The Therapeutic Efficacy of Psilocybin in a Preclinical Model of Depressive- and Anxiety-Like Symptomology

By

Henry Chafee

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Abstract

Depressive and anxiety disorders are debilitating psychiatric illnesses that affect a substantial portion of the world population. Current pharmaceutical interventions, such as selective serotonin reuptake inhibitors, are generally regarded as front-line treatments but are not universally effective at reducing symptomology. Psilocybin, the active component in Psilocybe cubensis mushrooms, is a potent hallucinogenic substance that has shown promise as a pharmacological intervention for depression and anxiety in clinical trials. However, the mechanisms by which psilocybin exerts a therapeutic impact have not been thoroughly investigated. Preclinical (i.e., animal) research provides the opportunity to systematically assess the utility of drugs that may have medicinal applications under tightly controlled methodological conditions. The aim of this thesis is to determine whether a single administration of psilocybin reduces pathological behavioural tendencies in a preclinical model of depressive- and anxiety-like symptomology.

Shortly after birth, rats were exposed to early maternal separation, which is an established preclinical analogue for early chronic perinatal stress. Baseline behaviour was assessed with the Affective Disorders Test (ADT), which is a novel preclinical assay developed to test for depressive- and anxiety-like behaviour in animals over time. After 5 days of consecutive baseline testing, animals were administered one of two active doses of psilocybin (8 or 16mg/kg) or saline. Behaviour was then assessed again with the ADT for 3 consecutive days directly after administration (i.e., acute effects), and then again for 6 consecutive days 12 days post-treatment (i.e., chronic effects). Although the results obtained were mixed and largely inconclusive, this research constitutes an important contribution to the limited number of preclinical investigations of psilocybin in the context of depression and anxiety. This thesis also suggests numerous alternative directions and critical methodological factors for future researchers to consider.

Introduction

Depression and Anxiety

Depression and anxiety (collectively referred to as affective disorders) are debilitating and highly prevalent psychiatric conditions. The Diagnostic and Statistical Manual of Mental Disorders – 5th edition (DSM-5) defines multiple types of depressive disorders, including disruptive mood dysregulation disorder, persistent depressive disorder, and major depressive disorder (MDD), which includes major depressive episodes (American Psychiatric Association [APA], 2013). Cardinal diagnostic features of MDD and major depressive episodes include the presence of persistent, depressive moods, insomnia or fatigue, and a lack of pleasure in activities that would usually be rewarding (i.e., anhedonia), among others (APA, 2013).

In the United States, an estimated 8.1% of adults experience at least one MDE each year as of 2019 (Substance Abuse and Mental Health Services Administration [SAMHSA], 2020). Furthermore, nearly two out of three individuals who experience a major depressive episode also report a significant degree of functional impairment (SAMHSA, 2020). In New Zealand, recent epidemiological data found the yearly prevalence of MDD diagnoses to be 16.4%, or 620,000 adults as of 2019 (Ministry of Health, 2020). The yearly global prevalence of MDD diagnoses among adults was reported by the World Health Organization (WHO) at a rate of 4.4%, or roughly 322 million people; the WHO also found depressive disorders in general to be among the leading cause of non-fatal health impairments worldwide (WHO, 2017).

As with depressive disorders, the DSM-5 also defines multiple types of anxiety disorders, including generalized anxiety disorder, social anxiety disorder, and numerous specific phobias (APA, 2013). All anxiety disorder subtypes share the common feature of extreme fear and anxiety which is experienced beyond normative levels or in situations that usually do not engender such a significant response. These pathological tendencies are often accompanied by various behavioural and physiological manifestations (APA, 2013). Roughly 15.6% of American adults experienced symptoms of some form of anxiety disorder in 2019 (Terlizzi & Villarroel, 2020). Prevalence estimates of yearly diagnoses of any anxiety disorder in New Zealand are estimated to be 11.3% of the adult population, or roughly 442,000 individuals (Ministry of Health, 2020). Finally, the annual global prevalence of any anxiety disorder is estimated to be 3.6%, or roughly 264 million adults worldwide (WHO, 2017).

Current Pharmacological Treatments

Given the high prevalence and significant cost to both societal and individual wellbeing, numerous psychosocial and pharmacological interventions that target affective disorders have been developed and trialled in the last 70 years. Results from randomized controlled trials indicate that behavioural therapy, cognitive-behavioural therapy, and interpersonal psychotherapy lead to significant reductions in the severity and persistence of MDD symptomology (Craighead et al., 2015). However, the current front-line treatment for MDD is antidepressant medications, specifically, selective serotonin reuptake inhibitors (SSRIs) (Anderson, 2001). SSRIs are a class of medication that, along with older antidepressant medications such as monoamine oxidase inhibitors and tricyclic antidepressants, modulate levels of serotonin (5-HT) and other neurotransmitters via direct and indirect mechanisms (Hillhouse & Porter, 2015). Initially, researchers hypothesized that the direct effects of the drugs (i.e., inducing a global increase in 5-HT concentration in the synaptic cleft) were primarily responsible for the antidepressant effects observed (Hillhouse & Porter, 2015). More recent research has suggested the importance of indirect effects, such as the role of region-specific 5-HT neurotransmission and serotonin 2A (5-HT_{2A}) receptors in promoting neurogenesis (Pilar-Cuéllar et al., 2012).

Despite the frequency with which SSRIs and other types of antidepressant medications are prescribed for MDD and other depressive disorders, large scale efficacy and meta-analytic studies frequently indicate a concerningly low rate of response and remission. An estimated 30-40% of depressed individuals fail to respond to antidepressant medications despite adherence to the treatment regimen, ongoing dosage adjustments, and long-term duration of treatment; furthermore, 60-70% fail to achieve complete remission, and nearly 20% fail to achieve recovery after two years of treatment (Kupfer, 2005). The Sequenced Treatment Alternatives to Relieve Depression (STAR*D) study, which is the largest depression treatment study ever conducted to date, reported that more individuals dropped out than achieved remission at each stage of treatment, largely due to intolerable side-effects caused by combined pharmacological interventions (Pigott, 2015). The results of this study also highlight another issue with antidepressant medications and SSRIs in particular: these drugs often take weeks to exert a therapeutic influence, with significant response or remission rates often observed as late as 12 weeks after initiation (Sinyor et al., 2010). This is a particularly salient issue in the case of severe MDD, where acute suicidality and ongoing treatment adherence are primary concerns.

SSRIs and a related class of medications, serotonin-norepinephrine reuptake inhibitors, are also front-line pharmacological interventions for a range of anxiety disorders, including generalized anxiety disorder, panic disorder/agoraphobia, and social anxiety disorder (Bandelow et al., 2017). Serotonin-norepinephrine reuptake inhibitors are similar to SSRIs in that they increase the concentration of 5-HT in the synaptic cleft by blocking reuptake but differ in that they also exert this effect on another neurotransmitter, norepinephrine. Like SSRIs, serotonin-norepinephrine reuptake inhibitors produce an anxiolytic effect only after 2-6 weeks of sustained treatment (Bandelow et al., 2017). Meanwhile, benzodiazepines are often prescribed to alleviate acute symptomology; in fact, roughly 55-94% of patients presenting with some form of anxiety disorder are initially treated with them (Starcevic, 2014). Benzodiazepines work pharmacologically by modulating gamma aminobutyric acid (GABA), the main inhibitory neurotransmitter. By binding to GABA A receptor sites, benzodiazepines exert an anxiolytic effect that occurs rapidly post-administration (Campo-Soria et al., 2006; Starcevic, 2014). Even though benzodiazepines are therapeutically effective and well-tolerated, prolonged usage is not recommended due to the high potential for tolerance, dependence, and abuse (Starcevic, 2014).

As with MDD, the results of treatment meta-analyses indicate that pharmacological interventions for anxiety disorders are not universally effective, although efficacy does seem to depend on the specific type of anxiety disorder being treated (Donovan et al., 2010). For instance, in the case of generalized anxiety disorder, fluoxetine (an SSRI) produces the greatest degree of response and remission (Baldwin et al., 2011). For the treatment of panic disorder, however, there is no difference in efficacy between antidepressant medications and benzodiazepines, and cognitive-behavioural therapy may be more effective than pharmacological interventions in the long term due to lower attrition rates (Gould et al., 1995). Because antidepressant medications are often used to treat anxiety as well as depressive disorders, many of the same issues with treatment efficacy exist, including latency of therapeutic efficacy, intolerable side effects, and the need for long-term usage to retain symptom reduction.

Background on Classic Hallucinogens

From the existing body of epidemiological and efficacy literature on the prevalence and treatment of affective disorders, there is a clear and immediate need for the development of novel pharmacological interventions. One such line of investigation is the use of classic

hallucinogens as a pharmacological adjunct to therapy. Classic hallucinogens are a class of substances that induce similar somatic symptoms, such as blurred vision and weakness; perceptual distortions, such as altered colours and shapes; and psychic symptoms, such as mood alterations, depersonalization, and visual hallucinations (Nichols, 2004). Some of these substances are synthesized compounds, such as lysergic acid diethylamide (LSD), 2,5-dimethoxy-4-iodoamphetamine (DOI), and 2,5-dimethoxy-4-methylamphetamine (DOM). Others occur naturally, such as psilocybin (derived from Psilocybe cubensis mushrooms), mescaline (derived from peyote cacti), and dimethyltryptamine (DMT, the psychoactive compound in the traditionally brewed ayahuasca tea) (Bogenschutz & Johnson, 2016). Structurally, classic hallucinogens are classified as either indoleamines (i.e., DMT and psilocybin), ergolines (i.e., LSD), or phenylalkylamines (i.e., mescaline, DOI, and DOM) (Bogenschutz & Johnson, 2016).

Many of the naturally occurring classic hallucinogens have an extremely long history of therapeutic and religious use by indigenous cultures worldwide. In Mexico and the American Southwest, where the peyote cactus grows naturally, natives have utilized its hallucinogenic properties to facilitate religious and spiritual enlightenment ceremonies for centuries (Nichols, 2004). Similarly, ayahuasca tea, which contains DMT, has an extremely long history of ceremonial use by indigenous peoples of the Amazon region in South America (Nichols, 2004). Beyond its use for spiritual purposes, several studies have found that ayahuasca and peyote have been successfully employed as adjunct treatments for substance use disorders (particularly alcohol use disorder) within native communities (Lu et al., 2009; Fábregas et al., 2010). However, despite nearly 5,000 years of documented use suggesting therapeutic applications, scientific interest in hallucinogens did not begin until the late 1800s, when Arthur Heffter successfully isolated mescaline and documented its effects (Bogenschutz & Johnson, 2016). Subsequently, Albert Hoffman synthesized and accidentally discovered the psychoactive effects of LSD in 1943, and successfully isolated psilocybin in 1958, at which point scientific inquiry into the use of classic hallucinogens in psychiatric treatment began in earnest (Bogenschutz & Johnson, 2016).

Classic hallucinogens are frequently referred to as serotonergic hallucinogens due to their shared mechanism of action. The characteristic perceptual alterations that they induce are largely due to partial or complete agonism of the 5-HT_{2A} receptor, although they also bind to many other types of serotonergic receptors, including 5-HT_{2C} and 5-HT_{1A} (Bogenschutz & Johnson, 2016; Carhartt-Harris & Guy, 2017). Support for this hypothesized mechanism of

action comes from clinical research that demonstrated that the selective 5-HT_{2A} antagonist ketanserin inhibits the subjective effects of psilocybin (Vollenweider et al., 1998). Additionally, findings from preclinical research demonstrated that repeated daily administration of classic hallucinogens leads to the rapid development of tolerance; this loss of drug sensitivity was correlated with 5-HT_{2A} receptor downregulation post-exposure (Buckholtz et al., 1990). Agonism of the 5-HT_{2A} receptor also has a direct downstream effect on the dopaminergic system by stimulating the release of dopamine (Moeller et al., 2001; Sholler et al., 2019). Thus, while a single administration of a classic hallucinogen might lead to 5-HT_{2A} receptor agonism and therefore dopamine release, repeated exposure may have the opposite effect (Gray & Roth, 2001).

The first research conducted with classic hallucinogens in the 1950s led to the observation that LSD seems to elicit psychotomimetic effects in healthy individuals (Rinkel et al., 1952). This finding led to an initial hypothesis that classic hallucinogens might be useful pharmacological agents to create models for schizophrenia and features of other psychotic disorders in preclinical paradigms (Bogenschutz & Forcehimes, 2016). However, promising preliminary results from studies utilizing classic hallucinogens in a therapeutic context quickly eclipsed research initiatives investigating the psychotomimetic model (Bogenschutz & Forcehimes, 2016). Research on the therapeutic efficacy of LSD in the context of addiction, particularly alcohol use disorder, constituted the bulk of this early scientific inquiry into psychiatric applications (Bogenschutz & Forcehimes, 2016). Other avenues of research during this time included the use of LSD, psilocybin, and occasionally mescaline as adjuncts to therapy in the treatment of opioid use disorder, as well as depressive disorders, anxiety disorders, and obsessive-compulsive disorder (Garcia-Romeu & Richards, 2018).

LSD was a popular target for pharmacological research because comparatively small amounts of the drug (i.e., micrograms) produce significant and long-lasting states of altered consciousness (Nichols, 2004). Like other classic hallucinogens, it is also extremely non-toxic; most importantly, there is no scientific evidence to suggest that LSD produces addiction or dependence, rendering it a safe option for the treatment of substance use disorders (Nichols, 2004). Early preclinical research confirmed this lack of addiction potential by demonstrating that animals could not be trained to self-administer the drug, and that it even acted as a negative reinforcer in some contexts (Nichols, 2004). Classic

hallucinogens were easily obtainable for clinical research until the late 1960s, which facilitated this initial surge in experimentation (Bogenschutz & Johnson, 2016).

Many of the groups that conducted the first research with classic hallucinogens, especially LSD, in the context of depression and anxiety noted significant improvements in clinician-judged symptomology, with an overall effect across studies estimated to be as high as 75% of participants (Rucker et al., 2016). However, many of these studies were methodologically unsound by modern standards: most did not include standard scientific measures such as control groups, standardized assessment protocols, and randomization (Rucker et al., 2016). The body of early research on alcohol use disorder was somewhat more methodologically robust. At least six randomized controlled trials reported that administration of LSD led to significant reductions in alcohol misuse that persisted for 2-3 months post-treatment (Krebs & Johansen, 2012). However, most of these early studies were fraught with significant design flaws, meaning that any reported effects can now only be construed as anecdotal evidence at best (Krebs & Johansen, 2012).

The initial clinical research that employed classic hallucinogens as an adjunct to therapy adhered to one of two therapeutic models: psycholytic or psychedelic (Bogenschutz & Johnson, 2016). First, the psycholytic model referred to administering multiple low to moderate doses of the drug to facilitate psychoanalytical sessions that attempted to address underlying factors such as unresolved trauma (Buckman, 1967; Bogenschutz & Johnson, 2016). Second, the psychedelic model referred to administering a high dose of the hallucinogenic drug within one or several sessions to facilitate a peak mystical experience that engendered lasting alterations in core thought patterns, emotional processing, and behavioural schemas (Sherwood et al., 1968; Bogenschutz & Johnson, 2016). This latter model was successfully employed as a standard treatment for alcohol use disorder with promising effects; however, tighter restrictions on the production of hallucinogenic drugs for scientific purposes ultimately led to a temporary moratorium on research and treatment (Krebs & Johansen, 2012; Bogenschutz & Pommy, 2012).

Current Clinical Research

Clinical research resumed in Europe and the United States in the 1990s when government restrictions on synthesizing and procuring classic hallucinogens for scientific purposes began to loosen (Carhart-Harris & Goodwin, 2017). These first studies attempted to replicate and extend findings on the psychotomimetic, pharmacodynamic, and

neurobiological effects of mescaline, DMT, and psilocybin in non-symptomatic individuals (Hermle et al., 1992; Strassman & Qualls, 1994; Vollenweider et al., 1998). As restrictions eased further, the scientific community resumed the investigation of classic hallucinogens in the context of psychiatric disorders (Carhart-Harris & Goodwin, 2017). Importantly, researchers also began to trace specific neurobiological mechanisms via neuroimaging and psychopharmacological methodologies, which has contributed to a far more comprehensive and nuanced understanding of how and why classic hallucinogens might be effective therapeutic agents (Carhart-Harris & Goodwin, 2017).

Recent clinical research conducted with symptomatic populations who were administered LSD has largely corroborated the promising initial findings with respect to certain therapeutic applications. For instance, a recent phase II trial found that LSD-assisted psychotherapy successfully reduced anxiety associated with life-threatening illness (Gasser et al., 2014). While the sample size of the study was limited (i.e., 10 participants), reductions in anxiety and increases in overall quality of life were sustained 12-months post-treatment; importantly, no major adverse psychological or physiological effects were reported (Gasser et al., 2015). More generally, in healthy individuals, administration of LSD produced self-reported feelings of well-being, closeness with others, trust, and openness without increasing baseline anxiety (Schmid et al., 2014). LSD also impaired the recognition of faces displaying negatively valanced emotional states, increased measures of empathetic emotional recognition, and led to a greater desire for sociality in a non-symptomatic sample population (Dolder et al., 2016).

Large-scale epidemiological studies have corroborated findings from clinical trials by consistently demonstrating a lack of association between lifetime use of classic hallucinogens and receiving or needing inpatient psychiatric treatment for mental health disorders (Johansen & Krebs, 2015). This line of research also debunked concerns of an association between classic hallucinogen use and suicidality; in fact, one study found that lifetime hallucinogen use was associated with a 29% reduction in the likelihood of suicidal planning and a 36% reduction in the likelihood of a suicide attempt (Hendricks et al., 2015). Despite the limitations inherent in self-report methodologies (i.e., response biases, etc.), these population studies provide compelling evidence for the safety of administering classic hallucinogens to symptomatic populations. Furthermore, they suggest that even when taken in an uncontrolled, naturalistic setting, these drugs can exert a therapeutic effect (Hendricks et al., 2015).

Despite the largely positive findings with regard to the therapeutic efficacy of LSD administered in controlled and uncontrolled environments, many researchers have begun to focus on psilocybin instead. This switch is due in part to the difference in duration of subjective effects between the drugs: while both have the same neurochemical mechanism of action and produce similar psychological effects, acute impairment caused by psilocybin dissipates 6 hours post-administration, whereas the effects of LSD are still present 12 or even 16 hours post-administration (Schmid et al., 2015). Research conducted with healthy volunteers has demonstrated that psilocybin has an extremely low abuse potential, produces significant alterations in cognitive and emotional processes, and engenders lasting positive alterations in values and attitudes (Studerus et al., 2010).

One of the first recent applications of psilocybin as a pharmacological therapeutic was conducted in the context of depression and anxiety experienced by individuals with late-stage cancer. The first investigation was a pilot study that evaluated whether a low dose (0.2mg/kg) could reduce anxiety in 12 participants with various forms of advanced cancer (Grob et al., 2011). In line with previous reports, the investigators reported no adverse long-term psychological effects or significant medical sequelae; furthermore, they reported significant reductions in validated measures of anxiety that persisted for two weeks post-administration (Grob et al., 2011). Although their findings are limited by methodological constraints such as a small sample size, this pilot study laid critical groundwork for more rigorously controlled studies to come.

In a follow-up randomized controlled trial, investigators administered a larger dose (0.3mg/kg) to 29 participants with various types of late-stage cancer who were randomly allocated to treatment conditions (Ross et al., 2016). The protocol utilized psilocybin as a pharmacological adjunct to psychotherapy and reported robust reductions in depressive and anxious symptomology for 60-80% of participants; furthermore, these significant reductions were still present at the follow-up assessment 6.5 months post-administration (Ross et al., 2016). The researchers also observed that self-reported mystical experiences mediated the therapeutic effects of the drug (Ross et al., 2016). These promising results were corroborated by a separate research group, which found that similar doses of psilocybin (0.3 or 0.4mg/kg) administered once in the context of psychotherapy led to significant reductions in clinician-rated and self-reported measures of depression and anxiety in 51 participants with life-threatening cancer diagnoses (Griffiths et al., 2016). Participants also reported significantly increased well-being and life satisfaction, along with corresponding improvements in

attitudes towards self, life, spirituality, and mood (Griffiths et al., 2016). Importantly, these therapeutic gains relative to baseline were present at 1- and 6-months post-treatment (Griffiths et al., 2016).

Simultaneously, other research groups have begun investigating the efficacy of psilocybin in the context of several forms of substance use disorder. In one proof-of-concept study, 10 individuals with diagnosed alcohol dependence received two doses of psilocybin in two sessions (0.3mg/kg in the first, and 0.4mg/kg in the second) in addition to sessions of motivational enhancement therapy (Bogenschutz et al., 2015). The investigators reported substantial improvements in drinking behaviour after the psilocybin sessions; furthermore, the degree of acute effects and the mystical quality of those effects were significant mediators of therapeutic efficacy (Bogenschutz et al., 2015). Psilocybin was also recently shown to be an effective smoking cessation aid as an adjunct to 15 weeks of cognitive-behavioural therapy (Johnson et al., 2014). Researchers found that 12 out of 15 participants (80%) demonstrated biologically verified 7-day point abstinence at 6 months post-treatment (Johnson et al., 2014). A long-term follow-up study with the same participant group found that 9 out of the 11 participants who returned for assessment (82%) were still smoking abstinent (the mean time since treatment was 30 months) (Garcia-Romeu et al., 2017). Finally, psilocybin-assisted treatment for cocaine use disorder is currently the subject of an ongoing clinical trial at the University of Alabama (NCT02037126).

Another related avenue of recent clinical research with psilocybin as a pharmacological adjunct to therapy is in the context of treatment-resistant depression. In an open-label feasibility study, researchers administered two oral doses (10 and 25mg 7 days apart) to 12 individuals with moderate to severe treatment-resistant depression as well as ongoing psychological support before, during, and after the administration sessions (Carhart-Harris et al., 2016). Out of the 12 participants, seven (58%) met criteria for remission after psilocybin treatment, and a further five (42%) remained in remission three months later (Carhart-Harris et al., 2016). The miniscule sample size precludes any substantial conclusions from being drawn, but the therapeutic benefit observed is significant enough to warrant research on a larger scale. Furthermore, the study demonstrated that psilocybin is well-tolerated and can be safely administered to this patient group (Carhart-Harris et al., 2016). Data collected from a follow-up study that assessed symptomology in the same group of 12 individuals 6 months after treatment suggested that psilocybin may prevent relapse at least as effectively as existing antidepressant medications (Carhart-Harris et al., 2018).

Beyond treatment-resistant depression, a recent randomized controlled trial found that psilocybin-assisted psychotherapy produced significant antidepressant effects in 24 individuals with MDD at both 1- and 4-weeks post-treatment (Davis et al., 2021).

Neurobiology and Personality

In addition to evaluating the efficacy of psilocybin in clinical trials, researchers have also investigated the potential neurobiological, cognitive, and emotional mechanisms responsible for the therapeutic effect. In their initial clinical research publications, Carhart-Harris and colleagues suggested that increased 5-HT_{2A} receptor signalling caused by psilocybin may induce a state of acute neural plasticity which, when coupled with an enriched and supportive context, may lead to the revision of certain primary cognitive schemas (Carhart-Harris et al., 2018). In support of this hypothesis, the same research group investigated changes in functional integrity within the default-mode network, which has been implicated as a critical region in depressive disorders, in a sample of depressed patients who were administered psilocybin in the context of psychotherapy (Carhart-Harris et al., 2017). While default-mode network functional integrity decreased directly after the drug was administered, increases were observed the day after (Carhart-Harris et al., 2017). Furthermore, increased resting-state functional connectivity measured in the default mode network and related areas predicted symptomology reduction at 5 weeks post-treatment (Carhart-Harris et al., 2017). Overall, these neurobiological findings suggest that psilocybin might "reset" these neural systems, thereby returning the cognitive processes they are responsible for back to normal functioning (Carhartt-Harris et al., 2017).

