but don't let them dry. Add AMP3 to each sample and incubate in a humid container (with wet paper and a lid) at 40°C for 15 MIN.

26. Wash twice with fresh 1x Wash Buffer at RT for 2 MIN.

1ST FLUOROPHORE

27. Remove excess liquid from the slides, but don't let them dry. Add HRP C1 to each sample and incubate in a humid container (with wet paper and a lid) at 40°C for 15 MIN.

PREPARE OPAL DYE FLUOROPHORES INTO SEPARATE TUBES (red into its own tube, and green into its own) BY DILUTING THE OPAL DYE FLUOROPHORE INTO TSA BUFFER (red = 620 → 1:750, green = 520 → 1:500). STORE AT 4°C UNTIL USE.

28. Wash twice with fresh 1x Wash Buffer at RT for 2 MIN.

29. Remove excess liquid from the slides, but don't let them dry. Add one of the diluted Opal Dye fluorophores (first the one for C1, 5-HT_{2A} = 620, red.) to each slide and incubate for 30 MIN at 40°C in a humid container (with wet paper and a lid).

30. Wash twice with fresh 1x Wash Buffer at RT for 2 MIN.

31. Remove excess liquid from the slides, but don't let them dry. Add HRP blocker to each sample and incubate in a humid container (with wet paper and a lid) for 15 MIN at 40°C.

32. Wash twice with fresh 1x Wash Buffer at RT for 2 MIN.

2ND FLUOROPHORE & DAPI

33. Repeat steps 29-34 by using HRP C2 for the second fluorophore (for the C2, 5-HT_{1A} = 520, green).

34. Remove excess liquid from the slides and add ~4 drops of DAPI to each slide and incubate at RT for 30 SEC, remove DAPI by flicking the slides and immediately place 1-2 drops mounting medium.