There is some evidence to suggest that psilocybin exerts a therapeutic impact by influencing other neural regions as well. The amygdala is a critical component in the emotional processing network, and hyperactivity in this region has been correlated with negative mood states and vulnerability to depressive disorders (Kraehenmann et al., 2015). In one fMRI imaging study, psilocybin reduced right amygdala reactivity to negative and neutral stimuli, which is an effect that also occurs after chronic administration of an SSRI (Kraehenmann et al., 2015). Furthermore, in a sample of patients diagnosed with treatment-resistant depression, psilocybin reduced functional connectivity between the ventromedial prefrontal cortex and the right amygdala after exposure to fearful and neutral faces; this reduction in functional connectivity was positively associated with reduced rumination 1 week post-treatment (Mertens et al., 2020). Finally, in support of these neurobiological

findings, psilocybin successfully remediated reaction times to emotional faces within a sample of patients with treatment-resistant depression to levels observed in healthy, untreated participants (Stroud et al., 2018). These faster reaction times were also positively associated with reduced anhedonia within the symptomatic group (Stroud et al., 2018).

Psilocybin treatment with psychological support also appears to have a lasting impact on elements of personality structure and underlying attitudes and beliefs, particularly in the context of treatment-resistant depression. For instance, a recent open-label study demonstrated that significant reductions in the NEO-PI-R 'Big Five' personality measure *Neuroticism*, which is frequently implicated as a risk factor for affective disorders, correlated with clinical improvement 3 months post-treatment (Erritzoe et al., 2018). Significant increases in *Extraversion*, which is associated with positive affect, were also observed (Erritzoe et al., 2018). Psilocybin-assisted psychotherapy significantly reduced measures of authoritarianism and increased measures of nature relatedness (an attitude that is associated with reduced anxiety and improved well-being) among a sample of patients with treatment-resistant depression (Lyons & Carhart-Harris, 2018). These differences were significant at both 1 week and 7-12 months post-treatment (Lyons & Carhart-Harris, 2018). Finally, this treatment paradigm also reduced the cognitive pessimism bias associated with depressive disorders: individuals with treatment-resistant depression were more optimistic and accurate at predicting future life events post-treatment, which correlated with reductions in the severity of depressive symptomatology (Lyons & Carhart-Harris, 2018).

Set and Setting

Beyond clinical efficacy with specific psychiatric disorders and relevant neurobiological/cognitive correlates, researchers have also sought to elucidate a more fundamental factor: the qualities of the hallucinogenic experience that predict therapeutic efficacy. From this body of research, two important components have emerged: set and setting and the so-called mystical experience (Gukasyan & Nayak, 2021; Roseman et al., 2018). Set and setting were terms first coined in the 1960s by the psychologist Timothy Leary, who believed that these extra-pharmacological components were critical in mediating the reaction to hallucinogenic drugs (Hartogsohn, 2016). Set refers to an individual's internal psychological state, and includes personality, expectation, and preparation. Setting refers to external environmental factors, such as physical, cultural, and interpersonal elements present during the hallucinogenic experience (Hartogsohn, 2016). If, as Leary and others have

argued, hallucinogens modulate certain elements of introspection and external perception, then the pharmacological effects of these drugs must be mediated by the individual's underlying subjective state and salient features of the immediate environment. Thus, it is clear why investigating potentially pivotal elements of set and setting and carefully controlling them in psychedelic-assisted treatment modalities is critically important to successfully achieving a therapeutic effect (Gukasyan & Nayak, 2021).

The other important component in promoting a therapeutic effect is the presence of a mystical experience. Mystical experiences are deeply profound and transformative psychological events and can be induced with psychedelic drugs and other non-pharmacological mechanisms (Roseman et al., 2018). Regardless of how the experience is created, numerous studies have correlated mystical experiences with positive clinical outcomes (Roseman et al., 2018). The altered states of consciousness questionnaire is a standardized measure designed to systematically assess factors that both contribute to and detract from the mystical experience caused by hallucinogens specifically (Dittrich, 1998). Measures include oceanic boundlessness, which is comprised of sub-factors such as bliss, unity, and insightfulness, as well as dread of ego dissolution, which includes sub-factors such as impaired control and anxiety (Studerus et al., 2010; Roseman et al., 2018). In a sample of 20 patients with treatment-resistant depression who were administered psilocybin, retrospectively reported high levels of oceanic boundlessness and low levels of dread of ego dissolution during the acute hallucinogenic experience correlated with favourable clinical outcomes (Roseman et al., 2018). Thus, engendering a mystical experience and controlling set and setting constitute critical extra-pharmacological components of psychedelic-assisted treatment paradigms, and highlight the quality of the acute hallucinogenic experience as a salient factor in mediating therapeutic benefits.

Current Preclinical Research

Methodologies that utilize animal subjects are particularly useful in pharmacological research as they provide the opportunity to directly test the neurobiological and behavioural effects of various substances in a controlled experimental setting. The body of research investigating psilocybin in preclinical models of depression and anxiety (or other psychiatric disorders for that matter) is considerably more limited than its clinical counterpart. In this sense, psilocybin research does not adhere to the traditional trajectory of scientific inquiry (i.e., preclinical to clinical) when investigating novel pharmacological therapeutics. With

that said, a limited number of studies have assessed the behavioural and neurobiological effects of psilocybin and psilocin (the psychoactive component of psilocybin) in animal models. For instance, one drug discrimination study demonstrated that a selective 5-HT_{2A} receptor antagonist only partially blocks the stimulus control of psilocybin, suggesting that the 5-HT_{2A} receptor is chiefly but not solely responsible for the subjective effects of the drug (Winter et al., 2007). The researchers also found that psilocybin fully generalized to DOM, LSD, and psilocin, which indicates that the effects of these drugs are highly similar in rodent models (Winter et al., 2007). In terms of behavioural measures, another study reported that psilocin dose-dependently impaired locomotion in the open field test as well as prepulse inhibition (Tylš et al., 2016). Administration of selective 5-HT_{1A} and 5-$HT_{2B/C}$ antagonists normalized locomotion, but not prepulse inhibition, which supports the implication that multiple serotonin receptor subtypes besides 5-HT_{2A} mediate the behavioural and subjective effects of the drug (Tylš et al., 2016). Finally, the researchers also found that the overall impairing effects of psilocin were more prominent in male animals than in females (Tylš et al., 2016).

In addition to pharmacological research, several studies have investigated the neurobiological effects of psilocybin/psilocin in rodent models. The cortical regions affected by psilocin in rodents and humans appear to be similar (Spain et al., 2015). However, in humans, blood-oxygen-level-dependent (BOLD) levels uniformly decreased post-administration, whereas region-specific increases and decreases were noted in the rodent brain (Spain et al., 2015). This divergence in BOLD signal directionality may suggest underlying interspecies differences or difficulties with translating similar effective doses from humans to animals (Spain et al., 2015). Psilocybin also dose-dependently increased the expression of multiple genes related to synaptic plasticity in the prefrontal cortex and hippocampus (Jefsen et al., 2020). Psilocybin modulated a greater number of these target genes in the prefrontal cortex, indicating that the drug may exert more of a transcriptional influence in that region compared to the hippocampus (Jefsen et al., 2020). These findings align with reports that multiple types of serotonergic hallucinogens, including DOI, DMT, and LSD, increase brain-derived neurotrophic factor (BDNF) and lead to increases in neuritogenesis and spinogenesis (Ly et al., 2018). The effects appear to be mediated by key receptor signalling hubs, including tropomyosin receptor kinase B (TkrB), the mechanistic target of rapamycin, and the 5-HT_{2A} receptor (Ly et al., 2018). Since prolonged use of traditional antidepressant medications is also associated with increased neuroplasticity in the

hippocampus via activation of BDNF and TrkB pathways, psilocybin may exert a therapeutic impact by modulating similar neurobiological processes (Artin et al., 2021).

Researchers have also investigated the effects of psilocybin on specific cognitive/behavioural measures in animal models. In a comparison of the effects of low dose psilocybin and ketamine, both drugs increased motivation and attention in rodents in a progressive ratio and a serial 5-choice task (Higgins et al., 2021). The impact was most prominent for animals who performed particularly poorly prior to treatment (Higgins et al., 2021). Similarly, a single low dose of psilocybin alleviated behavioural despair and cognitive deficits measured by immobility in the forced swim test (FST) and the object pattern separation task respectively (Hibicke et al., 2020). In this study, the researchers induced cognitive and behavioural deficits with an environmental paradigm of chronic restraint stress in adolescence (Hibicke et al., 2020). These results lend credence to the potential antidepressant properties of psilocybin in rodents and imply that environmental deficit models like early chronic stress are valid and viable options for assessing how the pharmacological effects of the drug impact symptomology.

The small number of preclinical studies that have assessed the therapeutic efficacy of psilocybin and other classic hallucinogens in animal models of specific disorders have yielded conflicting results. In an alcohol use disorder relapse model in rats, psilocybin had no significant impact even though the researchers tested multiple doses and dosing regimens similar to those that have proven effective in clinical trials (Meinhardt et al., 2020). Only sub-chronic dosing (i.e., five doses over 3 days) of 1mg/kg produced a transient anti-relapse effect (Meinhardt et al., 2020). These negative results were unexpected given that several years before, another research group reported that a single administration of LSD led to profound and sustained reductions in ethanol consumption in a genetic model of alcohol-preferring mice (i.e., C57BL/6J) (Alper et al., 2018). This discrepancy serves as an important reminder that comparatively small differences in preclinical methods used to model symptomology and the pharmacological agents used to modulate it can translate into fundamentally divergent results. Given that psilocybin has shown some therapeutic efficacy in the context of alcohol use disorder in clinical research, more preclinical work that investigates the impact of psilocybin in other rat models of drinking behaviour and relapse is warranted.

Besides alcohol use disorder and relapse, the utility of psilocybin in depression models has also been investigated by several research groups. One group utilized the Flinders Sensitive Line (FSL) as a genetic model of depressive-like symptomology to test for antidepressant effects of psilocybin and psilocin (Jefsen et al., 2019). FSL animals display increased immobility in the FST, which decreases after administration of standard antidepressant medications (Overstreet et al., 2005). Neurobiologically, FSL animals also exhibit serotonergic abnormalities, including reduced 5-HT_{1A} receptor sensitivity (Overstreet et al., 2005). Despite investigating multiple dosage groups as well as repeated and single dosing regimens, the researchers found no effect; no dosage or dosing regimen reduced immobility in the FST, nor was there an impact on overall locomotor behaviour measured with the open field test (Jefsen et al., 2019).

The authors offer a plausible hypothesis for the lack of effect which relates to the specific choice of animal model they decided to use. Standard antidepressant medications reliably reduce depressive tendencies in FSL rats; however, this animal strain also exhibits a significantly lower concentration of 5-HT_{2A} receptor mRNA in the frontal cortex and hippocampus, which might impact the neurochemical mechanism of action of psilocybin specifically (Jefsen et al., 2019). In support of this explanation, another recent study reported that a single administration of a relatively low dose of psilocybin (1mg/kg) yielded persistent antidepressant effects, which was assessed using the same behavioural measure (i.e., the FST) (Hibicke et al., 2020). Psilocybin also exerted a persistent anxiolytic effect, measured by increased time spent on the open arms of the elevated plus maze (Hibicke et al., 2020). The researchers utilized the Wistar-Kyoto (WKY) strain of rats, which, like the FSL line, are an established genetic animal model for depression and anxiety vulnerability; they show similar levels of immobility in the FST, as well as increased susceptibility to stress and anxiety-like behaviour (Hibicke et al., 2020). However, unlike the FSL line, WKY animals show similar levels of 5-HT_{2A} receptor mRNA to control animals, which may provide an explanation for the divergence in results between these two studies (De La Garza & Mahoney, 2004).

The Present Study

The current study extends from the limited body of preclinical research discussed above by attempting to determine whether a single high dose of psilocybin reduces depressive- and anxiety-like symptomology in an animal model. Several doses of psilocybin (8 and 16mg/kg) will be tested in order to determine whether any therapeutic effects observed

are dose dependent. In order to avoid any potential neurobiological confounds caused by altered functioning of the serotonergic system (and the 5-HT_{2A} receptor system in particular) in genetic models, an environmental model of chronic early perinatal stress (i.e., early maternal separation) will be used to induce a psychiatric phenotype.

After the early maternal separation protocol is complete and animals are left to develop normally, they will be tested for baseline behaviour in adolescence with the Affective Disorders Test (ADT). The ADT is a novel preclinical behavioural assay that measures depressive- and anxiety-like symptomology with a variety of operationalized variables over consecutive days of testing. Psilocybin (or a vehicle control) will be administered, and behavioural testing will resume to determine the presence of any acute effects. Finally, animals will be tested a third time several weeks post-administration to determine whether psilocybin modulates symptomatic behaviour chronically.

Regardless of whether psilocybin exerts a significant effect on symptomology, the results will add to the currently limited body of preclinical research on the efficacy of psilocybin in animal analogues for key features of specific psychiatric disorders. Given the promising findings from research involving human participants, preclinical investigations are necessary to determine whether the psychotherapeutic context or certain intrinsic qualities of human cognition are required for psilocybin to reduce pathology. If psilocybin reduces symptomology in animal models, an underlying pharmacological and/or neurobiological mechanism may be a sufficient explanation for the beneficial effects observed in humans. Alternatively, if psilocybin does not reduce symptomology in rodents, then the concurrent presence of psychotherapy or the capacity for critical introspection and cognitive self-reflection may be required for the drug to exert a therapeutic effect.

Given the dearth of published research on the subject and the conflicting nature of findings reported, hypotheses of effects should be made with caution. Despite this caveat, we predict that psilocybin will lead to dose-dependent reductions in depressive- and anxiety-like symptomology in animals that have been manipulated to display pathological behavioural tendencies. Furthermore, we predict that these reductions will be apparent immediately after treatment and persist for the duration of chronic testing. Finally, we predict that psilocybin will not lead to significant variations in baseline performance in animals that have not been subjected to early maternal separation, either directly after administration or after continued testing.

Method

Ethical Statement

The Victoria University of Wellington Animal Ethics Committee approved all animal procedures prior to initiating experimentation (application #29112, approved 03/12/2020).

Study Design

Animals were bred in five sequential cohorts. Three to four mating couples were paired every 23-30 days, producing 14-22 animals per cohort (Table 1). The first cohort was a pilot study and was therefore excluded from the final analysis. Once pregnancy was confirmed, litters were randomly allocated to either the early maternal separation (EMS) or control condition. The day of birth was postnatal day (PND) 0. All litters were weaned at PND21 and allowed to develop normally (i.e., *ad libitum* access to food and water) until preparations for experimentation commenced. As much as possible, the experimental timeline across cohorts matched such that all manipulations and treatments occurred within the same age range for all animals.

Table 1. *n* per experimental cohort.

Cohort	
1	$n = 17$
2	$n = 15$
3	$n = 14$
4	$n = 14$

Animals

For the experimental cohorts, a total of 60 male Sprague-Dawley rats were bred from 10 litters. Breeding males and females were selected based on non-relation from the colony maintained at the Victoria University of Wellington animal facility and paired for a period of 5-6 days before separation. Dams and litters were given *ad libitum* access to food and water and maintained at an ambient temperature of 21℃ at 55% humidity on a reversed 12-hour light/dark cycle (lights on at 7:00PM). All animals were housed in standard individually ventilated polycarbonate cages. On PND21, litters were weaned and the male animals were separated and housed in groups of two to three. Post-weaning, animals were maintained under the same conditions as previously described and given *ad libitum* access to water and standard rat chow until PND 48-52, at which point the food hoppers were removed in

preparation for food restriction. All animals continued to have unrestricted access to water throughout the duration of the experimental timeline.

Drugs

Psilocybin (BDG Synthesis, Wellington, New Zealand) was dissolved in 0.9% sterile saline at a concentration of 8mg/mL. Prior to the first injections, the pH was measured and deemed to carry a low risk of discomfort for the animals. For vehicle control injections, 0.9% sterile saline was used. To provide a better volume match with the two doses of psilocybin administered, control doses were split into low- and high-volume groups. The low volume was matched to the 8mg/kg group (1mL/kg) while the high volume was matched to the 16mg/kg group (2mL/kg).

Early Maternal Separation

Rationale

Early maternal separation (EMS) is an established environmental paradigm within the preclinical rodent literature that produces certain behavioural and neurobiological changes. The paradigm is an early life stressor and an animal analogue for early neglect and abuse in human children, which has been correlated with an increased risk of developing anxious and depressive symptomology in adulthood (Fonzo et al., 2015; LeMoult et al., 2020). EMS hinges on the importance of uninterrupted maternal care during the stress hyporesponsive period (SHRP), which occurs between PND4 and 14 in rodents (Kambali et al., 2019).

The SHRP is characterized by minimal stress responsiveness, low levels of adrenocorticotropic hormone (ACTH) and corticosterone throughout the brain, and minimal hypothalamic-pituitary-adrenal (HPA) axis activation (Oomen et al., 2009; Kambali et al., 2019). Specific maternal behaviours, such as licking and grooming, functionally maintain the reduction in HPA activation (Kambali et al., 2019). This period of hypo-responsiveness is critically important for synaptic pruning and the creation of neuronal networks that mediate stress responsiveness later in life (Kambali et al., 2019).

There is some variation in how EMS is performed, but the most common method involves separating pups from dams for 3 hours per day consecutively between PND2 or 4 and 14 (Rincel & Darnaudéry, 2019). Results from previous studies suggest that returned dams do not attempt to compensate for the period of separation with increased maternal care; indeed, dams appear less attentive to pups after the separation period (Vetulani, 2013).

Consequently, pups do not receive critical maternal care during the SHRP and frequently exhibit chronic HPA axis hyperresponsiveness in adolescence and adulthood (Vetulani, 2013).

The behavioural and neurobiological consequences of this paradigm are well-documented in the preclinical research. For instance, EMS rats exhibit increased anxiety-like behaviour and preference for alcohol (Huot et al., 2001). They also exhibit decreased consumption of highly anticipated rewards and impaired formation of Pavlovian conditioning involving reward-related stimuli (i.e., anhedonia-like behaviour). Furthermore, the administration of ADMs reverses these effects (Ladd et al., 2000; Matthews et al., 1996). Finally, EMS male rats (but not females) exhibit a greater startle response within an acoustic startle paradigm, which occurs as a direct result of hyperresponsiveness to stress (Kalinichev et al., 2002).

EMS also leads to certain neurobiological changes that correlate with the behavioural tendencies described above. Specifically, EMS rats show up-regulated corticotropin-releasing hormone (CRH) gene expression in the anterior pituitary and down-regulated glucocorticoid receptor (GR) gene expression in the hippocampus (Vetulani, 2013). EMS rats also exhibit changes in CRH and GR gene expression in the medial prefrontal cortex (mPFC) and amygdala, which contribute to hypervigilance, anxiety-like behaviour, and alcohol preference (Vetulani, 2013; Sánchez et al, 2001). The anhedonia-like tendencies exhibited by EMS rats, which are operationalized as a lack of motivating incentive induced by drug-related rewards, may be related to changes in dopaminergic and noradrenergic transmission in multiple cortical regions (Vetulani, 2013; Matthews & Robbins, 2003; Weinshenker & Shroeder, 2006).

Procedure

In the present study, dams were removed from the home cage and placed in a separate cage that was moved to a separate room. Separations occurred once daily for 3 hours from PND4-14. To maintain consistency, the separation period always began between 10:30AM and 12:00PM for all litters and all cohorts. After the 3-hour separation period concluded, dams were returned to the home cage. By design, these manipulations involved minimal handling of the dams and no handling of the pups. In contrast to the EMS condition, dams of litters designated to the control condition were handled for approximately 2 minutes per day from PND4-14. This active control procedure is in line with the previous literature, which

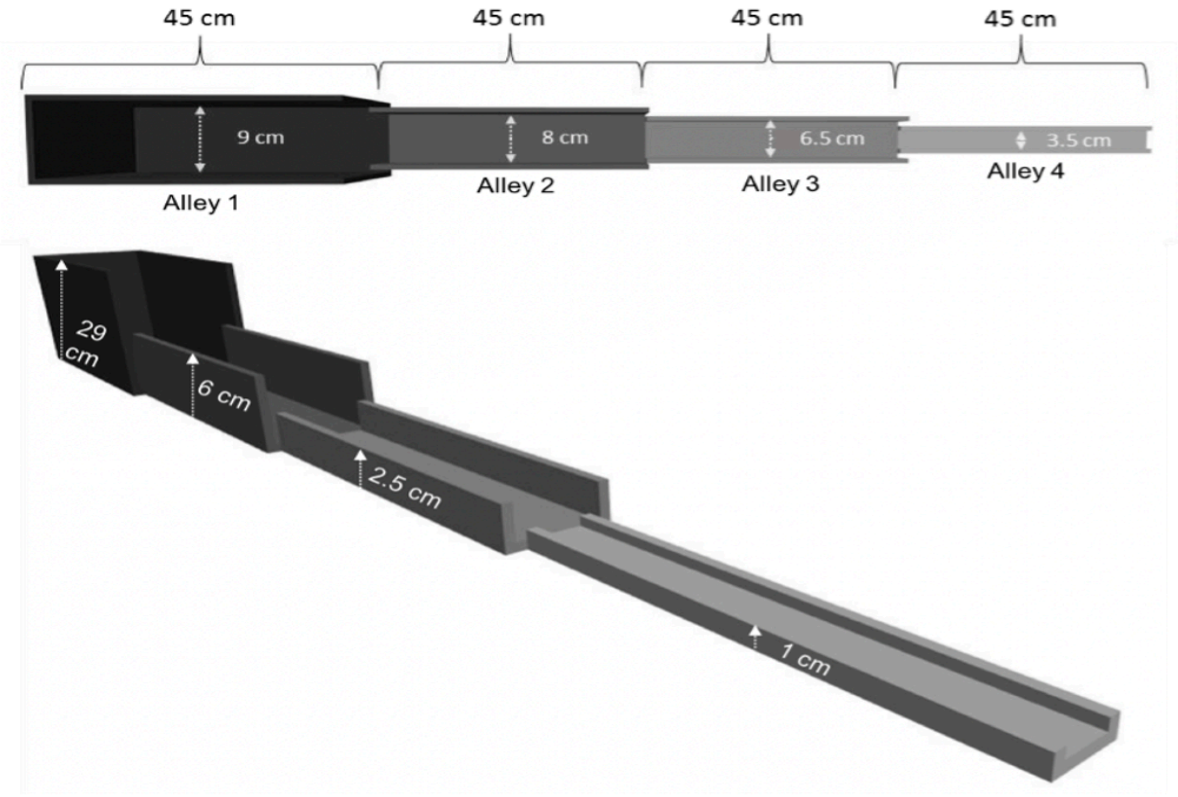
suggests that brief early handling mimics mothers leaving litters for short periods of time to forage for food in a naturalistic setting (Nishi et al., 2014).

Affective Disorders Test

Rationale

The Affective Disorders Test (ADT) is a novel behavioural assay that measures both depressive- and anxiety-like behaviour. The anxiety component is based on the Successive Alleys Test (SAT), which is an established measurement paradigm for anxiety-like symptomology in rodents (Deacon, 2013). The SAT consists of four connected alleys, each successively narrower and with lower walls (Figure 1). The increasing openness of each alley produces a progressively greater anxiogenic effect (Deacon, 2013). Although the SAT is a highly valid preclinical assay, it suffers from the one-trial tolerance effect: with repeated testing, animals learn that exploring the more anxiogenic alleys does not yield greater rewards. Thus, after multiple days of testing, even animals that initially ventured onto the fourth alley no longer do so, and the capacity for the test to measure anxiety-like behaviour is fatally compromised.

Figure 1. Dimensions of Successive Alleys Apparatus



The ADT ameliorates the one-trial tolerance effect by motivating animals to explore each alley in a standardized manner that is maintained over consecutive days of testing (Blackburne, 2021). In this modified version of the SAT, a highly anticipated sucrose reward (i.e., Froot Loops) is placed in the centre of each alley, thereby enticing the animal to explore the alley and retrieve the reward. Comparatively more anxious animals display a greater latency to enter the third and fourth alleys to retrieve the Froot Loop and consume them in the perceived safety of the first or second alley (Blackburne, 2021). As repeated testing over consecutive days continues, these animals exhibit a comparatively smaller decrease in latency to retrieve the Froot Loops in the third and fourth alleys, even as the actual safety of these alleys is learned and reinforced (Blackburne, 2021).

The depressive-like behaviour component of the ADT involves anticipatory pleasure, which is operationalized as 'wanting' a given reward (i.e., reward approach); in contrast, consummatory pleasure is the 'liking' of a reward (Sherdell et al., 2012). Clinical studies have consistently implicated decreased anticipatory pleasure (but not consummatory pleasure) in the aetiology of anhedonia, which is a cardinal component of depression (Liu et al., 2016). The ADT measures anhedonia-like behaviour by assessing anticipatory pleasure directly before animals are placed in the modified SAT (mSAT). By pairing the two tests temporally over consecutive days, animals begin to anticipate the Froot Loops they will receive in the mSAT as they are being assessed for anhedonia-like behaviour directly before. Animals that exhibit comparatively more anhedonia-like behaviour exhibit less anticipatory pleasure directly before the mSAT; as testing continues and the association is reinforced, these animals also exhibit less of an increase in anticipatory behaviour over time.

In rats, rearing onto the hindquarters and lifting both paws off the ground is a specific and reliable behaviour that is elicited in incentive motivation states (i.e., anticipatory behaviour) (Brenes & Schwarting, 2015). Rearing behaviour has also been correlated with 50 kHz ultrasonic vocalizations (USVs); these vocalizations are often referred to as appetitive calls, as they are reliably emitted by rats when presented with cues signalling the imminent presentation of a food reward (Brenes & Schwarting, 2015). Thus, in combination, rearing behaviour and 50 kHz USVs systematically evaluate the degree to which a rat anticipates food-related rewards in the presence of previously reinforced environmental cues.

Experimental Setting

The anticipatory box was constructed from four square black polycarbonate sheets (40x40x40cm) set into black plastic corner bolsters and reinforced with black masking tape (Figure 2). Clear sheet plastic awnings (40x5cm) were attached to the tops of the walls to prevent animals from attempting to jump out during testing. The floor of the box was a black plastic mat that covered the entire floor of the experimental room.

The walls and floors of the successive alleys were constructed from black polycarbonate panels slotted together and attached with commercial adhesive (Figures 1 and 2). The tops of the walls of the third and fourth alleys were covered with a thin layer of black masking tape to prevent damage from chewing. The first alley was secured to a tabletop with a vice grip, and the alleys projected out over the floor of the experimental room at a height of roughly one meter; tripods were placed under the second and fourth alleys to increase stability.

Figure 2. Experimental Setup of the Affective Disorders Test

Each session of habituation and testing (anticipatory box and successive alleys) for each animal was recorded with a USB camera (C170 Webcam, 640 x 480 resolution, 30 fps; Logitech, Lausanne, Switzerland) mounted to the ceiling of the experimental room. The camera was positioned to provide a bird's-eye view of both the anticipatory box and the alleys. The video footage was recorded in EthoVision® XT (Noldus, Wageningen, Netherlands) which tracked and analysed each animal's locomotor behaviour in real time.

Ultrasonic vocalizations (USVs) of animals in the anticipatory box were recorded with an UltraMic microphone (250K 16 bi; Java Sound). The microphone was attached to a metal stand positioned above and protruding down into the box slightly, thereby maximizing the sound quality and consistency of USVs recorded (Figure 2). All habituation and testing sessions in the anticipatory box were recorded in this manner.

The experimental chamber was dimly lit with soft fluorescent overhead lighting. During habituation and testing in the anticipatory box, a lamp near the box fitted with a shaded 75w flood light was also illuminated. This lamp was switched off as soon as the session in the anticipatory box was over, and the lamp was not illuminated for sessions in the successive alleys. The brightness inside the box (with the lamp on) was recorded at the beginning of each day of habituation and testing to ensure that experimental conditions were held as constant as possible across animals, days, and cohorts (habituation = 16.7-19.9 lux; testing = 16.5-19 lux). The brightness of each alley (with the lamp off) was also recorded at the beginning of each day of testing to maximize experimental consistency, as well as to ensure that a gradient in brightness from the first to the fourth alley was maintained (alley 1 = 0.5-0.9 lux; alley 2 = 4.3-5.2 lux; alley 3 = 8.7-10.9 lux; alley 4 = 13.1-18.2 lux).

Procedure

Food Restriction. In order to increase motivation for the sucrose reward in the ADT, all experimental animals were given limited access to food for 10 full days prior to initiating experimentation as well as for the duration of testing. Eleven days before testing began (on PND 47-51 depending on the cohort), food hoppers were removed in the evening and 13-15g of standard rat chow was scattered at the bottom of the cage. All animals were weighed daily to monitor the effects of the food restriction and allow for adjustments in feeding amount to be made if necessary. Because the animals were still growing at this point, they could not be uniformly food restricted to 85-90% of their starting weight and maintained, as is standard in the literature. Rather, the goal was to restrict food intake sufficiently to maintain increased

motivation while still allowing for some growth to occur at a reduced rate, which required periodic increases in feeding amount (Ganguly et al., 2018).

The decision to increase feeding was made by comparing average cohort weights to previous literature that reported on normal growth in adolescent Sprague-Dawley animals as well as average free-feeding weight charts from manufacturers (Turner & Burne, 2014; Charles River Laboratories, Wilmington, MA, U.S.A.). Throughout the duration of the experiment, animals were maintained between the average and two standard deviation below the average weight for normally developing Sprague-Dawley rats with *ad libitum* access to food at all age points. Within each cohort, all animals were fed the same amount to prevent differential levels of motivation; however, the dates at which food intake was increased differed slightly between cohorts due to different average weights. All animals were fed daily between 4:00PM and 5:30PM to maintain temporal variability between feeding and time of testing, as well as to avoid interrupting the animals during the light cycle. Animals were never fed within 15 minutes of the final test or treatment of the day and always had *ad libitum* access to water.

Handling. For 5 consecutive days before testing began, all animals were handled for roughly 2 minutes per day in the housing room before feeding in order to acclimate the animals to human interaction in a familiar environment prior to testing.

Habituation. For 3 consecutive days before testing began, animals were habituated to the testing protocol. One cage (two to three animals) at a time was transported to the testing room and animals remained in their home cage to habituate to the environment. Overhead lights were set to the dimmest setting and soft light from a lamp in the corner illuminated the anticipatory box. After 10 minutes had elapsed, the first animal to be habituated was removed from the cage, weighed, handled for 2 minutes, and placed in the anticipatory box for 10 minutes. Ultrasonic vocalizations (USVs) were recorded using the UltraMic microphone, the session was recorded in its entirety with the USB camera in EthoVision® XT, and rearing behaviour was scored manually. A rear was classified as such if both paws left the floor; if one paw remained on the floor, the movement was not classified as a rear. Rearing ended as soon as both paws returned to the floor. Rearing behaviour was divided into two types: supported (or "wall") rears and unsupported (or "free") rears. A rear was classified as supported if the animal supported itself with one or both paws against the wall, and a rear was categorized as unsupported if it did not involve bracing against the wall.

Once 10 minutes of recording had elapsed, the animal was returned to its cage and the floor and walls of the anticipatory box were sanitized with F10 SC veterinary disinfectant (Health and Hygiene, Roodepoort, Gauteng, South Africa); the second animal to be habituated was retrieved, and the process began again. In this manner, all boxes were habituated between the hours of 8:00AM and 4:30PM in a randomized counterbalanced order across all animals and cohorts. The first animal to be retrieved from the box for habituation was also reversed daily.

In addition to habituation to the testing room and apparatus, animals were also habituated to the sucrose reward (i.e., Froot Loops) for 3 consecutive days prior to initiating testing. After the last box of animals was habituated according to the counterbalanced schedule for that day, each animal was presented with three halved Froot Loops in their home cage in the breeding room. The experimenter ensured that each of the two or three animals in each cage ate all their allotted Froot Loops before animals were offered standard feed.

Testing. Testing was divided into three periods: baseline (days 1-5), acute effects post-treatment (days 6-8), and chronic effects post-treatment (days 9-14). Depending on the cohort and the exact date of birth for each litter, baseline testing began on PND58-62, acute testing began on PND64-68, and chronic testing began on PND75-79 (i.e. 10 days after psilocybin exposure). Between acute and chronic testing, animals were left undisturbed in their home cages for a period of 8 days and fed once daily between 4:00PM and 5:30PM according to the food restriction protocol to maintain motivation for subsequent testing. The goal of each testing period was to measure baseline behaviour, test whether psilocybin had an impact on behaviour directly after administration, and finally to determine whether any impact observed acutely persisted chronically (respectively).

The testing procedure was identical for all three periods. Starting at 8:00AM, the first cage of animals according to the day's counterbalanced order was carried into the experimental room. Animals were habituated to the testing room for 10 minutes under the same lighting conditions as previously described. After 10 minutes had elapsed, the first animal to be tested was removed from the cage, weighed, and placed in the anticipatory box. Rearing behaviour was scored manually as described previously, vocalizations were recorded with the UltraMic, and the session was recorded with the overhead USB camera in EthoVision® XT. After 5 minutes, the animal was removed from the box, and the lamp illuminating the box was switched off.

The animal was then placed in the first alley of the mSAT with the head pointed towards the back of the alley and recorded for the duration of the test (i.e., 5 minutes) with the overhead USB camera in EthoVision® XT. The experimenter manually recorded the alley in which each of the four Froot Loops was consumed, as well as the latency to consume the Froot Loop placed in the fourth alley (latency was reported as 300 seconds if the animal never consumed it). Animals sometimes consumed Froot Loops on the border between two alleys; when this occurred, the alley that the animal was facing while eating the Froot Loop was scored as the alley of consumption. Occasionally, animals were midway through consuming a Froot Loop when the test ended. If the test period ended before the entire Froot Loop was consumed, that consumption was not counted. In addition to latency to consume the Froot Loop in the fourth alley, each animal was assigned an SAT score for each day of testing. The SAT score was calculated by multiplying the number of Froot Loops eaten in a given alley by the alley's number and adding the values for each alley together; for example, if an animal ate two Froot Loops in the first alley, two Froot Loops in the second alley, and none in the third or fourth alleys, the SAT score would be six. Thus, a higher SAT score indicated reduced anxiety-like behaviour.

After the 5 minutes of testing had elapsed, the animal was retrieved from the mSAT and placed back in the home cage. The floors and walls of the anticipatory box and each alley were sanitized with F10, the alleys were baited with Froot Loops, the second animal to be tested was retrieved, and the process began again. This experimental protocol was repeated step by step for each cage according to the counterbalanced order for that given day until all animals from the cohort had been tested; all testing procedures were identical between cohorts. As with habituation, the first animal retrieved from the cage for testing was reversed daily.

Treatments

Within each cohort, animals were randomly allocated to one of three treatment conditions: 8mg/kg psilocybin, 16mg/kg psilocybin, or vehicle control (Table 2). Animals designated to the control condition were split into two subsequent groups: a low-volume saline condition (1mL/kg) and a high-volume saline condition (2mL/kg). All injections were administered interperitoneally (i.p.) between the hours of 10:00AM and 4:00PM across a single day. Injections took place in an experimental room with dimmed lighting that contained four locomotor activity boxes identical to the anticipatory box. The UltraMic

microphone was positioned above one of the boxes, and the USB camera was mounted to the ceiling in order to give a bird's-eye view of animal behaviour in all four. In groups of two to four, animals were weighed and habituated to the boxes for 10 minutes, after which they were immediately injected with one of the two volumes of saline or one of the two doses of psilocybin. Animals were then placed back into the same locomotor activity box they were habituated to for 30 minutes to assess acute effects of the drug. Ultrasonic vocalizations and locomotor behaviour during habituation and directly after injections were recorded with the UltraMic and USB camera in EthoVision® XT respectively. After 30 minutes of recording had elapsed post-injection, animals were placed back into their home cages and transported back to the housing room. This process was repeated until all animals had received an injection. As with habituation and testing, the order of administration was randomized and counterbalanced across all animals within each cohort.

Table 2. *n* per condition.

Condition	Saline-Low	Saline-High	8 mg/kg	16 mg/kg
Control	$n = 5$	$n = 5$	$n = 10$	$n = 10$
EMS	$n = 5$	$n = 5$	$n = 10$	$n = 10$

Data Collection and Critical Variables

Collected data were split into three categories: manually scored data, automatically scored data in EthoVision® XT, and USVs. In the interest of reducing any experimental inconsistency caused by falsely scoring a supported rear as an unsupported rear (or vice versa), these variables were collapsed into total rears for the purposes of analysis. Thus, manually scored data consisted of total rears in the anticipatory box, as well as the latency to consume the Froot Loop placed in the fourth alley and SAT score in the mSAT. Automatically scored data consisted of total distance travelled and average velocity in the anticipatory box, as well as total distance travelled, average velocity, frequency of visits to alleys 3 and 4, cumulative duration spent in alleys 3 and 4, and latency to first entry into alleys 3 and 4 in the mSAT. Finally, USVs consisted of the total number of calls within the 30-90 kHz range emitted in the anticipatory box. Data collection was split into four periods: habituation (days -2-0), baseline testing (days 1-5), acute effects (days 6-8), and chronic effects (days 9-14). For statistical analyses, day 5 of testing was also included in the

assessment of the acute effects period (i.e., days 5-8) for all measures to provide a comparison of performance directly before and after psilocybin was administered.

Data were collected and analysed for all 14 measures for each time period. However, several measures were selected as particularly relevant for the investigation of main effects and interactions of dosage and condition (i.e., measures of interest). For the manually scored data, total rears were deemed especially relevant in the assessment of anticipatory behaviour. SAT score was assumed to be the primary indicator of anxiety-like symptomology, as previous research has suggested that it is a more sensitive measure than latency to consume the Froot Loop in the fourth alley (Blackburne, 2021). For the automatically scored data in the anticipatory box, total distance moved and average velocity (i.e., anticipatory locomotor activity) were selected as particularly relevant measures. For automatically scored data in the mSAT, total distance moved and average velocity (i.e., mSAT locomotor activity), frequency of entries into alley 4, cumulative duration spent in alley 4, and latency to first entry into alley 4 were deemed priority measures. All animals from all cohorts explored alley 4 by the end of baseline testing, thereby rendering the alley 3 variables inferior as measures of anxiety-like behaviour. Finally, USVs were also deemed a measure of particular interest because previous research has indicated that they are a robust indicator of anticipatory pleasure (Knutson et al., 2002; Ahrens et al., 2013).

Statistical Analysis

Initially, an analysis was performed to determine whether performance on any measure differed significantly by cohort (i.e., testing for the presence of cohort effects). These analyses consisted of a series of repeated measures ANOVAs, one for each testing period, with day as a within-subjects factor and cohort as a between-subjects factor for each measure of interest: a 3 (day: -2-0) x 4 (cohort: 1-4) test for habituation, a 5 (day: 1-5) x 4 (cohort: 1-4) test for baseline behaviour, a 4 (day: 5-8) x 4 (cohort: 1-4) for acute effects, and a 6 (day: 9-14) x 4 (cohort: 1-4) test for chronic effects. Secondly, an analysis was performed to determine whether saline volume had a significant impact on performance within the vehicle treatment group across all measures. For the saline analysis, only the acute effects period was considered because treatment occurred after baseline testing and any observed effects of the different saline dosages would presumably not persist past the acute testing period. Thus, each measure was analysed with a 4 (day: 5-8) x 2 (condition: EMS, control) x

2 (dosage: saline-high, saline-low) repeated measures ANOVA, with day as a within-subjects factor and condition and saline treatment as a between-subjects factor.

Habituation data (i.e., rearing behaviour, total distance moved, and average velocity) were analysed with a 3 (day: -2-0) x 2 (condition: EMS, control) repeated measures ANOVA, with day as a within-subjects factor and condition as a between-subjects factor. Baseline data (manually scored anticipatory measures, automatically scored anticipatory measures, USVs, manually scored mSAT measures, and automatically scored mSAT measures) were analysed with a 5 (day: 1-5) x 2 (condition: EMS, control) repeated measures ANOVA, with day as a within-subjects factor and condition as a between-subjects factor. Acute effects data (same measures as baseline) were analysed with a 4 (day: 5-8) x 2 (condition: EMS, control) x 3 (dosage: saline, 8mg/kg, 16mg/kg) repeated measures ANOVA, with day as a within-subjects factor and condition and dosage as a between-subjects factor. Finally, chronic effects data (same measures as baseline and acute effects) were analysed with a 6 (day: 9-14) x 2 (condition: EMS, control) x 3 (dosage: saline, 8 mg/kg, 16 mg/kg) repeated measures ANOVA, with day as a within-subjects factor and condition and dosage as between-subjects factors.

Results

The results from the analyses mentioned previously are reported in Tables 3-22. Due to the large number of measures collected and analysed, this presentation of results will focus on those measures deemed particularly relevant in the assessment of depressive- (total rears, total distance moved and average velocity, and USVs) and anxiety-like behaviour (SAT score, total distance moved and average velocity on the mSAT, frequency of visits to alley 4, cumulative duration in alley 4, and latency to first entry into alley 4). Descriptive statistics for all measures can be found in Appendix A, and the results of all analyses for all measures not included in the results section can be found in Appendix C.

Before analyses for main effects and interactions of condition and dosage were performed, each measure was assessed for the presence of cohort and saline dosage effects within each time period as well as over all testing days. The results of these checks for unintended main effects are discussed below for each measure of interest. The results of the analyses for cohort and saline dosage effects for all measures not presented below can be found in Appendix B.

Cohort Effects

Analyses for cohort effects revealed that three measures of interest differed significantly by cohort: SAT score, total distance moved on the mSAT, and average velocity on the mSAT (Table 3). For each of these measures, the cohort effect was only significant at baseline; the effect was not significant within any other time period, nor when all testing days were considered within a single model. Main effects of cohort did not approach the level of significance for any other variables of interest (Appendix B). Given that the main interest was in the effects of psilocybin on depressive- and anxiety-like symptomology and there were no cohort effects for the acute and chronic time periods or all time periods combined, cohorts were combined together to increase statistical power.

Table 3. Summary of Cohort Effects Tests for Total Rears, SAT Score, Total Distance Moved on SAT, and Average Velocity on SAT

Measure	Time Period	F Statistic	p-value
SAT Score	Baseline	F (3, 56) = 4.56	.006**
	Acute	F (3, 56) = 1.10	.357
	Chronic	F (3, 56) = 2.18	.101
	All Testing Days	F (3, 56) = 2.03	.120

SAT Total Distance	Baseline	F (3, 56) = 6.77	< .001***
	Acute	F (3, 56) = 1.09	.360
	Chronic	F (3, 56) = 1.96	.130
	All Testing Days	F (3, 56) = 1.78	.162
SAT Average Velocity	Baseline	F (3, 56) = 5.83	.002**
	Acute	F (3, 56) = 1.09	.362
	Chronic	F (3, 56) = 2.11	.109
	All Testing Days	F (3, 56) = 1.85	.148

Note. *p < .05, **p < .01, ***p < .001

Saline Dosage Effects

Saline dosage effects (i.e., significant differences between animals allocated to the saline-low and saline-high conditions) were not significant for any measure (of interest or otherwise) at acute testing (Appendix B). Since significant differences did not arise directly after administration, it was assumed that differences between saline conditions would remain insignificant at chronic testing as well. Therefore, for all subsequent analyses, the two saline conditions were combined and considered as a single condition.

Manually Scored Anticipatory Box Data

Total Rears

Tests for main effects and interactions of day, condition, and dosage within the total rears dataset revealed several significant results (Table 4). A significant main effect of day was discovered at habituation and baseline, but not acute or chronic testing. At habituation, animals reared significantly less on days -1 and 0 compared with day -2 (Table 5). At baseline, animals reared significantly more on day 5 compared to days 2, 3, and 4 (Table 6). Interactions of day with the other terms (i.e., day*condition, day*dosage, and day*condition*dosage) were not significant at any time period. Main effects of condition and dosage were also non-significant at all time periods, but a significant condition*dosage interaction was found at chronic testing.

Post hoc analysis of the significant condition*dosage interaction at chronic testing revealed that the interaction was significant or approaching significance between multiple groups (Figure 3). The difference in total rears between the control/8mg/kg group and the EMS/8mg/kg group approached significance, with the former rearing more than the latter (p

= .053). The difference in total rears between the control/16mg/kg and the EMS/16mg/kg also approached significance, with the control animals rearing more (p = .059). Animals in the EMS/saline group reared significantly more than animals in the EMS/16mg/kg group (p = .027), and finally, the difference in rears between EMS animals designated to the two active dosage conditions (EMS/8mg/kg and EMS/16mg/kg) approached the level of significance, with animals who received the lower dose rearing more (p = .050). Overall, these data indicate that psilocybin reduced rearing and provide some evidence that control rats reared more than rats designated to the EMS condition (particularly after psilocybin administration).