35. Insert coverslip and let dry overnight in the dark at 2–8°C.

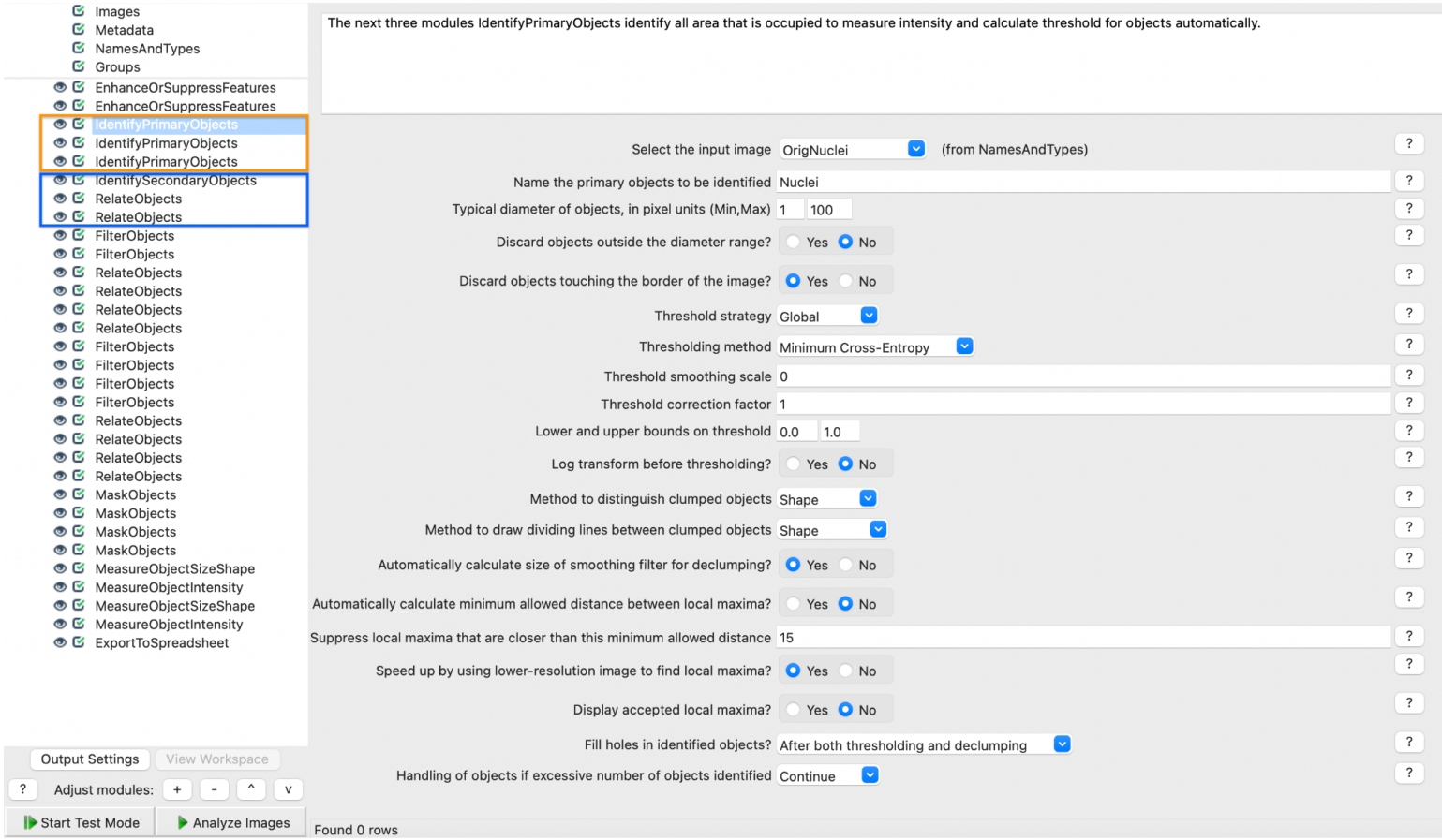

FIGURE 14 The CellProfiler pipeline. The pipeline first recognizes primary objects from the micrographs (orange rectangle), such as blue dots representing nuclei (diameter 1-100 px), green dots representing 5-HT_{1A} receptors (diameter 1-10 px) a, and red dots representing 5-HT_{2A} receptors (diameter 1-10 px). Next, the pipeline creates artificial cell membranes around the nuclei (expands 20 px around the nuclei) and relates green and red dots to the cell (blue rectangle). The step followed is for looking at the outcome parameter we were interested in, such as calculating the cells with three or more dots ("cells exclusively expressing the receptor of interest"). Finally, the pipeline exports all calculations to a spreadsheet.