Table 4. Summary of Analyses for Total Rears

Time Period	Model Predictor	F Statistic	p-value
Habituation	Day	F (2, 116) = 91.30	< .001***
	Condition	F (1, 58) = 1.59	.212
	Day*Condition	F (2, 116) = 0.20	.820
Baseline	Day	F (4, 232) = 3.68	.006**
	Condition	F (1, 58) = 1.01	.320
	Day*Condition	F (4, 232) = 0.81	.520
Acute	Day	F (3, 162) = 2.42	.068
	Day*Condition	F (3, 162) = 0.20	.930
	Day*Dosage	F (6, 162) = 0.96	.457
	Day*Condition*Dosage	F (6, 162) = 0.61	.726
	Condition	F (1, 54) = 0.17	.680
	Dosage	F (2, 54) = 0.43	.650
	Condition*Dosage	F (2, 54) = 2.45	.096
Chronic	Day	F (5, 270) = 1.54	.177
	Day*Condition	F (5, 270) = 1.21	.304
	Day*Dosage	F (10, 270) = 0.99	.453
	Day*Condition*Dosage	F (10, 270) = 0.75	.678
	Condition	F (1, 54) = 0.22	.643
	Dosage	F (2, 54) = 1.10	.339
	Condition*Dosage	F (2, 54) = 4.01	.024*

Note. *p < .05, **p < .01, ***p < .001

Table 5. Summary of Significant Post Hoc Results for Day Effect at Habituation for Total Rears

Comparison					
Day	Day	Mean Difference	SE	*t*(58)	p-value
-2	-1	33.52	3.17	10.59	< .001***
-2	0	32.92	2.90	11.34	< .001***

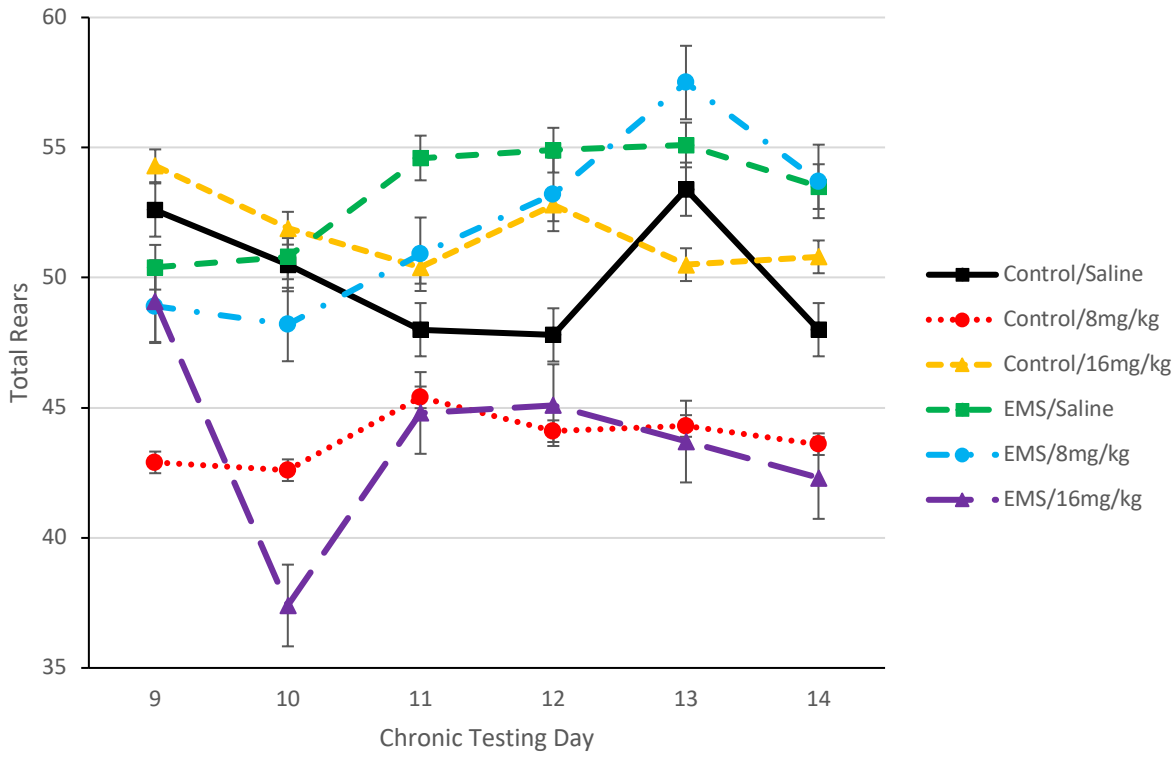
Note. *$p < .05$, **$p < .01$, ***$p < .001$

Table 6. Summary of Significant Post Hoc Results for Day Effect at Baseline Testing for Total Rears

Comparison					
Day	Day	Mean Difference	SE	*t*(58)	p-value
2	5	-5.90	1.82	-3.24	.002**
3	5	-4.00	1.47	-2.72	.009**
4	5	-3.58	1.13	-3.19	.002**

Note. *$p < .05$, **$p < .01$, ***$p < .001$

Figure 3. Condition*Dosage Interaction for Total Rears at Chronic Testing



USVs

The analysis of the USV dataset did not reveal any significant main effects or interactions at any time period (Table 7).

Table 7. Summary of Analyses for USVs

Time Period	Model Predictor	F Statistic	p-value
Baseline	Day	F (1, 58) = 0.50	.481
	Condition	F (1, 58) = 1.12	.295
	Day*Condition	F (1, 58) = 0.02	.880
Acute	Day	F (1, 54) = 3.68	.060
	Day*Condition	F (1, 54) = 1.61	.210
	Day*Dosage	F (2, 54) = 0.92	.406
	Day*Condition*Dosage	F (2, 54) = 0.08	.922
	Condition	F (1, 54) = 0.34	.561
	Dosage	F (2, 54) = 0.36	.698
	Condition*Dosage	F (2, 54) = 0.82	.448
Chronic	Condition	F (1, 54) = 0.30	.585
	Dosage	F (2, 54) = 1.92	.156
	Condition*Dosage	F (2, 54) = 1.32	.276

Note. *$p < .05$, **$p < .01$, ***$p < .001$

Manually Scored mSAT Data

SAT Score

Analysis of the SAT score dataset revealed minimal main effects and interactions across all terms and time periods (Table 8). A significant main effect of day was discovered at baseline: SAT score on day 1 was significantly less than SAT score on all other days of testing (Table 9). Additionally, SAT score on days 2 and 3 were both significantly less than on day 4 (Table 9). A day*dosage interaction also reached the level of significance at chronic testing. Closer inspection of the SAT dataset at chronic testing revealed that animals who received saline had higher SAT scores than their active dosage counterparts, particularly after day 11 of testing (Figure 4).

Table 8. Summary of Analyses for SAT Score

Time Period	Model Predictor	F Statistic	p-value
Baseline	Day	F (4, 232) = 13.23	< .001***
	Condition	F (1, 58) = 0.02	.881
	Day*Condition	F (4, 232) = 1.53	.193
Acute	Day	F (3, 162) = 0.19	.901
	Day*Condition	F (3, 162) = 0.91	.440
	Day*Dosage	F (6, 162) = 0.46	.837
	Day*Condition*Dosage	F (6, 162) = 0.55	.768
	Condition	F (1, 54) = 0.25	.617
	Dosage	F (2, 54) = 0.38	.687
	Condition*Dosage	F (2, 54) = 0.43	.650
Chronic	Day	F (5, 270) = 1.65	.147
	Day*Condition	F (5, 270) = 0.55	.545
	Day*Dosage	F (10, 270) = 1.98	.035*
	Day*Condition*Dosage	F (10, 270) = 0.35	.967
	Condition	F (1, 54) = 0.11	.745
	Dosage	F (2, 54) = 0.72	.494
	Condition*Dosage	F (2, 54) = 0.37	.691

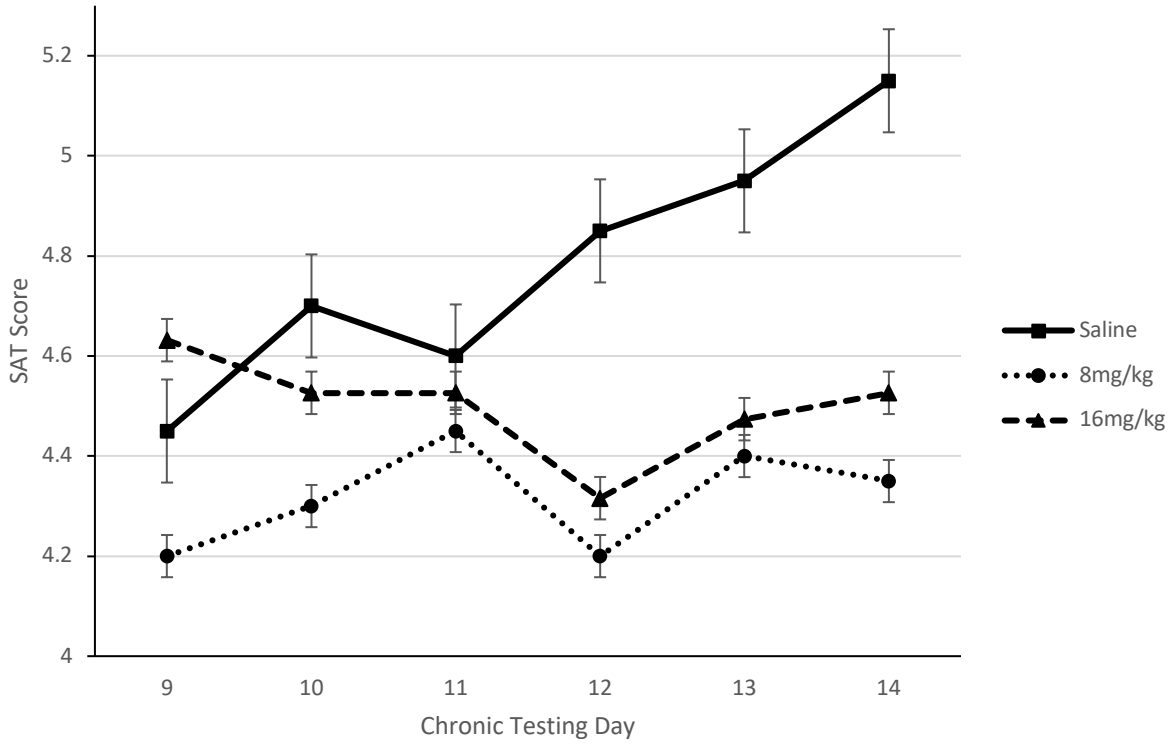
Note. *p < .05, **p < .01, ***p < .001

Table 9. Summary of Significant Post Hoc Results for Day Effect at Baseline Testing for SAT Score

Comparison					
Day	Day	Mean Difference	SE	t(58)	p-value
1	2	-1.00	0.29	-3.44	.001**
	3	-1.17	0.30	-3.86	< .001***
	4	-1.62	0.27	-6.02	< .001***
	5	-1.55	0.29	-5.38	< .001***
2	4	-0.62	0.23	-2.73	.008**
3	4	-0.45	0.19	-2.34	.023*

Note. *p < .05, **p < .01, ***p < .001

Figure 4. Day*Dosage Interaction for SAT Score at Chronic Testing



Automatically Scored Anticipatory Box Data

Total Distance Moved and Average Velocity

Analysis of the total distance moved and average velocity (i.e., locomotor behaviour) in the anticipatory box revealed a few main effects and interactions that reached the level of significance (Table 10). The specific terms that proved to be significant were very similar between the two measures, which indicates that total distance moved and average velocity are measuring similar aspects of the same behaviour (i.e., locomotor activity). Day exerted a significant main effect at habituation and baseline testing for both measures. At habituation, total distance moved and average velocity decreased significantly between day -2 and days -1 and 0 (Table 11). At baseline, total distance moved and average velocity decreased significantly between day 1 and 2, but increased significantly between each successive day of baseline testing thereafter (Table 12). A significant day*dosage interaction at acute testing also reached the level of significance for both measures. Animals designated to the saline condition exhibited more locomotor activity than either active dosage group until the last day of the acute testing period, at which point total distance moved and average velocity exhibited by the 16mg/kg group increased to reach a similar level (Figures 5 and 6).

Table 10. Summary of Analyses for Total Distance Moved and Average Velocity in the Anticipatory Box

Measure	Time Period	Model Predictor	F Statistic	p-value
Distance Moved	Habituation	Day	F (2, 116) = 86.89	< .001***
		Condition	F (1, 47) = 1.78	.187
		Day*Condition	F (2, 116) = 0.51	.605
	Baseline	Day	F (4, 232) = 5.18	< .001***
		Condition	F (1, 58) = 2.13	.149
		Day*Condition	F (4, 232) = 2.36	.054
	Acute	Day	F (3, 162) = 0.32	.810
		Day*Condition	F (3, 162) = 0.76	.518
		Day*Dosage	F (6, 162) = 2.70	.016*
		Day*Condition*Dosage	F (6, 162) = 0.48	.825
		Condition	F (1, 54) = 1.88	.176
		Dosage	F (2, 54) = 1.27	.289
		Condition*Dosage	F (2, 54) = 0.54	.589
	Chronic	Day	F (5, 270) = 0.45	.812
		Day*Condition	F (5, 270) = 0.38	.862
		Day*Dosage	F (10, 270) = 1.16	.316
		Day*Condition*Dosage	F (10, 270) = 0.79	.637
		Condition	F (1, 54) = 0.93	.339
		Dosage	F (2, 54) = 1.02	.367
		Condition*Dosage	F (2, 54) = 0.09	.910
Velocity	Habituation	Day	F (2, 116) = 89.77	< .001***
		Condition	F (1, 58) = 2.92	.093
		Day*Condition	F (2, 116) = 0.53	.592
	Baseline	Day	F (4, 232) = 5.57	< .001***
		Condition	F (1, 58) = 2.56	.115
		Day*Condition	F (4, 232) = 2.09	.083
	Acute	Day	F (3, 162) = 0.22	.880
		Day*Condition	F (3, 162) = 0.73	.538
		Day*Dosage	F (6, 162) = 2.54	.022*
		Day*Condition*Dosage	F (6, 162) = 0.42	.863

	Condition	F (1, 54) = 2.16	.147
	Dosage	F (2, 54) = 1.33	.273
	Condition*Dosage	F (2, 54) = 0.49	.615
Chronic	Day	F (5, 270) = 0.37	.868
	Day*Condition	F (5, 270) = 0.42	.838
	Day*Dosage	F (10, 270) = 1.12	.347
	Day*Condition*Dosage	F (10, 270) = 0.85	.586
	Condition	F (1, 54) = 0.82	.368
	Dosage	F (2, 54) = 1.05	.356
	Condition*Dosage	F (2, 54) = 0.08	.925

Note. *p < .05, **p < .01, ***p < .001

Table 11. Summary of Significant Post Hoc Results for Day Effect at Habituation Testing for Total Distance Moved and Average Velocity in the Anticipatory Box

Measure	Comparison		Mean Difference	SE	t(58)	p-value
	Day	Day				
Distance Moved	-2	-1	927.10	89.90	10.32	< .001***
	-2	0	947.00	88.90	10.66	< .001***
Velocity	-2	-1	1.65	0.16	10.53	< .001***
	-2	0	1.67	0.15	10.82	< .001***

Note. *p < .05, **p < .01, ***p < .001

Table 12. Summary of Significant Post Hoc Results for Day Effect at Baseline Testing for Total Distance Moved and Average Velocity in the Anticipatory Box

Measure	Comparison		Mean Difference	SE	t(58)	p-value
	Day	Day				
Distance Moved	1	2	90.90	42.20	2.15	.036*
	2	5	-154.10	43.70	-3.53	< .001***
	3	5	-112.50	34.10	-3.30	.002**
	4	5	-127.20	32.20	-3.95	< .001***
Velocity	1	2	0.36	0.15	2.41	.019*
	2	5	-0.54	0.15	-3.64	< .001***
	3	5	-0.40	0.12	-3.43	.001**

4	5	-0.43	0.11	-3.80	< .001***

Note. *$p < .05$, **$p < .01$, ***$p < .001$

Figure 5. Day*Dosage Interaction for Total Distance Moved in Anticipatory Box at Acute Testing

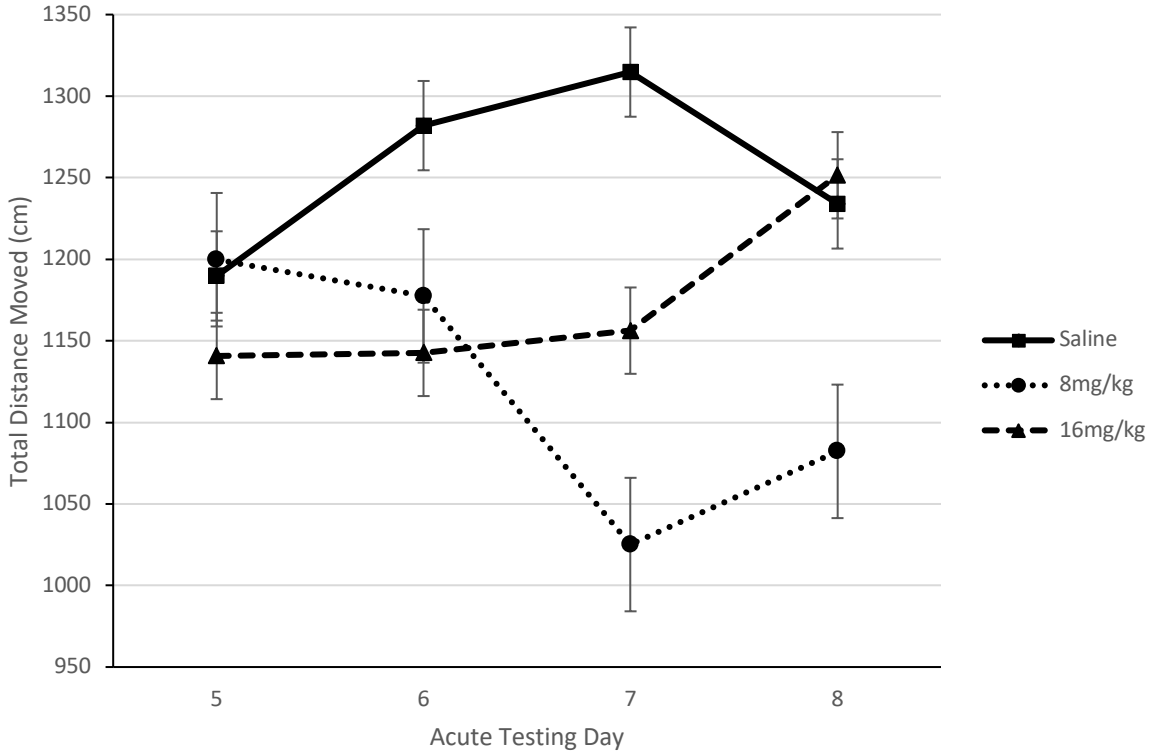
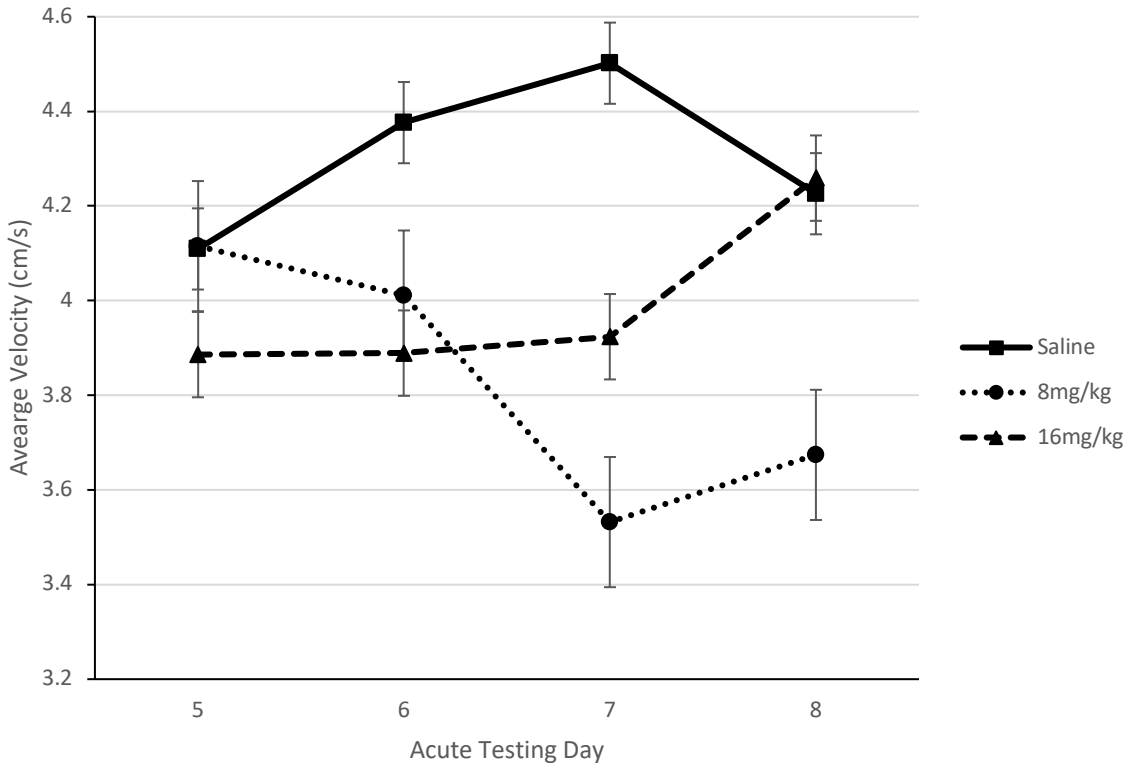


Figure 6. Day*Dosage Interaction for Average Velocity in Anticipatory Box at Acute Testing



Automatically Scored mSAT Data

Frequency of Entries into Alley 4

Analysis of the frequency of entries into alley 4 dataset revealed several significant effects (Table 13). Day exerted a significant main effect at both baseline and acute testing. At baseline, animals exhibited significantly more entries into alley 4 every day compared to day 1; additionally, animals entered significantly more on days 3 and 4 in comparison to day 2 and on days 4 and 5 in comparison to day 3 (Table 14). At acute testing, animals entered alley 4 significantly more on days 7 and 8 compared to days 5 and 6 (Table 15). A day*condition*dosage interaction also reached the level of significance at acute testing. Closer inspection of the alley 4 frequency dataset revealed that animals designated to the control/saline condition entered alley 4 more frequently than animals in all other conditions, particularly on day 8 of testing (Figure 7). No significant main effects or interactions were observed at chronic testing.

Table 13. Summary of Analyses for Frequency of Entries into Alley 4

Time Period	Model Predictor	F Statistic	p-value
Baseline	Day	F (4, 232) = 25.51	< .001***

	Condition	F (1, 58) = 0.44	.509
	Day*Condition	F (4, 232) = 0.70	.590
Acute	Day	F (3, 162) = 6.31	< .001***
	Day*Condition	F (3, 162) = 0.81	.492
	Day*Dosage	F (6, 162) = 0.70	.652
	Day*Condition*Dosage	F (6, 162) = 2.63	.019*
	Condition	F (1, 54) = 0.88	.351
	Dosage	F (2, 54) = 1.27	.290
	Condition*Dosage	F (2, 54) = 1.12	.335
Chronic	Day	F (5, 270) = 0.86	.506
	Day*Condition	F (5, 270) = 0.60	.700
	Day*Dosage	F (10, 270) = 0.58	.833
	Day*Condition*Dosage	F (10, 270) = 1.22	.276
	Condition	F (1, 54) = 0.12	.727
	Dosage	F (2, 54) = 0.10	.906
	Condition*Dosage	F (2, 54) = 0.66	.520

Note. *p < .05, **p < .01, ***p < .001

Table 14. Summary of Significant Post Hoc Results for Day Effect at Baseline Testing for Frequency of Entries into Alley 4

Comparison					
Day	Day	Mean Difference	SE	t(58)	p-value
1	2	-1.03	0.28	-3.65	< .001***
1	3	-1.47	0.24	-6.08	< .001***
1	4	-2.03	0.24	-8.47	< .001***
1	5	-2.25	0.24	-9.36	< .001***
2	4	-1.00	0.25	-3.99	< .001***
2	5	-1.22	0.28	-4.30	< .001***
3	4	-0.57	0.24	-2.39	.020*
3	5	-0.78	0.25	-3.18	.002**

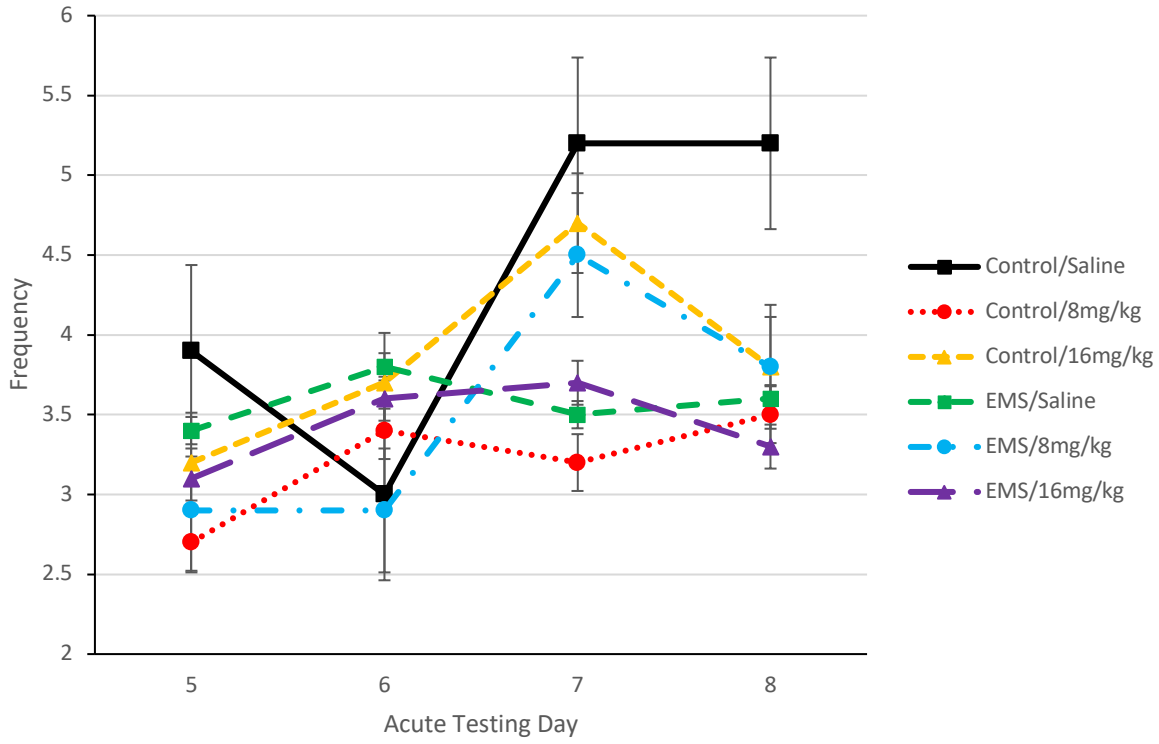
Note. *p < .05, **p < .01, ***p < .001

Table 15. Summary of Significant Post Hoc Results for Day Effect at Acute Testing for Frequency of Entries into Alley 4

Comparison		Mean Difference	SE	*t*(54)	p-value
Day	Day				
5	7	-0.93	0.28	-3.38	.001**
5	8	-0.67	0.24	-2.77	.008**
6	7	-0.73	0.24	-3.04	.004**
6	8	-0.47	0.21	-2.21	.031*

Note. *$p < .05$, **$p < .01$, ***$p < .001$

Figure 7. Day*Condition*Dosage Interaction for Frequency of Entries into Alley 4 at Acute Testing



Cumulative Duration in Alley 4

Analysis of the cumulative duration in alley 4 dataset revealed several significant main effects and interactions (Table 16). A main effect of day was significant at baseline testing and approached significance at chronic. At baseline, cumulative duration in alley 4 increased significantly between every successive day except days 4 and 5 (Table 17). At acute testing, a day*condition*dosage interaction reached the level of significance. Closer inspection of the alley 4 cumulative duration dataset did not provide any clear explanations for the significant interaction (Figure 8).