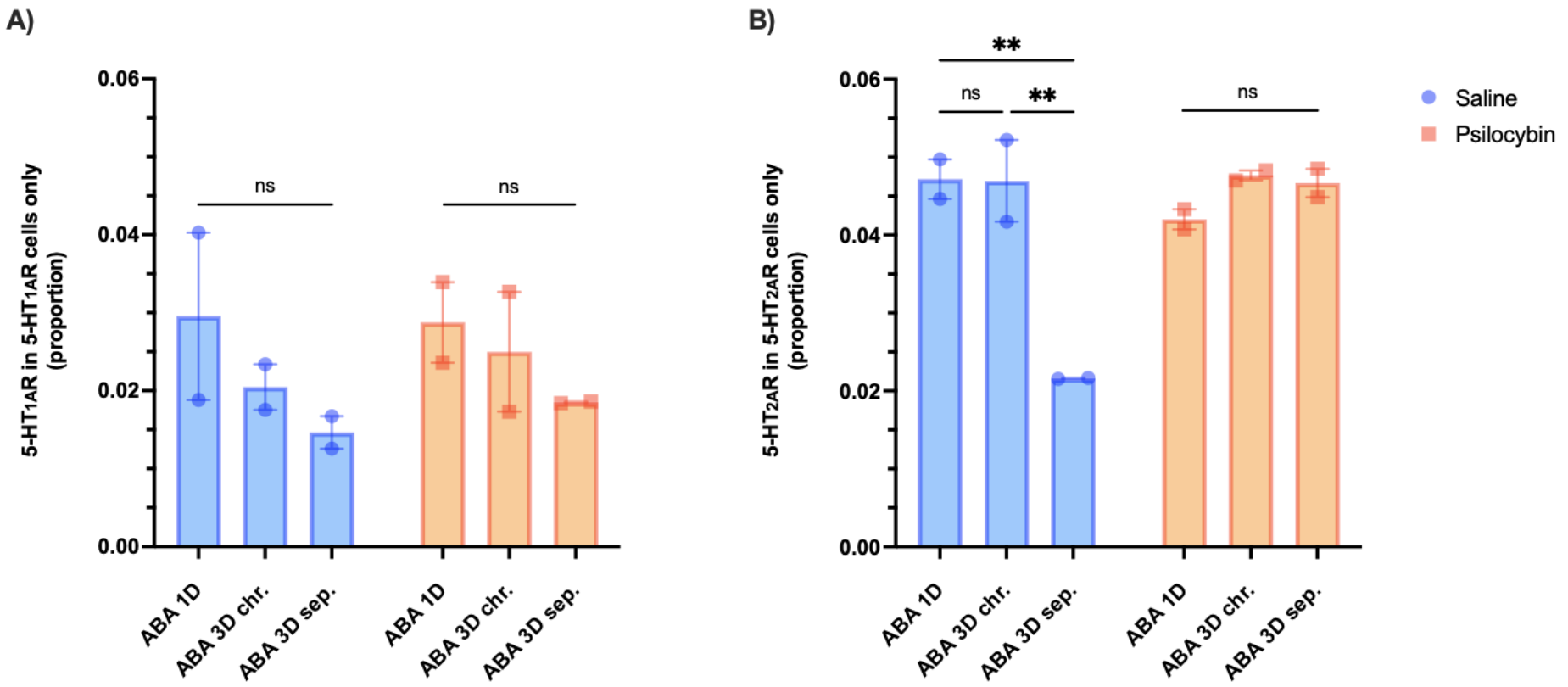

FIGURE 15 The proportion of cells exclusively expressing 5-HT$_{1A}$ or 5-HT$_{2A}$ receptors in the mPFC within ABA cohorts and treatment groups. Two-way ANOVA showed no significant differences in **(A)** cells exclusively expressing 5-HT$_{1A}$ receptors between cohorts within treatment groups (*F (2, 6) = 2.218, p = 0.190*), neither in **(B)** cells exclusively expressing 5-HT$_{2A}$ receptors between psilocybin-treated cohorts (all *p's > .5*), but significant differences between saline-treated cohorts (*F (2, 6) = 14.70, p < .005*), and was followed by Tukey's Multiple comparisons (both *p's = .003*). The significant results in 5-HT$_{2A}$ receptors between saline-treated animals with different dosing paradigms is unclear whether this represents underlying biological differences, or whether these differences reflect litter or cohort effects. All data is represented with the mean ± SEM. *n* (ABA, each saline cohort) = 2, *n* (ABA, each psilocybin cohort) = 2.