Table 16. Summary of Analyses for Cumulative Duration in Alley 4

Time Period	Model Predictor	F Statistic	p-value
Baseline	Day	F (4, 232) = 45.44	< .001***
	Condition	F (1, 58) = 0.12	.726
	Day*Condition	F (4, 232) = 1.58	.180
Acute	Day	F (3, 162) = 2.28	.081
	Day*Condition	F (3, 162) = 0.66	.578
	Day*Dosage	F (6, 162) = 1.29	.266
	Day*Condition*Dosage	F (6, 162) = 2.49	.025*
	Condition	F (1, 54) = 0.36	.552
	Dosage	F (2, 54) = 0.49	.613
	Condition*Dosage	F (2, 54) = 0.50	.608
Chronic	Day	F (5, 270) = 2.25	.050
	Day*Condition	F (5, 270) = 0.58	.712
	Day*Dosage	F (10, 270) = 1.02	.428
	Day*Condition*Dosage	F (10, 270) = 1.61	.102
	Condition	F (1, 54) = 0.22	.638
	Dosage	F (2, 54) = 0.44	.644
	Condition*Dosage	F (2, 54) = 0.04	.963

Note. *p < .05, **p < .01, ***p < .001

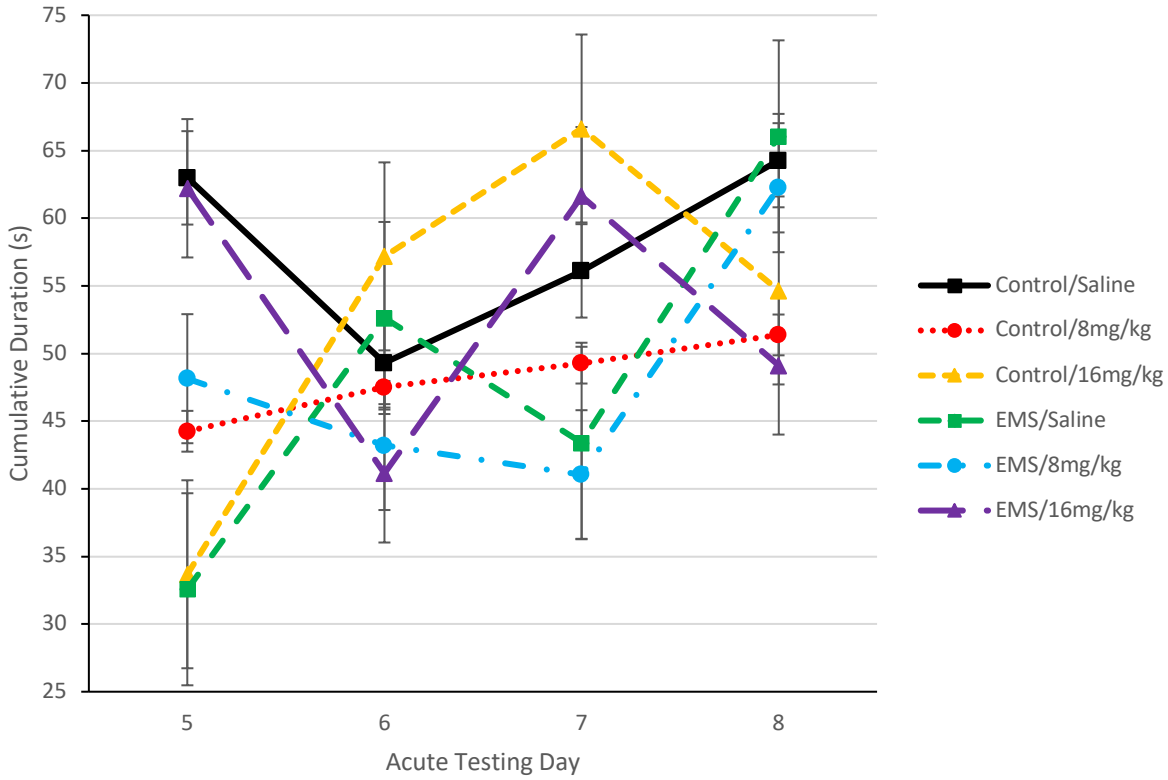
Table 17. Summary of Significant Post Hoc Results for Day Effect at Baseline Testing for Cumulative Duration in Alley 4

Comparison					
Day	Day	Mean Difference	SE	t(58)	p-value
1	2	-8.67	2.51	-3.45	.001**
1	3	-17.10	2.95	-5.79	< .001***
1	4	-32.82	3.79	-8.66	< .001***
1	5	-40.12	3.83	-10.47	< .001***
2	3	-8.43	2.92	-2.89	.005**
2	4	-24.15	3.30	-7.32	< .001***
2	5	-31.45	3.93	-8.00	< .001***
3	4	-23.02	4.03	-5.72	< .001***

3	5	-23.02	4.03	-5.72	< .001***

Note. *$p < .05$, **$p < .01$, ***$p < .001$

Figure 8. Day*Condition*Dosage Interaction for Cumulative Duration in Alley at Acute Testing



Latency to First Entry into Alley 4

In the latency to first entry into alley 4 dataset, analysis revealed that day exerted a significant main effect at all time periods (Table 18). At baseline, the decrease in latency was significant between every successive day except days 1 and 2 (Table 19). At acute, latency decreased significantly between days 5 and 7, 5 and 8, and 6 and 8 (Table 20). Finally, at chronic testing, latency significantly decreased between days 9 and 13, 10 and 11, 10 and 12, 10 and 13, 10 and 14, and 11 and 13 (Table 21). No other main effects or interactions reached the level of significance.

Table 18. Summary of Analyses for Latency to First Entry into Alley 4

Time Period	Model Predictor	F Statistic	p-value
Baseline	Day	F (4, 232) = 31.55	< .001***
	Condition	F (1, 58) = 0.36	.553

	Day*Condition	F (4, 232) = 2.02	.092
Acute	Day	F (3, 162) = 5.59	.001**
	Day*Condition	F (3, 162) = 0.44	.723
	Day*Dosage	F (6, 162) = 0.26	.955
	Day*Condition*Dosage	F (6, 162) = 0.52	.794
	Condition	F (1, 54) = 0.00	.944
	Dosage	F (2, 54) = 0.36	.697
	Condition*Dosage	F (2, 54) = 2.50	.092
Chronic	Day	F (5, 270) = 3.35	.006**
	Day*Condition	F (5, 270) = 0.51	.766
	Day*Dosage	F (10, 270) = 0.62	.800
	Day*Condition*Dosage	F (10, 270) = 1.15	.324
	Condition	F (1, 54) = 0.76	.388
	Dosage	F (2, 54) = 0.34	.712
	Condition*Dosage	F (2, 54) = 0.78	.463

Note. *p < .05, **p < .01, ***p < .001

Table 19. Summary of Significant Post Hoc Results for Day Effect at Baseline Testing for Latency to First Entry into Alley 4

Comparison					
Day	Day	Mean Difference	SE	t(58)	p-value
1	3	71.30	15.69	4.54	< .001***
1	4	105.20	12.23	8.60	< .001***
1	5	122.30	13.28	9.01	< .001***
2	3	40.80	12.93	3.16	.003**
2	4	74.70	11.53	6.48	< .001***
2	5	91.80	12.47	7.36	< .001***
3	4	33.90	11.96	2.83	.006**
3	5	51.00	12.78	3.99	< .001***
4	5	17.1	8.16	2.10	.040*

Note. *p < .05, **p < .01, ***p < .001

Table 20. Summary of Significant Post Hoc Results for Day Effect at Acute Testing for Latency to First Entry into Alley 4

Comparison					
Day	Day	Mean Difference	SE	*t*(54)	p-value
5	7	21.26	6.11	3.48	< .001***
5	8	25.92	8.11	3.20	.002**
6	8	16.00	7.54	2.12	.039*

Note. *$p < .05$, **$p < .01$, ***$p < .001$

Table 21. Summary of Significant Post Hoc Results for Day Effect at Chronic Testing for Latency to First Entry into Alley 4

Comparison					
Day	Day	Mean Difference	SE	*t*(54)	p-value
9	13	6.40	2.41	2.66	.010*
10	11	5.83	2.70	2.16	.035*
10	12	11.19	4.32	2.59	.012*
10	13	10.22	2.61	3.91	< .001***
10	14	9.66	4.29	2.25	.028*
11	13	4.39	2.17	2.02	.048*

Note. *$p < .05$, **$p < .01$, ***$p < .001$

Total Distance Moved and Average Velocity

The analysis of the total distance moved and average velocity in the mSAT revealed several significant main effects and interactions (Table 22). Like the data from the anticipatory box, these points of significance were nearly identical, which lends further support to the conclusion that these variables measure the same aspect of behaviour (i.e., locomotor activity). Day exerted a significant main effect at all time periods for both measures. At baseline, distance moved and average velocity increased significantly over each successive day except between days 1 and 2 and days 4 and 5 (Table 23). At acute, distance moved and average velocity similarly increased significantly over each successive day except between days 5 and 6 and days 7 and 8 (Table 24). Interestingly, at chronic testing, distance moved and average velocity decreased significantly between day 9 and all other days of chronic testing (Table 25). A main effect of condition was also found at acute testing for both measures: animals designed to the control condition moved significantly

more and at a significantly higher average velocity in the mSAT than EMS animals (Figures 9 and 10).

Table 22. Summary of Analyses for Total Distance Moved and Average Velocity in the mSAT

Measure	Time Period	Model Predictor	F Statistic	p-value
Distance Moved	Baseline	Day	F (4, 232) = 16.03	< .001***
		Condition	F (1, 58) = 3.15	.081
		Day*Condition	F (4, 232) = 0.60	0.665
	Acute	Day	F (3, 162) = 10.65	< .001***
		Day*Condition	F (3, 162) = 1.05	.374
		Day*Dosage	F (6, 162) = 1.71	.121
		Day*Condition*Dosage	F (6, 162) = 0.71	.642
		Condition	F (1, 54) = 5.62	.021*
		Dosage	F (2, 54) = 1.89	.162
		Condition*Dosage	F (2, 54) = 0.09	.914
	Chronic	Day	F (5, 270) = 4.57	< .001***
		Day*Condition	F (5, 270) = 0.87	.504
		Day*Dosage	F (10, 270) = 0.57	.837
		Day*Condition*Dosage	F (10, 270) = 0.61	.804
		Condition	F (1, 54) = 2.58	.114
		Dosage	F (2, 54) = 0.01	.988
		Condition*Dosage	F (2, 54) = 1.05	.358
Velocity	Baseline	Day	F (4, 232) = 16.50	< .001***
		Condition	F (1, 58) = 3.05	.086
		Day*Condition	F (4, 232) = 0.57	.683
	Acute	Day	F (3, 162) = 10.44	< .001***
		Day*Condition	F (3, 162) = 1.09	.354
		Day*Dosage	F (6, 162) = 1.63	.141
		Day*Condition*Dosage	F (6, 162) = 0.71	.639
		Condition	F (1, 54) = 5.55	.022*
		Dosage	F (2, 54) = 1.86	.165
		Condition*Dosage	F (2, 54) = 0.09	.916

Chronic	Day	F (5, 270) = 4.53	< .001***
	Day*Condition	F (5, 270) = 0.85	.514
	Day*Dosage	F (10, 270) = 0.53	.866
	Day*Condition*Dosage	F (10, 270) = 0.63	.791
	Condition	F (1, 54) = 2.55	.116
	Dosage	F (2, 54) = 0.01	.989
	Condition*Dosage	F (2, 54) = 0.98	.382

Note. *p < .05, **p < .01, ***p < .001

Table 23. Summary of Significant Post Hoc Results for Day Effect at Baseline Testing for Total Distance Moved and Average Velocity in the mSAT

Measure	Comparison					
	Day	Day	Mean Difference	SE	t(58)	p-value
Distance Moved	1	3	-123.00	44.10	-2.79	.007**
	1	4	-205.30	51.50	-3.99	< .001***
	1	5	-235.20	38.20	-6.15	< .001***
	2	3	-142.20	35.40	-4.02	< .001***
	2	4	-224.60	41.00	-5.47	< .001***
	2	5	-254.40	39.50	-6.44	< .001***
	3	4	-82.30	35.20	-2.34	.023*
	3	5	-112.20	38.90	-2.88	.006**
Velocity	1	3	-0.43	0.15	-2.88	.006**
	1	4	-0.71	0.17	-4.10	< .001***
	1	5	0.81	0.13	-6.35	< .001***
	2	3	-0.48	0.12	-4.00	< .001***
	2	4	-0.76	0.14	-5.50	< .001
	2	5	-0.86	0.13	-6.51	< .001***
	3	4	-0.28	0.12	-2.34	.023*
	3	5	-0.38	0.13	-2.90	.005**

Note. *p < .05, **p < .01, ***p < .001

Table 24. Summary of Significant Post Hoc Results for Day Effect at Acute Testing for Total Distance Moved and Average Velocity in the mSAT

Measure	Comparison

	Day	Day	Mean Difference	SE	*t*(54)	p-value
Distance Moved	5	7	-231.60	54.60	-4.24	< .001***
	5	8	-176.10	47.70	-3.69	< .001***
	6	7	-176.90	45.20	-3.69	< .001***
	6	8	-121.30	36.60	-3.32	.002**
Velocity	5	7	-0.75	0.18	-4.16	< .001***
	5	8	-0.57	0.15	-3.70	< .001***
	6	7	-0.59	0.15	-3.94	< .001***
	6	8	-0.41	0.12	-3.30	.002*

Note. *$p < .05$, **$p < .01$, ***$p < .001$

Table 25. Summary of Significant Post Hoc Results for Day Effect at Chronic Testing for Total Distance Moved and Average Velocity in the mSAT

Measure	Comparison					
	Day	Day	Mean Difference	SE	*t*(58)	p-value
Distance Moved	9	10	157.31	41.20	3.82	< .001***
	9	11	100.47	39.60	2.54	.014*
	9	12	144.31	41.60	3.47	.001**
	9	13	97.36	45.20	2.15	.036*
	9	14	122.97	39.80	3.09	.003**
Velocity	9	10	0.53	0.14	3.89	< .001***
	9	11	0.34	0.13	2.59	.012*
	9	12	0.48	0.14	3.41	.001**
	9	13	0.32	0.15	2.09	.042*
	9	14	0.40	0.13	3.01	.004**

Note. *$p < .05$, **$p < .01$, ***$p < .001$

Figure 9. Main Effect of Condition for Total Distance Moved in the mSAT at Acute Testing

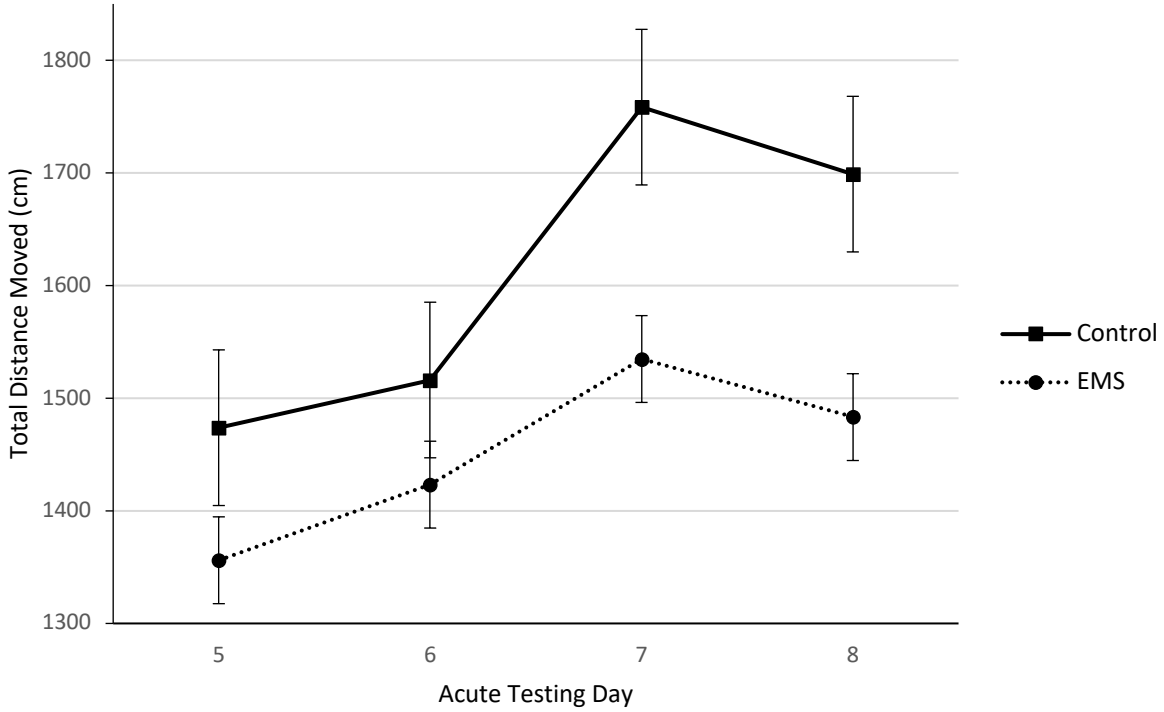
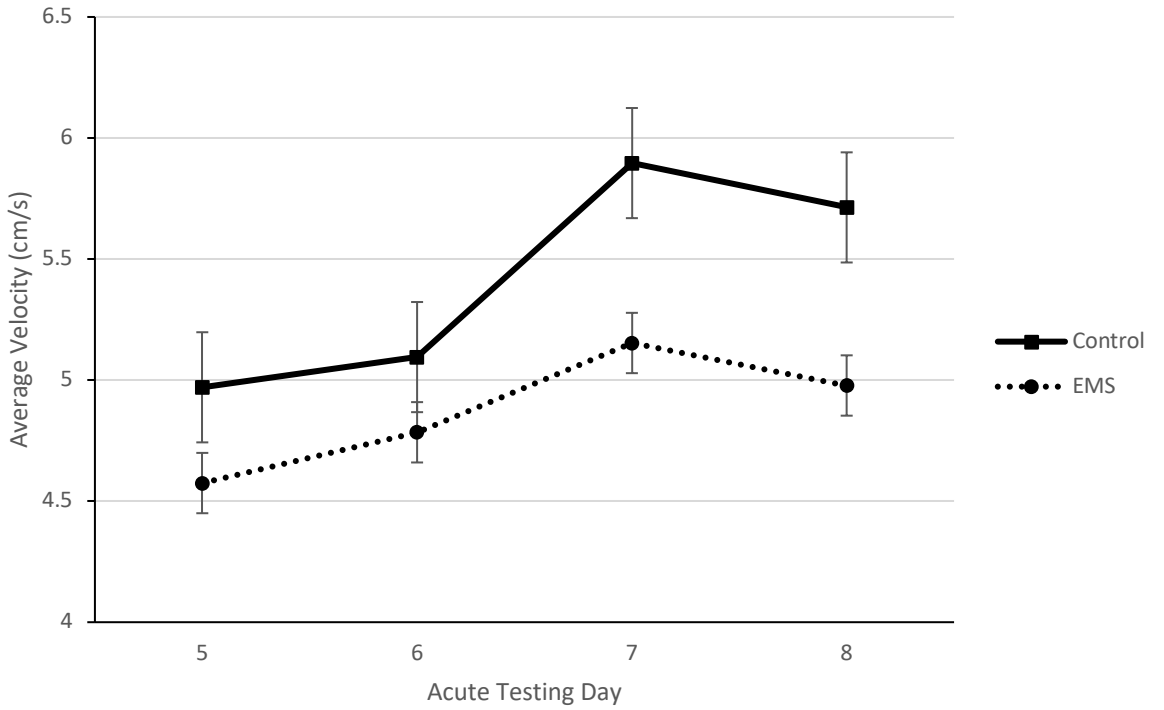


Figure 10. Main Effect of Condition for Average Velocity in the mSAT at Acute Testing



Discussion

The purpose of the present study was to assess whether psilocybin reduces depressive- and anxiety-like symptomology in an animal model. Instead of a genetic model, an environmental paradigm involving early chronic perinatal stress was used to induce symptomology. Animals were tested for pathological tendencies with the Affective Disorders Test (ADT) prior to psilocybin administration, and for several weeks after to determine whether the drug impacted symptomology. If psilocybin reduced depressive- and anxiety-like behaviour in the current study, then the drug may achieve a therapeutic impact in humans via an underlying neurological mechanism that is conserved between species. However, if psilocybin had no effect in the animal model, perhaps the therapeutic utility of the drug relies on the context of psychotherapy or a unique facet of human cognition and perception. Alternatively, the animal model employed may not have produced symptomology that was robust enough to be impacted by the pharmacological intervention.

In total, 14 different measures were collected: 4 measured depressive-like symptomology in the form of anticipatory pleasure (i.e., total rears, total distance moved and average velocity in the anticipatory box, and total USVs) and 10 measured anxiety-like symptomology in the modified SAT (mSAT) (i.e., SAT score, latency to consume 4th Froot Loop, total distance moved and average velocity in the mSAT, frequency of entries, cumulative duration, and latency to first entry into alley 3, and frequency of entries, cumulative duration, and latency to first entry into alley 4). The goal of including multiple measures (both manually and automatically scored) for both behaviours was to capture as many manifestations of symptomology as possible. However, the downside of collecting data for so many measures was that it increased the likelihood of false positives (i.e., type 1 error) in the statistical analyses: a main effect or interaction could have reached the level of significance purely by chance. Since only a few main effects and interactions of the critical terms reached the level of statistical significance, it is possible that they did so by chance. However, if false positives did occur, then significant effects and interactions would have presumably occurred across all variables. The data obtained indicates that points of significance were found in only a few specific, related variables at a higher rate than any others. Thus, it is unlikely that false positives were in fact the cause of the significant results reported previously.