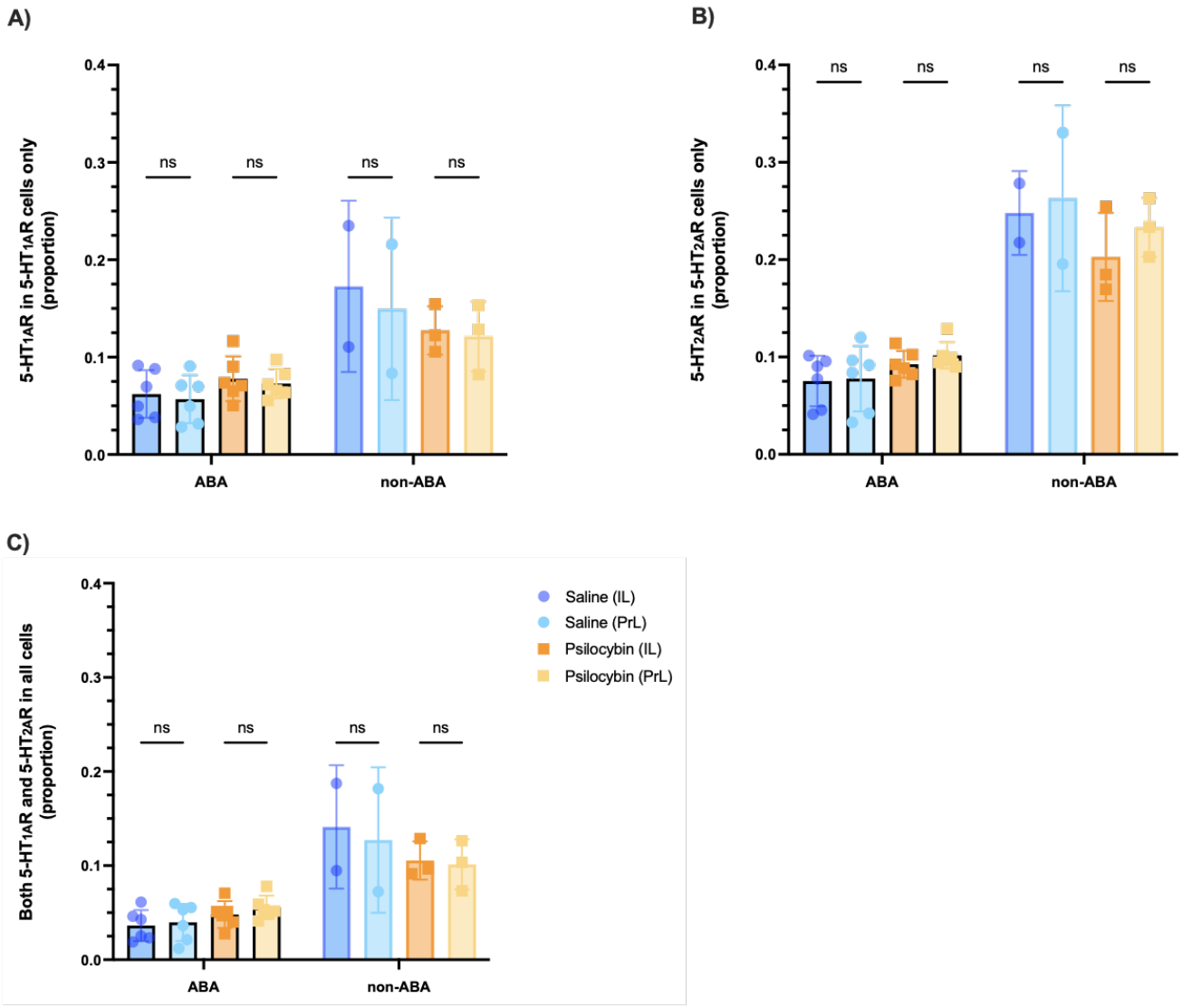

FIGURE 16 The proportion of cells exclusively expressing 5-HT_{1A} or 5-HT_{2A} receptors and the proportion of co-localized 5-HT_{1A} and 5-HT_{2A} receptors in cells expressing both receptor subtypes in the subregions of mPFC within cohorts and treatment groups. Two-way ANOVA showed no significant differences in **(A)** cells exclusively expressing 5-HT_{1A} receptors (*F (3, 29) = .141; p = .935*) neither in **(B)** cells exclusively expressing 5-HT_{2A} receptors between the regions of the mPFC within the treatments of each cohort (*F (3, 29) = .409; p = .748*), or in **(C)** in co-localized 5-HT_{1A} and 5-HT_{2A} receptors in cells expressing both receptor subtypes between the regions of the mPFC within the treatments of each cohorts (*F (3, 26) = .292, p = .831*). All data is represented with the mean ± SEM. *n* (ABA, total saline) = 6, *n* (non-ABA, total saline) = 2, *n* (ABA, total psilocybin) = 6, *n* (non-ABA, total psilocybin) = 3.