Summary and Interpretation of Key Results

One of the most consistently significant terms found across all measures and time periods was day of testing. Although this result does little in terms of suggesting a meaningful conclusion in terms of the main hypothesis, it does suggest some important conclusions with regard to how the ADT functioned as an assay. The ADT was designed specifically to circumvent issues with one-trial tolerance in the measurement of anxiety-like symptomology (Blackburne, 2021). Given that each animal was exposed to the mSAT protocol for 14 days and the anticipatory box for 17, some effect caused by increasing familiarity with the testing protocol was bound to occur. However, the most consistent day effects were found within the habituation and baseline testing periods for each measure, which suggests that performance on the ADT stabilized as testing continued into the acute and chronic periods.

The significant main effects of day found for several of the anticipatory behaviour measures suggest an important conclusion with regard to the ADT. Day exerted a significant effect for total rears, total distance moved, and average velocity at habituation and baseline. Post hoc analysis revealed that the pattern of effect was identical for each measure: activity initially decreased during habituation, and then increased after the first day of baseline testing. The consistency of this pattern across most of the anticipatory measures implies that initial exposure to the box led to novelty exposed activity and the habituation period reduced it, thereby highlighting the importance of including the habituation procedure before the anticipatory phase. More importantly, by temporally pairing impending access to a sucrose reward (i.e., Froot Loops) with the contextual stimuli of the box, the ADT successfully induced anticipatory behaviour. Furthermore, these results suggest that total rears and locomotor activity (i.e., total distance moved and average velocity) are robust behavioural measures that capture anticipatory pleasure.

The pattern of significant day effects across the mSAT measures are more ambiguous in terms of what can be concluded about how the anxiety-like behaviour portion of the ADT functioned. Day exerted a significant effect at baseline testing for all measures of interest, and at acute for most; across the board, post hoc analyses showed that anxiety-like behaviour decreased across baseline and acute testing (and chronic testing for latency to first entry into alley 4). However, locomotor activity appeared to decrease with prolonged exposure: total distance moved and average velocity both decreased significantly across chronic testing days. There are several possible interpretations of this finding. Perhaps animals were no longer incentivised to explore the alleys with the same verve as they did initially after so many days

of repeated exposure (i.e., a ceiling effect for exploratory behaviour). Alternatively, perhaps locomotor activity is not as robust an indicator of anxiety-like behaviour in the mSAT as it is for anticipatory behaviour in the box.

Unlike day, condition proved to be a universally poor predictor of group differences. The only measures for which condition yielded a significant main effect were total distance moved and average velocity in the mSAT at acute testing. As mentioned previously, these variables seemed to measure the same elements of behaviour, and therefore produced nearly identical results. It is therefore unsurprising that condition emerged as a significant term for both measures. Control animals scored higher on both measures than EMS animals at every day of acute testing, and particularly on day 7, which was two days after animals received either saline or one of two active doses of psilocybin (i.e., 8 or 16mg/kg); however, no significant differences between conditions emerged at baseline testing. In combination, these results could be interpreted several ways: perhaps EMS failed to create a reliable phenotype for depressive- and anxiety-like symptomology, or perhaps the ADT was insufficiently sensitive to capture differences between these groups. Alternatively, perhaps EMS did exert a significant effect, but differences only materialized in acute testing and were not ameliorated by the pharmacological intervention. However, the lack of significant differences between conditions for other time periods and measures provides greater support for one of the two former interpretations, which will be expanded upon further in the limitations section.

A main effect of dosage was not found for any measure at any time period. This lack of effect implies that, irrespective of condition, the dose of psilocybin that animals were exposed to (and even whether they were exposed to psilocybin at all) had no impact on behaviour in the ADT. From a pharmacological perspective, these results are unexpected given observations of the animals that received active doses versus those that received saline. Animals injected with either the 8 or 16mg/kg dose of psilocybin uniformly exhibited reduced locomotor activity and flat body posture (FBP), whereas animals that received saline behaved normally (Beer, 2021). FBP is an established marker of high 5-HT receptor stimulation, often considered part of the so-called ‘serotonin syndrome’ (Canal & Morgan, 2012). Rodents exhibit FBP after exposure to hallucinogenic serotonin agonists, such as 2,5-Dimethoxy-4-iodoamphetamine (DOI), as well as non-hallucinogenic substances that stimulate the serotonergic system, such as 1-5-hydroxytryptophan (5-HTP) (Geyer & Krebs, 1994; Colpaert et al., 1989). Furthermore, ritanserin, a 5-HT_{2A} antagonist, reduces FBP

induced by serotonin agonists (Colpaert et al., 1989). The consistency of FBP in animals treated with either active dose indicates that psilocybin had a substantial acute effect, but the lack of any main effects of dose indicates that this acute pharmacological impact did not translate into a prolonged change in behaviour in the ADT. Potential reasons for this discrepancy between the acute pharmacological and chronic behavioural effects of psilocybin will be discussed further in the limitations section.

The analysis of the total rears dataset revealed a significant interaction of condition and dosage at chronic testing. Post hoc analysis of the interaction revealed that the difference in rears approached significance between several groups. Animals designated to the control/8mg/kg group reared more than animals in the EMS/8mg/kg group (this difference approached significance, $p = 0.53$), and the difference in rears between animals in the control/16mg/kg group and EMS/16mg/kg group also approached significance ($p = .059$), with control/16mg/kg animals rearing more. In combination, these results suggest that EMS may have induced some degree of anhedonia-like symptomology, and that neither the 8 nor the 16mg/kg dose significantly reduced that behavioural tendency. However, it is somewhat surprising that these effects only appeared at chronic testing. It is also important to note that there was no significant difference between animals designated to the control/saline group and control animals exposed to either dose of psilocybin, which provides further support for the conclusion that psilocybin did not exert a substantial impact on long-term behaviour. Interestingly, animals designated to the EMS/saline and EMS/8mg/kg conditions reared more than EMS/16mg/kg animals ($p = .027$ and $p = .052$, respectively), which suggests a dose-dependent relationship in the opposite direction of what was expected. However, given the lack of consistent dose dependent or condition effects, formulating a clear conclusion from the post hoc results for total rears is not possible.

The analysis of total USVs did not reveal any significant main effects or interactions of any terms at any time period. Unlike the other measures, only data collected on days 1, 5, 6, and 9 were analysed. Only these select days were considered because analysing the raw sound files with DeepSqueak proved to be extremely time-consuming; so time-consuming, in fact, that analysing all raw sound files collected would have been far beyond the scope of the current study. Thus, days 1 and 5 were analysed as a measure of anticipatory behaviour at baseline testing, day 6 (i.e., the day after injections) was analysed as a measure of acute behaviour, and day 9 was analysed as a measure of chronic behaviour. It is possible that analysing USV data for all testing days might have revealed a few more significant

differences between groups, and a complete analysis would have certainly produced a more comprehensive picture of how USVs represent anticipatory pleasure at the very least. However, it is unlikely that choosing to analyse a select few days critically endangered the assessment of anticipatory pleasure because many other measures of that same behaviour were analysed across all testing days and painted a very similar picture. Furthermore, it is important to note that the number of USVs emitted is extremely variable between individual subjects under any circumstances, and this inherent variability may have prevented differences between experimental groups from reaching the level of significance. A large degree of individual variance in USVs is a common feature in outbred rat strains. An alternative strategy for the future might be to pre-select animals on the basis of their baseline call frequency.

Analysis of the automatically scored data from the anticipatory box (i.e., total distance moved and average velocity) indicated that the same model predictors that reached significance did so across the same testing periods for both measures. As mentioned previously with regard to total distance moved and average velocity in the mSAT, this similarity in results indicates that the two measures are assessing the same behaviour: locomotor activity. A day*dosage interaction reached the level of significance at acute testing for both measures. Animals who were administered saline moved more and at a higher average velocity than either dosage group. The 16mg/kg group showed a slow increase in locomotor activity, ultimately reaching similar levels of movement to the saline group by the end of the acute testing period. Meanwhile, the 8mg/kg animals exhibited comparatively low levels of locomotor activity across acute testing days. Taken together, these results suggest that the higher dose of psilocybin may have reduced anhedonia-like behaviour to a greater degree than the 8mg/kg dose, at least in the short-term. However, the lack of consistent dosage group trends across the acute testing period renders this conclusion somewhat tentative.

For the SAT score, a day*dosage interaction reached the level of significance at chronic testing. The interaction seemed to arise from the fact that animals treated with saline had significantly higher scores than animals in either active dosage condition, particularly after day 11 of testing. Contrary to the original hypothesis, this result implies that both doses of psilocybin exacerbated anxiety-like symptomology towards the end of testing, while non-treated animals appeared to exhibit less anxious behaviour. However, it should be noted that the difference between the least anxious and most anxious groups was less than 1 point (on a

scale of maximally 10) and hence the functional importance of this significance may not be very substantial.

The analysis of the frequency of entries into alley 4 revealed a significant day*condition*dosage interaction at acute testing: animals designated to the control/saline condition exhibited the greatest increase in frequency of entries between days 6 and 7, and entered alley 4 more than all other experimental groups across days 7 and 8 of testing (injections were given between days 5 and 6). However, it should also be noted that animals designated to the EMS/8mg/kg group showed a dramatic increase in frequency of entries between days 6 and 7, although they did not exceed the control/saline group. These results are somewhat contradictory: they suggest that psilocybin may have exacerbated anxiety-like symptomology in the days directly after administration, but also that the lower dose may have somewhat ameliorated anxiety-like tendencies for EMS animals specifically. The analysis of the cumulative duration in alley 4 also revealed a significant day*condition*dosage interaction at acute testing, with the control/16mg/kg group showing the greatest increase in duration between days 5 and 7. However, it was not clear from the trend lines at precisely what point this 3-way interaction reached significance. Finally, the analysis of the latency to first entry into alley 4 revealed that day was the only significant term; no other main effects or interactions reached the level of significance.

The measures for alley 3 in the mSAT (i.e., frequency of entries, cumulative duration, and latency to first entry) yielded no significant effects or interactions beyond some main effects of day at specific time periods. These results are unsurprising given that most animals exhibited low enough levels of anxiety-like behaviour to visit alley 4 multiple times by the end of the baseline testing period. Although there were a few more main effects and interactions for the alley 4 measures, the lack of clear direction or consistency implies two potential issues. As mentioned previously, perhaps EMS animals were not significantly more anxious than control animals; alternatively, perhaps the mSAT was not sufficiently anxiogenic to elicit consistently different behaviour between the two conditions, or the mSAT measures were insufficiently sensitive to capture it. These potential explanations for the lack of significant differences between groups in the mSAT measures will be explored at greater length in the limitations section.

Methodological Considerations

The current study involved three critical methodological components: EMS to induce depressive- and anxiety-like symptomology, the ADT to measure it, and comparatively high doses of psilocybin as a pharmacological intervention to counteract the behavioural alterations induced by EMS. Careful consideration of precisely how the methodological planning and execution of these critical components may have led to the dearth of significant findings and consistent directionality, either solely or in combination, is warranted.

EMS

EMS is a well-established manipulation within the preclinical literature. However, the precise parameters used (i.e., the duration of daily separations, the age at which separation begins, etc.) vary wildly between experimenters and studies (Lehmann & Feldon, 2000). These differences in precisely how EMS is executed are critical because, according to the literature, EMS does not universally produce a robust phenotype. There is some indication that protocols employing consecutive days of separation require the separation period to be at least 2 hours to produce a chronic effect on the hypothalamic-pituitary-adrenal (HPA) axis (Lehmann & Feldon, 2000). The majority of researchers that use consecutive days of EMS to induce symptomology employ daily 3-hour periods of separation between postnatal days (PND) 2 and 14, although the stress hyporesponsiveness period (SHRP) in the developing rodent brain occurs between PND4 and 14 (Vetulani, 2013; Sapolsky et al., 1986). There is considerable evidence to suggest that rat pups exposed to this version of EMS exhibit HPA hyperresponsiveness which leads to myriad neurobiological changes in adulthood, including increased corticotropin-releasing hormone (CRH) and noradrenaline transmission as well as downregulation of CRH and glucocorticoid receptor (GR) mRNA in the amygdala and medial prefrontal cortex (Vetulani, 2013). These neurochemical changes purportedly correlate with behavioural differences measured with standard preclinical tests, such as the elevated plus maze and open field test to measure anxiety-like symptomology, and the forced swim test and sucrose preference test to measure depressive-like symptomology. When EMS is successful, pups exposed to the manipulation exhibit distinct behavioural tendencies including increased fear responsiveness, decreased anticipatory pleasure, and increased anhedonia-like behaviour; although, again, these findings are mixed and seem to depend heavily on the specific form of measurement employed (Lehmann & Feldon, 2000).

The present study adhered to the standard practice of 3-hour separation over consecutive days, but pups were separated from PND4-14 rather than PND2-14. The

decision to slightly shorten the window of separation was made in the interest of animal welfare: there is a fine line between inducing a robust preclinical psychiatric analogue and causing significant and unnecessary harm to developing animals. Because the SHRP occurs from PND4-14, separating the animals for an extra two days before the SHRP window began was deemed an unnecessarily harsh extension of an already stressful paradigm. Furthermore, there is evidence to suggest that separating for additional days does not lead to an increased likelihood of inducing symptomology. Long-Evans pups separated from dams for 3 hours per day between PND3-14 did not show any significant difference from control animals in sucrose preference (a measure of anhedonia-like behaviour and anticipatory pleasure) or exploratory behaviour (a measure of anxiety-like behaviour) (Shalev & Kafkafi, 2002). Similarly, separating Wistar pups from PND1-14 for 3 hours per day did not lead to any significant neurobiological or behavioural changes in either male or female animals (Farkas et al., 2009). Even separating animals for 4 hours and an additional day (i.e., PND1-15) did not produce significant differences in measures of pathological tendencies, such as exploration, locomotor activity, or proclivity towards substance dependence (Marmendal et al., 2004).

One possibility could be that environmental manipulations on their own are insufficient to produce a robust phenotype. The potential for EMS to impact the behaviour of separated pups in adulthood is highly dependent on maternal behaviour directly before and after the period of separation (Schmidt et al., 2011). Disruptions in maternal care soon after birth have been shown to produce neurobiological changes related to HPA-axis regulation, such as increased methylation of the GR gene (Nishi et al., 2013). Within standard EMS protocols, there is no method to directly control for differences in maternal care before and after the period of separation. Patterns of maternal care seem to vary significantly between strains of rodents and even between individual dams (Schmidt et al., 2011). One method to ensure that maternal care is in fact disrupted by EMS is to combine it with an established genetic model of depression: Flinders Sensitive Line (FSL) animals separated for 3 hours per day from PND2-14 exhibited a significant increase in depressive-like behaviour in adulthood compared to Flinders Resistant Line (FRL) animals (El Khoury et al., 2006). Furthermore, these pathological tendencies were ameliorated in adulthood when animals were treated with a selective serotonin reuptake inhibitor (SSRI), which suggests that the epigenetic manipulation did in fact yield an animal analogue for depressive-like symptomology (El Khoury et al., 2006). Thus, it is entirely possible that an environmental and genetic ‘double

hit' is required to disrupt maternal care sufficiently to produce a robust phenotype (Schmidt et al., 2011). It is also important to note that the effects of EMS are assessed in adulthood weeks after the procedure concludes, so everything that occurs within that intervening time period inevitably impacts the long-term consequences of the paradigm. Maintaining methodological consistency across as many salient variables (i.e., housing, light/dark cycles, food type, etc.) as possible is critical to the success of any EMS protocol.

The ADT

As mentioned previously, the ADT is a novel preclinical behavioural assay that was designed to measure both depressive- and anxiety-like symptomology. The mSAT measures anxiety-like behaviour by juxtaposing the rewarding opportunity to explore with the inherently anxiogenic properties of exposed spaces. The original SAT is similar to the mSAT in that it consists of four connected alleys that are successively more exposed (Deacon, 2013). However, the original test suffers from the one-trial tolerance effect: after initial exposure, animals habituate to the testing apparatus and lose motivation to enter the latter alleys (File et al., 1990; Blackburn, 2021). The mSAT ameliorates this effect by incentivising animals to continue to explore the more anxiogenic alleys with a sucrose reward (i.e., Froot Loops), thereby permitting repeated testing and assessment of anxiety-like behaviour as a stable trait. Meanwhile, the anticipatory box measures anhedonia-like behaviour directly before the mSAT: by temporally pairing the box with the mSAT repeatedly, animals begin to associate the box with impending access to Froot Loops and display anticipatory behaviour (i.e., rears and anticipatory USVs).

Even though the ADT was only recently developed and tested, preliminary findings suggest that it is a valid and reliable measure for depressive- and anxiety-like behaviour. The serotonin transporter knockout strain of rats (SERT KO) exhibit behavioural tendencies in a variety of preclinical assays that are consistent with depressive- and anxiety-like symptomology, particularly the homozygous strain ($SERT^{-/-}$) (Olivier et al., 2008). When tested with the ADT, these animals exhibit less anticipatory behaviour in the anticipatory box and greater anxiety-like behaviour in the mSAT than their control counterparts (Blackburne, 2021). Furthermore, standard pharmacological interventions lead to a significant change in performance. After treatment with ketamine (which has shown promise as a pharmacological treatment for depressive disorders), $SERT^{-/-}$ animals rear significantly more in the anticipatory box compared with baseline testing (Blackburne, 2021; Murrough et al., 2013).

Similarly, SERT$^{-/-}$ animals treated with diazepam (an established anxiolytic medication) spend significantly more time in alley 3 and consume Froot Loops in the more anxiogenic alleys more often compared with baseline performance (Blackburne, 2021). While these initial findings support the validity of both components of the ADT, it is possible that the assay is not suited to elucidate differences between experimental groups when the divergence in behaviour is less pronounced or the effects of the pharmacological intervention are less substantial.

Recent findings with the ADT have also established that food restriction is a critical component of the methodology. Compared with free-feeding counterparts, animals maintained at 85-90% of their free-feeding weight prior to and throughout the duration of ADT testing exhibit significantly more anticipatory USVs and rearing behaviour; they also retrieve Froot Loops in the mSAT faster and tend to consume them in the more anxiogenic alleys more often (Blackburne, 2021). In the present study, food restriction proved to be extremely difficult to maintain and standardize across individual rats and cohorts because animals were still growing when restriction commenced. Therefore, rather than maintaining animals at a certain percentage of their free-feeding weight, the goal was to adjust feeding to allow some growth to occur, but at a slower rate than would be exhibited under free-feeding conditions. Inevitably, this was a process of constant adjustment and re-evaluation based on daily weight averages.

As mentioned in the method section, the decision to increase or decrease daily food rations was made by comparing the average weight of each cohort with average free-feeding weight charts for normally developing Sprague-Dawley rats at regular age intervals. Every effort was made to keep the average weight of each cohort at the very edge of two standard deviations below the free-feeding average at all age points. Considerable variability between cohorts in average free-feeding weight at the beginning of the food deprivation period, as well as variability in how rapidly animals gained or lost weight during food restriction, rendered efforts to temporally standardize increases or decreases in feeding between cohorts impossible. Despite efforts made to reduce inter-individual variability by feeding each animal in each cohort the same amount, individual weights varied wildly. Similarly, despite efforts to minimize inter-cohort variability by comparing average weight to the same standard metric and keeping feeding amounts between 13 and 19 grams for each animal in each cohort throughout the duration of experimentation, there was considerable variation in average weight between cohorts. The food restriction protocol proved to be the single most

challenging component of the entire experiment, and potentially the greatest source of methodological inconsistency. However, it is promising that despite this potential confound, there were very few significant cohort effects and the variability in performance across measures between individual animals was relatively low (with the exception of USVs).

Dosage

In the current study, animals were administered one of two doses of psilocybin (8 or 16mg/kg) or a vehicle control. Within the existing (albeit small) body of both clinical and preclinical research, two distinct types of doses have been investigated: full doses and microdoses. Although there is considerable variability in the precise amount ingested, microdoses are generally defined as a dose that is one tenth of what would ordinarily induce significant hallucinogenic effects (Kuypers, 2020). There is some evidence from uncontrolled population studies involving human participants that microdoses of psilocybin may have some therapeutic value in the context of depression, although controlled trials involving psychiatric populations have yet to be conducted and published (Kuypers, 2020). Similarly, there are a few examples from preclinical research that have reported positive results of psilocybin microdosing regimens, albeit modest. For instance, psilocin, the active compound in psilocybin mushrooms, modestly decreased anxiety-like behaviour in rats at a dose of 0.05-0.075mg/kg administered on three occasions (Horsley et al., 2018). Similarly, a single low dose of 0.05-0.1mg/kg of psilocybin administered to rats mildly improved measures of motivation and attention (Higgins et al., 2021). Interestingly, however, another study found that rats administered 0.1mg/kg of psilocybin exhibited significantly greater reductions in depressive- and anxiety-like symptomology than animals administered 0.025 or 0.05mg/kg (Risca, 2021). This final finding suggests that there might be a floor effect for the therapeutic utility of microdosed psilocybin, at least in rodents. Moreover, the modest effect sizes of these results across the board suggest that preclinical models might not be the best tools to investigate the therapeutic utility of microdosing psilocybin.

Preclinical investigations of the effects of full doses of psilocybin are unfortunately just as limited in number as those investigating microdoses, and even more conflicted in terms of effect. In rats, research suggests that 1mg/kg is the minimal dose required to induce behavioural effects (Meinhardt et al., 2020). While one study found that 1mg/kg of psilocybin administered once produced a persistent antidepressant and anxiolytic effect in Wistar-Kyoto (WKY) animals, another found no effect whatsoever of 2, 3, or 10mg/kg

administered once to FSL rats (Hibicke et al., 2020; Jefsen et al., 2019). Although these studies utilized different animal models of symptomology, both used the forced swim test and open field test to measure depressive- and anxiety-like symptomology (respectively) and both gave injections via the same route of administration (i.e., intraperitoneally) (Hibicke et al., 2020; Jefsen et al., 2019). Taken together, these results might suggest a ceiling effect of psilocybin dosage from a therapeutic efficacy perspective. However, another study that investigated the neurobiological effects of a variety of doses of psilocybin ranging from 0.5 to 20mg/kg found that it increased plasticity-related genes, particularly in the prefrontal cortex and hippocampus, in a dose-dependent manner (Jefsen et al., 2020). Since reduced plasticity in both regions has been implicated in the neuropathogenesis of depression, these results suggest that higher doses of psilocybin may be more effective at ameliorating depressive-like symptomology in preclinical models (Duman et al., 2016).

By all accounts, the two active doses used in the present study were substantial. The decision to use comparatively higher rather than lower doses was made based on consideration of the few and conflicting findings from the studies described above. Additionally, in comparative neurobiological studies, psilocin was found to have 15 times the affinity for human 5-HT_2 receptors than it did for the same receptors in rodents, which suggests that a higher dose is required to produce the same neurochemical and behavioural effects in rodents (Gallaher et al., 1993; Klein et al., 2018). At the very least, the claim that the doses administered were too low to produce an effect cannot be reasonably made. As described previously, animals exposed to psilocybin consistently displayed behaviour indicative of an acute pharmacological effect (i.e., FBP), and this behaviour was not exhibited by any animal administered saline (Beer, 2021). However, it is possible that the doses chosen were in fact too high (i.e., a ceiling effect). More preclinical research that investigates a variety of doses while keeping all other experimental variables constant will inevitably help to determine the correct dose to optimize symptom reduction.

Implications

Given the lack of significant effects, as well as the limitations discussed above, potential implications of the current study mostly involve methodological considerations for future research investigating the therapeutic efficacy of psilocybin in preclinical models of depressive- and anxiety-like symptomology. The original null hypothesis stipulated that if psilocybin did not reduce pathological behavioural tendencies in animals, the therapeutic

utility might depend on the context of ongoing psychotherapy before, during, and after the acute effects of the drug are experienced, or on some unique and intrinsic quality of the human psyche. Other researchers who have investigated the effects of psilocybin in animal models of depressive- and anxiety-like behaviour have expressed scepticism that rats possess sufficient self-awareness to critically introspect during the acute hallucinogenic experience (Hibicke et al., 2019). However, the same researchers that expressed that scepticism also found a significant therapeutic effect of psilocybin in an animal model. They attribute their positive results to fundamental underlying neurological mechanisms that are affected by psilocybin and implicated in depression and anxiety, such as the 5-HT_{2A} receptor and the serotonergic system (Hibicke et al., 2019). Indeed, there is evidence to suggest that a single administration of psilocybin is sufficient to exert a significant impact on genes related to neuroplasticity in the rodent brain (Jefsen et al., 2020). However, the results from the present study alone are insufficient to reliably conclude that psilocybin exerts a therapeutic effect only in the context of psychotherapy and human perception, via a universal neurobiological mechanism, or a combination of both.

What can be implied from the present study, however, is that far more preclinical research involving psilocybin and animal models of depressive- and anxiety-like behaviour is needed before any reliable conclusions regarding the therapeutic mechanism of psilocybin can be made. Serotonergic hallucinogens induce extremely complex constellations of effects, both neurochemically and behaviourally; preclinical models offer the opportunity to tease apart these potential mechanisms by reducing other sources of variability through tightly controlled experimental settings. Furthermore, preclinical paradigms allow for the opportunity to directly assess and correlate in vivo neurological assays with observed behaviour. The conflicting results of the few other preclinical studies that have investigated psilocybin in the context of depression and anxiety also imply the need for methodological consistency: even with more publications, comparison of results is impossible if researchers do not use the same animal models of pathology, the same methods of testing symptomology, and the same doses and dosing regimens.

Future Directions

As the discussion of limitations suggests, there are a plethora of potential avenues for future research to take. From the results obtained in the present study, it is apparent that the specific EMS protocol employed was insufficiently stressful to produce a robust phenotype.

Although it would require sacrificing some degree of animal wellbeing, utilizing a more severe version of EMS that has been validated by previous research might increase the chances of inducing symptomology. However, given reports of null effects even after subjecting pups to relatively more severe paradigms (i.e., 4-hour separations from PND1-15), perhaps a better alternative would be a genetic model, such as FSL, WKY, or SERT KO animals (Marmendal et al., 2004).

While genetic manipulations tend to produce far more reliable and consistent models of pathology than environmental manipulations, genetically altered animals also exhibit significantly greater neurochemical and neurobiological abnormalities, especially within the serotonergic system (Hasegawa et al., 2006; De La Garza 2nd et al., 2004; Homberg et al., 2007). These abnormalities could render genetic models unsuitable for testing the effects of serotonergic drugs; on the other hand, given that many of these models are validated with SSRIs, perhaps the inherent disruptions to the serotonergic system would not preclude a potential therapeutic effect. The environmental paradigms that are reported to produce the most robust phenotypes do so in the presence of a concurrent genetic manipulation (i.e., EMS in FSL rats) (El Khoury et al., 2006). Thus, perhaps a sensible direction for future research extending from the current study to take would be to execute a slightly more severe version of EMS (i.e., 3-hour separations from PND2-14) in SERT$^{+/-}$ animals. Using the heterozygous genotype rather than animals with complete SERT knockout (i.e., SERT$^{-/-}$) might prevent a floor effect for induced symptomology; also, the heterozygous animals do not exhibit the 5-HT receptor downregulation that could prevent psilocybin from exerting a therapeutic effect neurochemically (Homberg et al., 2007). Moreover, the SERT$^{+/-}$ rats have about a 50% reduction in SERT activity, which is analogous to humans with the short version of the allele of the serotonin-transporter-linked promotor region (5-HTTLPR) gene (Homberg et al., 2007). This short version is a well-established genetic risk factor for major depressive and anxiety disorders in the clinical literature (Pezawas et al., 2005).

In addition to alternative preclinical models of symptomology, future animal research involving psilocybin should include carefully selected behavioural tests. As mentioned previously, the benefit of the ADT is that it assesses both depressive- and anxiety-like symptomology within a single assay. Furthermore, it provides the opportunity to assess the effects of manipulations and treatments both acutely and chronically. However, while the ADT has received some initial validation, it has not been replicated nearly as much as older behavioural assays, such as the forced swim test and the open field test. The food deprivation

requirement is also cumbersome and a potential source of added variability if, as in the current study, testing for symptomology begins in adolescence or early adulthood. Finally, future research should ideally include neurobiological/neurochemical assays to corroborate and validate the observed effects of psilocybin on behaviour.

Although it would be more labour-intensive, a future study could involve behavioural testing with the ADT, as well as several other more validated behavioural assays for depressive- and anxiety-like symptomology to increase reliability. After behavioural testing is complete, fluorescence microscopy and electrophysiology could be used to assess changes in neuronal structure and neurotransmitter release in cortical regions implicated in the neuropathogenesis of affective disorders (i.e., the hippocampus and prefrontal cortex, among others), with a particular focus on 5-HT_{2A} signalling pathways (Ly et al., 2018). After sacrifice and brain extraction, a Western blot analysis could be conducted to determine how the drug impacts the expression of genes related to neuroplasticity, such as c-Fos, in the same cortical regions (Jefsen et al., 2020).

Future research should also systematically investigate full dose response curves for psilocybin, including low and intermediate doses. The original research proposal for the current study involved an additional 4mg/kg dosage group; however, it was determined that testing the additional number of animals required to include the lower dose was beyond the scope of the project. Given essentially unlimited time and resources, comparing behaviour between animals that are administered 0, 1, 2, 4, 8, and 16mg/kg would provide an extremely comprehensive picture of any differential effects of dosage. It would also be interesting to investigate the therapeutic utility of several intermittent administrations versus a single exposure in terms of symptom reduction, and whether dosing regimen impacts the longevity of therapeutic effects.

Investigating the potential for differential sex effects and extending the duration of testing are also important components for future researchers to consider. In addition to the 4mg/kg group, the original research protocol also called for female counterparts for all experimental conditions, since previous research has suggested that some acute behavioural effects of the active compound (i.e., psilocin) differ by sex (Tylš et al., 2016). Although undoubtedly important, this variable was similarly jettisoned in the interest of ensuring that the scope of the project was practical given the timeframe. Future researchers should also carefully consider the duration of testing for chronic effects, especially if the ADT is used as

the primary behavioural assay. Clinical research with psychiatric populations has found that symptom reductions are sustained for at least 6 months post-treatment, and potentially longer (Griffiths et al., 2016). Although testing animals every day for 6 months is practically beyond the scope of any project, extending the period of chronic testing beyond the 6-day period employed in the current study would shed light on the stability of behavioural effects observed directly after treatment.

In addition to psilocybin, researchers should also consider investigating the therapeutic efficacy of other serotonergic hallucinogens in preclinical models, such as LSD. In clinical research, LSD in the context of psychotherapy has been found to successfully reduce anxiety associated with a life-threatening illness (Gasser et al., 2014). In rodents, one study found that repeated administration of LSD rescued surgically induced impaired learning behaviour and increased hippocampal 5-HT_{2A} signalling (Buchborn et al., 2014). Another preclinical study found that a single administration of LSD led to sustained reductions in depressive- and anxiety-like behaviour, although the effect was stronger in rats treated with psilocybin (Hibicke et al., 2020). Both hallucinogens share an initial neurochemical mechanism of action via 5-HT_{2A} receptor stimulation, but behavioural pharmacology studies suggest that LSD exerts subjective effects during a secondary temporal phase as well. This second phase of effects appears to be mediated by the dopamine D2 receptor (Marona-Lewicka et al., 2005). Since the dopaminergic system is heavily implicated in motivation and reward seeking, future preclinical studies that investigate LSD in the context of depressive- and anxiety-like symptomology could assess whether LSD exerts a therapeutic effect by increasing the motivational incentive of reward-related stimuli in addition to investigating the impact of the initial serotonergic effect (Wise, 2004).

Finally, it would be interesting to attempt to replicate findings from clinical studies that highlight the salience of set and setting during the acute hallucinogenic experience in a preclinical model. As mentioned previously, set refers to the internal psychological state of the individual, while setting refers to elements of the physical environment (Hartogsohn, 2016). Clinical studies consistently report that carefully controlling these extra-pharmacological components is critically important for optimizing the therapeutic impact of treatment modalities that involve a hallucinogenic component (Carhart-Harris, 2018). In rodents, elements of set could be manipulated by exposing rats to positively or negatively valanced stimuli (i.e., a sucrose reward or the opportunity to socialize versus a mild shock or exposure to distress USVs) directly before the hallucinogenic substance is administered.

Setting could be manipulated by administering the hallucinogen to animals in a familiar, enriched housing condition versus an unfamiliar, standard housing condition. As discussed previously, several clinical studies have suggested that the therapeutic benefit of psilocybin requires the acute neurobiological effects (i.e., increased neural plasticity) to occur in the context of a subjectively positive environment (Carhartt-Harris et al., 2018; Gukasyan & Nayak, 2021). Determining whether this combination is critical to modulate symptomatic behavioural tendencies in preclinical models would add another explanatory layer to understanding precisely how hallucinogenic substances might reduce symptomology, both in humans and in animals.

Concluding Remarks

In conclusion, the current study investigated whether psilocybin reduces depressive- and anxiety-like symptomology in an animal model. The results were inconclusive, potentially due to the fact that the environmental paradigm used did not produce a robust phenotype. Given the number of relatively or wholly novel methodological components involved, this research should be regarded as a large pilot study. This specific combination of environmental manipulation, behavioural assay, and pharmacological intervention has never been tested until now. Even though significant effects were not found, the sheer number of potential directions for future research suggested by the study render it an important contribution to the community of psychological science.

The present study also helps to address the decided lack of preclinical research investigating the utility of psilocybin in the context of depression and anxiety specifically. The exponential growth of clinical studies that have found positive therapeutic effects of psilocybin in humans for a variety of psychiatric disorders could be a harbinger of an eventual push for FDA approval as a psychiatric medication. Thus, it is incumbent upon the preclinical research community to thoroughly evaluate and replicate the behavioural and neurological effects of psilocybin in animal models concurrently with clinical research, as is standard practice for any compound with the potential for therapeutic applications.

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Appendix A

Means and Standard Deviations for Total Rears

Condition	Treatment		D-2	D-1	D0	D1	D2	D3	D4	D5*	D6	D7	D8	D9	D10	D11	D12	D13	D14
Control		*Mean*	77.53	45.60	46.13	40.17	34.83	38.07	36.87	40.57									
		SD	18.21	22.97	21.50	11.73	12.07	12.03	12.01	9.87									
EMS		*Mean*	74.13	39.03	39.70	34.63	33.23	33.80	35.83	39.30									
		SD	19.54	24.38	19.03	14.75	13.96	16.35	13.18	14.36									
Control	Saline	*Mean*								38.50	44.80	44.30	45.80	52.60	50.50	48.00	47.80	53.40	48.00
		SD								9.03	11.61	12.59	15.98	8.36	12.08	7.72	11.89	10.48	12.04
	8mg/kg	*Mean*								38.50	39.80	38.20	37.30	42.90	42.60	45.40	44.10	44.30	43.60
		SD								12.65	6.14	7.24	10.12	13.27	9.09	9.66	7.77	8.92	9.01
	16mg/kg	*Mean*								44.70	43.60	48.00	49.90	54.30	51.90	50.40	52.80	50.50	50.80
		SD								6.62	13.12	10.15	8.82	8.21	8.45	14.39	10.09	12.01	8.48
EMS	Saline	*Mean*								42.20	45.10	46.50	43.80	50.40	50.80	54.60	54.90	55.10	53.50
		SD								15.65	16.97	14.26	20.25	13.35	16.02	15.40	16.29	16.57	15.12
	8mg/kg	*Mean*								42.40	45.00	41.10	44.60	48.90	48.20	50.90	53.20	57.50	53.70
		SD								16.17	7.94	13.97	17.96	13.26	11.03	11.83	13.72	6.95	14.90
	16mg/kg	*Mean*								33.30	38.20	38.40	39.50	49.10	37.40	44.80	45.10	43.70	42.30
		SD								9.91	7.96	9.07	8.06	4.86	15.76	10.24	15.33	11.32	10.27

Note. *Animals were treated between days 5 and 6, but groups are split by dosage starting on day 5 to show differences pre- and post-treatment.

Means and Standard Deviations for SAT Score

Condition	Treatment		D1	D2	D3	D4	D5*	D6	D7	D8	D9	D10	D11	D12	D13	D14
Control		*Mean*	2.63	3.47	4.17	4.37	4.50									
		SD	2.01	2.13	1.29	1.30	1.38									
EMS		*Mean*	2.93	4.10	3.73	4.43	4.17									
		SD	2.39	1.88	1.87	1.14	1.37									
Control	Saline	*Mean*					4.50	4.50	4.60	4.50	4.80	4.90	4.70	5.00	5.10	5.30
		SD					0.85	1.08	1.26	0.97	1.62	1.60	1.57	1.63	1.52	1.95
	8mg/kg	*Mean*					4.30	4.10	4.10	4.00	4.00	4.20	4.20	4.10	4.20	4.30
		SD					1.95	1.29	1.91	1.94	1.89	1.69	1.93	1.66	1.93	1.95
	16mg/kg	*Mean*					4.70	5.00	4.50	4.70	5.10	4.90	4.80	4.50	4.80	4.90
		SD					1.25	1.41	0.85	1.25	1.60	1.91	1.62	0.71	1.32	1.60
EMS	Saline	*Mean*					4.30	4.20	4.30	4.20	4.10	4.50	4.50	4.70	4.80	5.00
		SD					0.95	0.63	0.82	0.63	0.74	0.97	0.85	0.95	1.23	1.41
	8mg/kg	*Mean*					4.20	4.30	4.30	4.60	4.40	4.40	4.70	4.30	4.60	4.40
		SD					1.48	0.67	0.48	0.97	0.52	0.52	0.67	0.82	0.70	0.52
	16mg/kg	*Mean*					4.00	4.40	4.50	4.50	4.60	4.50	4.70	4.50	4.60	4.50
		SD					1.70	1.35	1.27	1.27	1.58	1.27	1.57	1.27	1.58	1.27

Note. *Animals were treated between days 5 and 6, but groups are split by dosage starting on day 5 to show differences pre- and post-treatment.

Means and Standard Deviations for Latency to Consume 4th Froot Loop (sec)

Condition	Treatment		D1	D2	D3	D4	D5*	D6	D7	D8	D9	D10	D11	D12	D13	D14
Control		*Mean*	286.87	247.17	214.03	151.93	127.20									
		SD	32.42	64.79	80.52	75.99	65.20									
EMS		*Mean*	288.97	249.90	207.00	145.80	135.83									
		SD	22.58	67.28	76.09	65.77	81.64									
Control	Saline	*Mean*					97.50	89.90	79.20	78.40	72.40	77.80	78.90	76.40	70.40	83.20
		SD					22.64	20.04	13.69	15.00	16.83	13.79	13.92	17.56	11.59	38.07
	8mg/kg	*Mean*					151.20	142.20	110.00	130.80	108.00	110.20	100.80	120.50	92.50	94.20
		SD					80.78	85.46	68.01	89.62	68.94	69.35	71.12	94.77	73.36	72.97
	16mg/kg	*Mean*					132.90	114.10	83.70	95.50	77.60	115.60	75.10	72.90	76.40	83.60
		SD					70.72	42.15	17.04	31.58	15.78	71.14	18.37	21.00	22.27	20.71
EMS	Saline	*Mean*					153.50	136.30	125.70	91.50	96.50	102.10	100.20	80.50	92.30	78.50
		SD					90.49	89.39	86.15	26.80	73.40	73.60	72.76	22.02	74.12	17.33
	8mg/kg	*Mean*					136.40	107.30	95.90	95.20	76.60	78.70	77.60	91.30	73.50	73.70
		SD					86.92	33.60	35.57	73.28	18.00	14.47	25.01	74.91	16.27	23.22
	16mg/kg	*Mean*					117.60	106.90	88.60	90.80	85.10	89.20	78.30	78.40	70.90	79.20
		SD					70.82	69.87	20.23	33.10	23.93	28.07	19.80	21.09	19.94	16.63

Note. *Animals were treated between days 5 and 6, but groups are split by dosage starting on day 5 to show differences pre- and post-treatment.

Means and Standard Deviations for Total Distance Moved in Anticipatory Box (cm)

Condition	Treatment		D-2	D-1	D0	D1	D2	D3	D4	D5*	D6	D7	D8	D9	D10	D11	D12	D13	D14
Control		*Mean*	2407.88	1561.63	1515.68	1220.59	1068.82	1143.36	1059.05	1194.85									
		SD	421.16	578.43	602.89	292.89	285.91	266.61	262.80	270.21									
EMS		*Mean*	2355.85	1347.97	1354.15	1006.35	976.38	985.02	1040.01	1158.62									
		SD	474.90	657.44	546.96	374.98	352.72	392.71	345.78	429.76									
Control	Saline	*Mean*								1232.37	1392.51	1410.62	1326.97	1427.58	1371.81	1352.17	1341.52	1486.73	1364.51
		SD								285.63	316.92	332.30	394.44	266.93	376.38	323.18	334.01	311.39	284.09
	8mg/kg	*Mean*								1143.70	1174.18	1076.43	1088.28	1134.14	1204.05	1218.14	1277.81	1256.54	1288.09
		SD								358.02	159.63	207.83	242.78	271.26	197.29	182.90	315.88	222.37	197.61
	16mg/kg	*Mean*								1208.47	1155.56	1223.77	1332.80	1331.44	1380.36	1344.25	1392.29	1330.03	1329.95
		SD								144.32	239.68	264.38	258.35	243.75	328.93	340.21	287.96	404.75	149.11
EMS	Saline	*Mean*								1147.16	1171.34	1218.91	1140.89	1378.42	1369.41	1419.41	1477.27	1469.71	1487.71
		SD								346.10	379.32	300.34	397.28	369.95	339.44	315.06	288.08	347.97	356.44
	8mg/kg	*Mean*								1255.74	1180.85	973.74	1076.17	1273.59	1280.73	1287.83	1380.22	1459.12	1391.52
		SD								584.69	204.00	353.52	447.02	263.57	301.28	260.22	309.67	207.77	338.73
	16mg/kg	*Mean*								1072.94	1129.63	1088.70	1170.17	1542.15	1367.83	1483.20	1341.11	1249.79	1450.55
		SD								338.67	309.05	226.47	333.88	443.75	711.25	671.94	523.68	333.27	656.70

Note. *Animals were treated between days 5 and 6, but groups are split by dosage starting on day 5 to show differences pre- and post-treatment.

Means and Standard Deviations for Average Velocity in Anticipatory Box (cm/s)

Condition	Treatment		D-2	D-1	D0	D1	D2	D3	D4	D5*	D6	D7	D8	D9	D10	D11	D12	D13	D14
Control		*Mean*	4.27	2.77	2.71	4.23	3.66	3.91	3.66	4.13									
		SD	0.67	1.05	1.13	1.03	0.97	0.89	0.88	0.90									
EMS		*Mean*	4.11	2.32	2.34	3.47	3.33	3.36	3.55	3.95									
		SD	0.82	1.15	0.94	1.23	1.21	1.33	1.19	1.48									
Control	Saline	*Mean*								4.30	4.76	4.85	4.56	4.86	4.69	4.58	4.54	5.04	4.61
		SD								0.98	1.09	1.20	1.42	0.96	1.30	1.09	1.13	1.07	0.97
	8mg/kg	*Mean*								3.97	3.99	3.76	3.70	3.89	4.07	4.11	4.32	4.24	4.37
		SD								1.16	0.55	0.82	0.84	0.92	0.67	0.62	1.10	0.78	0.67
	16mg/kg	*Mean*								4.11	3.94	4.16	4.52	4.51	4.68	4.57	4.72	4.52	4.51
		SD								0.47	0.84	0.94	0.89	0.86	1.16	1.22	1.00	1.38	0.51
EMS	Saline	*Mean*								3.92	3.99	4.16	3.89	4.66	4.66	4.83	4.99	4.96	5.03
		SD								1.23	1.28	1.00	1.38	1.24	1.19	1.13	0.99	1.17	1.21
	8mg/kg	*Mean*								4.26	4.03	3.31	3.64	4.30	4.31	4.34	4.65	4.92	4.70
		SD								1.99	0.72	1.21	1.54	0.89	1.01	0.88	1.04	0.71	1.15
	16mg/kg	*Mean*								3.66	3.84	3.69	3.99	5.26	4.64	5.05	4.52	4.21	4.95
		SD								1.16	1.05	0.77	1.19	1.59	2.49	2.38	1.78	1.12	2.31

Note. *Animals were treated between days 5 and 6, but groups are split by dosage starting on day 5 to show differences pre- and post-treatment.

Means and Standard Deviations for Total Distance Moved in SAT

Condition	Treatment		D1	D2	D3	D4	D5*	D6	D7	D8	D9	D10	D11	D12	D13	D14
Control		*Mean*	1237.87	1205.98	1371.30	1489.33	1473.77									
		SD	336.20	374.91	421.40	455.24	348.35									
EMS		*Mean*	1121.68	1115.16	1234.25	1280.91	1356.09									
		SD	243.34	277.32	381.11	329.86	328.47									
Control	Saline	*Mean*					1538.78	1450.41	1871.21	1942.98	1923.34	1746.10	1738.00	1733.79	1722.69	1662.15
		SD					318.25	277.20	349.52	329.01	402.96	235.15	434.59	327.35	321.76	388.90
	8mg/kg	*Mean*					1419.74	1492.69	1592.13	1519.06	1783.19	1594.20	1597.42	1641.77	1718.68	1685.56
		SD					336.93	241.65	437.23	292.87	234.77	226.37	225.35	273.66	282.09	159.63
	16mg/kg	*Mean*					1462.80	1605.29	1811.74	1634.66	1984.72	1669.41	1890.72	1741.38	1829.86	1793.55
		SD					410.03	447.20	501.54	389.29	537.50	388.33	607.75	534.11	372.36	348.73
EMS	Saline	*Mean*					1371.92	1457.59	1576.88	1595.41	1717.49	1610.15	1584.97	1614.71	1624.87	1619.85
		SD					414.65	363.57	382.75	267.77	357.75	434.93	461.48	317.06	435.65	360.13
	8mg/kg	*Mean*					1301.60	1329.67	1463.60	1410.74	1713.86	1677.87	1761.65	1617.50	1666.09	1654.83
		SD					332.83	369.40	377.79	341.22	339.64	452.68	414.47	365.40	410.59	367.64
	16mg/kg	*Mean*					1394.76	1482.54	1563.81	1443.41	1599.89	1480.90	1546.93	1507.48	1576.15	1568.74
		SD					244.33	194.60	196.38	226.79	242.26	246.77	195.85	296.50	255.74	196.25

Note. *Animals were treated between days 5 and 6, but groups are split by dosage starting on day 5 to show differences pre- and post-treatment.

Means and Standard Deviations for Average Velocity in SAT

Condition	Treatment		D1	D2	D3	D4	D5*	D6	D7	D8	D9	D10	D11	D12	D13	D14
Control		*Mean*	4.16	4.06	4.61	5.02	4.97									
		SD	1.13	1.28	1.42	1.51	1.15									
EMS		*Mean*	3.78	3.76	4.17	4.33	4.57									
		SD	0.82	0.93	1.27	1.10	1.08									
Control	Saline	*Mean*					5.20	4.89	6.26	6.51	6.47	5.85	5.84	5.84	5.80	5.61
		SD					1.03	0.91	1.17	1.10	1.38	0.79	1.47	1.14	1.10	1.33
	8mg/kg	*Mean*					4.78	5.00	5.33	5.13	6.01	5.39	5.38	5.56	5.83	5.69
		SD					1.10	0.81	1.46	1.01	0.79	0.76	0.75	0.98	1.01	0.57
	16mg/kg	*Mean*					4.93	5.39	6.10	5.50	6.66	5.64	6.37	5.85	6.17	6.03
		SD					1.37	1.51	1.75	1.36	1.81	1.38	2.12	1.83	1.30	1.19
EMS	Saline	*Mean*					4.63	4.91	5.30	5.35	5.78	5.43	5.34	5.45	5.49	5.48
		SD					1.36	1.21	1.28	0.89	1.19	1.46	1.56	1.07	1.48	1.22
	8mg/kg	*Mean*					4.40	4.46	4.93	4.74	5.78	5.62	5.92	5.44	5.60	5.59
		SD					1.08	1.24	1.24	1.14	1.15	1.51	1.39	1.23	1.38	1.26
	16mg/kg	*Mean*					4.69	4.98	5.23	4.84	5.37	4.99	5.19	5.06	5.30	5.29
		SD					0.81	0.66	0.66	0.76	0.80	0.82	0.65	0.99	0.86	0.66

Note. *Animals were treated between days 5 and 6, but groups are split by dosage starting on day 5 to show differences pre- and post-treatment.

Means and Standard Deviations for Frequency of Entries into Alley 3

Condition	Treatment		D1	D2	D3	D4	D5*	D6	D7	D8	D9	D10	D11	D12	D13	D14
Control		*Mean*	4.73	5.47	6.47	8.43	8.67									
		SD	3.17	3.14	3.28	3.50	2.72									
EMS		*Mean*	4.40	5.80	6.83	6.87	8.20									
		SD	3.04	3.14	3.45	3.15	3.75									
Control	Saline	*Mean*					9.40	9.00	11.90	12.10	12.10	10.70	11.90	11.10	11.60	10.90
		SD					1.58	1.94	3.45	3.51	5.51	2.63	6.15	4.28	3.47	4.33
	8mg/kg	*Mean*					8.30	8.50	9.20	8.90	11.30	8.80	9.90	10.00	10.20	11.20
		SD					2.98	2.22	2.86	4.23	3.71	3.55	1.85	2.16	5.47	2.35
	16mg/kg	*Mean*					8.30	9.20	10.20	9.20	12.30	9.70	11.10	10.90	9.60	10.90
		SD					3.40	3.01	3.39	2.49	4.81	4.03	4.07	3.90	1.90	3.28
EMS	Saline	*Mean*					7.90	10.00	9.20	8.70	11.20	11.00	9.70	11.80	11.40	10.50
		SD					5.57	5.08	2.94	2.75	4.69	4.59	4.40	4.57	4.09	4.06
	8mg/kg	*Mean*					9.30	7.00	9.90	9.70	12.90	11.20	11.70	10.90	11.10	11.80
		SD					2.75	3.30	3.31	3.92	7.69	3.77	5.60	4.65	3.96	4.71
	16mg/kg	*Mean*					7.40	9.20	8.90	8.50	10.20	9.40	9.50	9.70	10.80	11.40
		SD					2.12	3.39	3.14	1.96	3.79	3.63	2.37	3.50	2.94	4.22

Note. *Animals were treated between days 5 and 6, but groups are split by dosage starting on day 5 to show differences pre- and post-treatment.

Means and Standard Deviations for Cumulative Duration Spent in Alley 3

Condition	Treatment		D1	D2	D3	D4	D5*	D6	D7	D8	D9	D10	D11	D12	D13	D14
Control		*Mean*	34.36	37.36	39.56	40.72	44.78									
		SD	19.90	17.42	19.61	15.20	21.70									
EMS		*Mean*	33.99	40.28	33.56	36.59	41.72									
		SD	18.69	22.46	15.49	21.92	20.63									
Control	Saline	*Mean*					47.33	45.20	56.30	56.11	56.03	44.29	59.76	51.37	55.92	67.80
		SD					17.90	8.66	15.09	26.42	26.89	10.14	28.20	19.87	23.10	32.96
	8mg/kg	*Mean*					37.88	50.29	44.37	56.68	61.59	51.87	61.66	47.93	46.31	54.23
		SD					15.42	22.14	16.59	21.81	10.70	28.52	15.27	17.04	13.55	16.03
	16mg/kg	*Mean*					49.13	34.28	41.88	48.14	42.18	51.52	47.30	47.82	54.05	46.87
		SD					29.64	6.67	18.76	26.03	17.00	20.78	17.42	27.17	27.41	20.43
EMS	Saline	*Mean*					31.13	51.01	51.38	47.40	58.01	56.09	52.61	57.38	49.01	45.56
		SD					23.76	17.12	13.75	17.17	28.92	23.51	25.56	21.23	27.49	15.86
	8mg/kg	*Mean*					53.49	42.47	54.81	55.54	54.38	56.62	43.58	54.23	59.03	55.38
		SD					18.24	22.18	18.14	27.99	15.65	15.73	18.37	14.46	21.16	20.25
	16mg/kg	*Mean*					40.54	45.89	56.07	53.64	43.66	56.19	55.36	52.81	49.71	44.85
		SD					13.93	17.32	18.51	29.48	23.41	22.73	23.43	28.46	18.03	16.97

Note. *Animals were treated between days 5 and 6, but groups are split by dosage starting on day 5 to show differences pre- and post-treatment.

Means and Standard Deviations for Latency to First Entry into Alley 3

Condition	Treatment		D1	D2	D3	D4	D5*	D6	D7	D8	D9	D10	D11	D12	D13	D14
Control		*Mean*	81.37	95.40	65.03	67.96	56.23									
		SD	67.93	80.47	55.52	36.53	43.80									
EMS		*Mean*	103.36	103.91	86.97	76.47	61.15									
		SD	66.48	60.71	70.23	71.93	47.15									
Control	Saline	*Mean*					53.38	44.29	38.65	40.14	36.33	42.83	42.00	44.12	42.66	34.71
		SD					23.95	17.47	18.49	18.02	14.77	9.65	13.86	8.27	8.24	15.25
	8mg/kg	*Mean*					63.71	61.02	61.48	42.95	49.58	51.93	45.05	37.66	42.45	38.50
		SD					70.49	53.48	36.05	22.91	8.59	9.95	7.20	10.10	10.91	11.43
	16mg/kg	*Mean*					51.59	45.23	48.89	48.64	42.07	37.49	35.04	34.68	40.14	37.45
		SD					23.35	31.95	19.64	12.95	14.90	22.45	20.13	16.18	18.76	18.55
EMS	Saline	*Mean*					81.15	53.63	48.71	50.67	51.21	34.79	48.08	45.85	44.22	43.25
		SD					72.55	46.13	27.91	15.62	35.92	23.47	19.66	12.99	19.33	9.89
	8mg/kg	*Mean*					49.27	57.50	52.15	52.45	43.12	47.52	41.93	39.22	39.65	40.46
		SD					20.99	14.38	8.46	33.15	10.44	9.98	11.67	16.87	15.28	13.93
	16mg/kg	*Mean*					53.02	43.10	45.45	47.78	53.52	47.74	45.85	42.36	41.67	43.78
		SD					27.99	22.26	19.90	25.61	16.69	23.69	17.14	11.36	18.16	9.78

Note. *Animals were treated between days 5 and 6, but groups are split by dosage starting on day 5 to show differences pre- and post-treatment.

Means and Standard Deviations for Frequency of Entries into Alley 4

Condition	Treatment		D1	D2	D3	D4	D5*	D6	D7	D8	D9	D10	D11	D12	D13	D14
Control		*Mean*	1.00	2.10	2.37	3.33	3.27									
		SD	1.23	1.90	1.75	1.84	1.64									
EMS		*Mean*	0.90	1.87	2.47	2.63	3.13									
		SD	1.30	1.85	1.96	1.87	1.96									
Control	Saline	*Mean*					3.90	3.00	5.20	5.20	5.00	4.70	5.20	4.20	4.60	4.10
		SD					0.88	0.47	2.04	1.32	2.75	1.89	3.46	1.40	1.90	1.91
	8mg/kg	*Mean*					2.70	3.40	3.20	3.50	3.70	3.60	4.20	3.80	4.90	4.70
		SD					1.57	1.58	1.62	2.07	1.06	1.35	1.32	1.23	2.56	1.34
	16mg/kg	*Mean*					3.20	3.70	4.70	3.80	4.90	4.40	4.40	4.40	4.30	4.70
		SD					2.15	0.95	1.89	1.55	2.33	3.03	1.58	1.65	0.95	1.89
EMS	Saline	*Mean*					3.40	3.80	3.50	3.60	4.70	3.60	3.60	4.60	4.40	4.60
		SD					2.95	1.81	1.72	1.51	2.26	2.32	2.17	2.01	2.01	2.37
	8mg/kg	*Mean*					2.90	2.90	4.50	3.80	4.70	4.40	5.20	4.60	4.20	4.90
		SD					1.20	1.45	2.01	1.32	2.31	1.71	3.46	2.22	1.40	2.56
	16mg/kg	*Mean*					3.10	3.60	3.70	3.30	4.00	3.90	4.10	3.90	3.60	4.20
		SD					1.45	1.78	0.67	1.16	1.56	1.66	1.60	1.79	1.26	1.69

Note. *Animals were treated between days 5 and 6, but groups are split by dosage starting on day 5 to show differences pre- and post-treatment.

Means and Standard Deviations for Cumulative Duration Spent in Alley 4

Condition	Treatment		D1	D2	D3	D4	D5*	D6	D7	D8	D9	D10	D11	D12	D13	D14
Control		*Mean*	8.93	16.80	20.68	44.92	46.97									
		SD	14.35	20.70	20.57	25.45	30.36									
EMS		*Mean*	5.44	14.91	27.89	35.10	47.64									
		SD	9.11	17.03	27.06	31.42	27.95									
Control	Saline	*Mean*					62.98	49.31	56.11	64.26	49.13	52.33	53.15	43.85	46.78	43.83
		SD					22.09	33.08	25.32	9.48	24.17	27.57	26.18	21.20	23.85	20.87
	8mg/kg	*Mean*					44.25	47.51	49.29	51.37	44.73	42.88	52.56	51.05	47.21	62.57
		SD					35.37	21.16	23.31	35.26	19.12	16.01	25.96	23.84	25.52	21.84
	16mg/kg	*Mean*					33.68	57.18	66.63	54.66	62.54	46.14	56.25	53.36	55.06	53.51
		SD					27.24	28.64	32.75	23.57	27.13	17.51	21.69	22.97	26.72	26.78
EMS	Saline	*Mean*					32.57	52.62	43.40	66.05	45.24	36.54	58.16	51.05	55.76	55.65
		SD					25.16	34.18	31.51	43.47	24.06	28.13	36.79	22.24	31.67	27.61
	8mg/kg	*Mean*					48.14	43.20	41.04	62.25	51.98	56.31	59.90	57.51	45.47	53.29
		SD					24.82	27.68	24.33	31.30	30.76	27.77	27.13	31.92	22.63	27.29
	16mg/kg	*Mean*					62.21	41.14	61.63	49.12	51.50	42.75	53.30	48.14	66.57	68.65
		SD					27.93	22.99	23.06	25.09	17.11	21.99	19.60	26.02	26.49	33.21

Note. *Animals were treated between days 5 and 6, but groups are split by dosage starting on day 5 to show differences pre- and post-treatment.

Means and Standard Deviations for Latency to First Entry into Alley 4

Condition	Treatment		D1	D2	D3	D4	D5*	D6	D7	D8	D9	D10	D11	D12	D13	D14
Control		*Mean*	205.75	199.00	167.76	123.30	112.46									
		SD	111.90	80.65	95.40	59.96	70.30									
EMS		*Mean*	256.76	202.58	152.18	128.88	105.51									
		SD	70.06	88.25	82.82	65.37	51.92									
Control	Saline	*Mean*					87.64	80.50	66.33	65.79	66.45	72.26	65.83	70.13	64.59	66.71
		SD					23.35	14.84	23.07	25.54	16.45	13.11	21.68	14.59	11.21	21.53
	8mg/kg	*Mean*					133.04	112.87	102.77	104.50	76.34	77.83	73.70	60.54	64.93	64.54
		SD					90.46	78.46	70.36	69.09	17.32	12.09	13.52	20.18	8.46	15.35
	16mg/kg	*Mean*					116.70	104.89	81.60	84.35	71.75	86.99	70.74	67.28	69.04	78.40
		SD					77.61	37.06	13.89	25.31	15.73	26.63	15.45	18.55	22.70	19.56
EMS	Saline	*Mean*					127.48	110.25	102.98	85.64	92.82	88.35	95.05	73.76	88.54	73.11
		SD					66.44	70.99	76.79	25.83	74.38	79.84	74.15	21.34	75.30	16.07
	8mg/kg	*Mean*					84.09	99.41	90.04	81.56	70.65	73.06	68.13	71.13	68.00	68.16
		SD					19.68	34.75	32.10	45.50	17.20	12.61	19.76	31.17	15.87	22.42
	16mg/kg	*Mean*					104.95	86.45	82.63	76.56	79.92	82.36	72.43	70.85	64.43	71.95
		SD					53.27	81.97	19.71	40.85	23.40	26.57	19.07	15.94	20.26	14.71

Note. *Animals were treated between days 5 and 6, but groups are split by dosage starting on day 5 to show differences pre- and post-treatment.

Means and Standard Deviations for Total USVs

Condition	Treatment		D1*	D5	D6	D9
Control		*Mean*	53.60	60.23		
		SD	59.45	90.82		
EMS		*Mean*	2.50	36.10		
		SD	1.14	68.18		
Control	Saline	*Mean*		66.90	77.10	140.30
		SD		90.84	78.89	124.22
	8mg/kg	*Mean*		65.20	55.40	58.20
		SD		93.91	76.59	80.42
	16mg/kg	*Mean*		48.60	59.60	97.80
		SD		96.34	80.62	125.64
EMS	Saline	*Mean*		30.30	49.20	68.20
		SD		36.61	55.92	73.30
	8mg/kg	*Mean*		24.40	34.80	50.20
		SD		35.23	52.27	64.99
	16mg/kg	*Mean*		66.50	93.30	133.10
		SD		122.73	135.28	138.54

Note. *Animals were treated between days 5 and 6, but groups are split by dosage starting on day 5 to show differences pre- and post-treatment.

Appendix B

Summary of Analyses for Cohort Effects for Latency to Consume 4th Froot Loop, Frequency of Entries into Alley 3, Cumulative Duration in Alley 3, Latency of First Entry into Alley 3, Frequency of Entries into Alley 4, Cumulative Duration in Alley 4, Latency to First Enter Alley 4, and Total USVs

Measure	Time Period	F Statistic	p-value
Total Rears	Habituation	F (3, 56) = 0.83	.486
	Baseline	F (3, 56) = 1.14	.342
	Acute	F (3, 56) = 2.16	.103
	Chronic	F (3, 56) = 1.36	.265
	All Testing Days	F (3, 56) = 1.66	.186
Latency to Consume 4th	Baseline	F (3, 56) = 1.95	.133
	Acute	F (3, 56) = 0.71	.551
	Chronic	F (3, 56) = 2.10	.111
	All Testing Days	F (3, 56) = 0.59	.621
Distance Moved in AB	Habituation	F (3, 56) = 0.41	.745
	Baseline	F (3, 56) = 0.48	.699
	Acute	F (3, 56) = 1.68	.181
	Chronic	F (3, 56) = 0.18	.912
	All Testing Days	F (3, 56) = 0.59	.622
Average Velocity in AB	Habituation	F (3, 56) = 0.53	.662
	Baseline	F (3, 56) = 0.32	.812
	Acute	F (3, 56) = 1.46	.236
	Chronic	F (3, 56) = 0.17	.918
	All Testing Days	F (3, 56) = 0.46	.710
Alley 3 Frequency	Baseline	F (3, 56) = 0.44	.723
	Acute	F (3, 56) = 0.71	.552
	Chronic	F (3, 56) = 3.72	.016*
	All Testing Days	F (3, 56) = 2.10	.110
Alley 3 Cumulative Duration	Baseline	F (3, 56) = 4.20	.009**
	Acute	F (3, 56) = 2.07	.114
	Chronic	F (3, 56) = 2.16	.103
	All Testing Days	F (3, 56) = 3.32	.026*

Alley 3 Latency to First Entry	Baseline	F (3, 56) = 0.85	.475
	Acute	F (3, 56) = 2.05	.117
	Chronic	F (3, 56) = 0.46	.708
	All Testing Days	F (3, 56) = 0.89	.454
Alley 4 Frequency	Baseline	F (3, 56) = 0.27	.847
	Acute	F (3, 56) = 0.35	.791
	Chronic	F (3, 56) = 2.65	.058
	All Testing Days	F (3, 56) = 1.07	.369
Alley 4 Cumulative Duration	Baseline	F (3, 56) = 0.61	.611
	Acute	F (3, 56) = 0.69	.561
	Chronic	F (3, 56) = 0.83	.483
	All Testing Days	F (3, 56) = 0.24	.869
Alley 4 Latency to First Entry	Baseline	F (3, 56) = 0.24	.866
	Acute	F (3, 56) = 0.75	.528
	Chronic	F (3, 56) = 0.43	.731
	All Testing Days	F (3, 56) = 0.08	.969
Total USVs	Baseline	F (3, 56) = 1.71	.175
	Acute	F (3, 56) = 1.29	.287
	Chronic	F (3, 56) = 1.39	.256
	All Testing Days	F (3, 56) = 1.29	.286

Note. *p < .05, **p < .01, ***p < .001

Summary of Analyses for Saline Dosage Effects at Acute Testing for All Measures Collected

Measure	F Statistic	p-value
Total Rears	F (1, 18) = 0.26	.619
SAT Score	F (1, 18) = 0.11	.741
Latency to Consume 4th	F (1, 18) = 2.08	.166
Distance Moved in AB	F (1, 18) = 0.44	.517
Average Velocity in AB	F (1, 18) = 0.43	.518
Distance Moved in SAT	F (1, 18) = 2.06	.168
Average Velocity in SAT	F (1, 18) = 2.13	.161
Alley 3 Frequency	F (1, 18) = 0.56	.465
Alley 3 Cumulative Duration	F (1, 18) = 0.00	.996

Alley 3 Latency to First Entry	F (1, 18) = 1.84	.192
Alley 4 Frequency	F (1, 18) = 0.39	.541
Alley 4 Cumulative Duration	F (1, 18) = 0.67	.423
Alley 4 Latency to First Entry	F (1, 18) = 1.25	.278
Total USVs	F (1, 18) = 1.02	.325

Note. *$p < .05$, **$p < .01$, ***$p < .001$

Appendix C

Summary of Analyses for Latency to Consume 4th Froot Loop

Time Period	Model Predictor	F Statistic	p-value
Baseline	Day	F (4, 232) = 115.19	< .001***
	Condition	F (1, 58) = 0.00	.996
	Day*Condition	F (4, 232) = 0.29	.886
Acute	Day	F (3, 162) = 12.31	< .001***
	Day*Condition	F (3, 162) = 0.99	.398
	Day*Dosage	F (6, 162) = 0.60	.730
	Day*Condition*Dosage	F (6, 162) = 0.55	.767
	Condition	F (1, 54) = 0.06	.800
	Dosage	F (2, 54) = 0.67	.517
	Condition*Dosage	F (2, 54) = 2.16	.125
Chronic	Day	F (5, 270) = 2.83	0.016*
	Day*Condition	F (5, 270) = 0.63	.678
	Day*Dosage	F (10, 270) = 1.73	.074
	Day*Condition*Dosage	F (10, 270) = 0.99	.450
	Condition	F (1, 54) = 0.21	.653
	Dosage	F (2, 54) = 0.32	.729
	Condition*Dosage	F (2, 54) = 1.32	.275

Note. *p < .05, **p < .01, ***p < .001

Summary of Analyses for Frequency of Entries into Alley 3

Time Period	Model Predictor	F Statistic	p-value
Baseline	Day	F (4, 232) = 23.27	< .001***
	Condition	F (1, 58) = 0.30	.588
	Day*Condition	F (4, 232) = 1.51	.200
Acute	Day	F (3, 162) = 4.24	.007**
	Day*Condition	F (3, 162) = 0.54	.657
	Day*Dosage	F (6, 162) = 1.07	.384
	Day*Condition*Dosage	F (6, 162) = 1.94	.077
	Condition	F (1, 54) = 1.24	.271

	Dosage	F (2, 54) = 0.93	.403
	Condition*Dosage	F (2, 54) = 0.74	.481
Chronic	Day	F (5, 270) = 2.37	.040*
	Day*Condition	F (5, 270) = 0.77	.576
	Day*Dosage	F (10, 270) = 0.56	.848
	Day*Condition*Dosage	F (10, 270) = 0.79	.635
	Condition	F (1, 54) = 0.02	.902
	Dosage	F (2, 54) = 0.21	.811
	Condition*Dosage	F (2, 54) = 0.49	.613

Note. *p < .05, **p < .01, ***p < .001

Summary of Analyses for Cumulative Duration in Alley 3

Time Period	Model Predictor	F Statistic	p-value
Baseline	Day	F (4, 232) = 2.46	.046*
	Condition	F (1, 58) = 0.43	.516
	Day*Condition	F (4, 232) = 0.66	.619
Acute	Day	F (3, 162) = 4.09	.008**
	Day*Condition	F (3, 162) = 0.92	.433
	Day*Dosage	F (6, 162) = 0.72	.631
	Day*Condition*Dosage	F (6, 162) = 1.93	.078
	Condition	F (1, 54) = 0.15	.697
	Dosage	F (2, 54) = 0.32	.729
	Condition*Dosage	F (2, 54) = 1.20	.308
Chronic	Day	F (5, 270) = 0.04	.999
	Day*Condition	F (5, 270) = 1.57	.170
	Day*Dosage	F (10, 270) = 0.87	.565
	Day*Condition*Dosage	F (10, 270) = 1.34	.208
	Condition	F (1, 54) = 0.00	.949
	Dosage	F (2, 54) = 0.84	.436
	Condition*Dosage	F (2, 54) = 0.16	.852

Note. *p < .05, **p < .01, ***p < .001

Summary of Analyses for Latency to First Entry into Alley 3

Time Period	Model Predictor	F Statistic	p-value
Baseline	Day	F (4, 232) = 35.01	< .001***
	Condition	F (1, 58) = 1.63	.206
	Day*Condition	F (4, 232) = 0.55	.698
Acute	Day	F (3, 162) = 2.18	.094
	Day*Condition	F (3, 162) = 0.24	.869
	Day*Dosage	F (6, 162) = 1.11	.357
	Day*Condition*Dosage	F (6, 162) = 0.56	.763
	Condition	F (1, 54) = 0.23	.633
	Dosage	F (2, 54) = 0.46	.635
	Condition*Dosage	F (2, 54) = 0.92	.403
Chronic	Day	F (5, 270) = 2.00	.079
	Day*Condition	F (5, 270) = 0.88	.498
	Day*Dosage	F (10, 270) = 1.43	.168
	Day*Condition*Dosage	F (10, 270) = 0.95	.490
	Condition	F (1, 54) = 1.30	.259
	Dosage	F (2, 54) = 0.06	.938
	Condition*Dosage	F (2, 54) = 1.05	.356

Note. *p < .05, **p < .01, ***p < .001