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Use of subanesthetic doses of ketamine on a chronic variable stress-induced depression model in female Sprague Dawley rats

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Abstract

Major depressive disorder (MDD) affects approximately 20 million adults in the United States. The prevailing theory suggests that MDD results from imbalances in monoamines such as serotonin, norepinephrine, and dopamine; however, many MDD patients do not respond to drugs that target these systems, indicating that the neurochemical causes of MDD may be more diverse than originally suspected. Ketamine, an NMDA receptor antagonist with hallucinogenic and dissociative properties, has been suggested as a treatment for patients who do not respond to typical antidepressants. As a method of inducing depression-like behavior in rodents, the chronic variable stress (CVS) protocol consists of exposing rodents to frequent, non-painful, unpredictable stressors over an extended period and has been shown to result in depression-like behavior in tests like the forced swim test (FST) that responds well to typical antidepressants. Although affective disorders are more common in females, this population is underrepresented in both human and animal trials, indicating a need for more studies using female subjects. This study investigates if CVS would increase immobility and decrease latency to immobility in the FST, and if ketamine would prevent these changes.

Female Sprague Dawley rats were exposed to CVS for three weeks to induce a model of depression, then depression-like behaviors were measured using the FST, anxiety-like behaviors were measured using the open field test (OFT) and elevated zero maze (EZM), and blood samples were collected to measure the stress hormone corticosterone. Ketamine (10 mg/kg) or saline was then administered via i.p. injection weekly for three weeks, and the FST was administered multiple times during the same period. After three weekly injections of ketamine, the OFT, EZM, and blood draws were repeated. Exposure to CVS had no effect on time spent

immobile in the FST at all time points, conversely to what was expected. There was also a significant main effect of ketamine, but not CVS, in reducing the latency to immobility in the FST. There was an effect of CVS in the OFT, but not the EZM; however, a comparison of behavior in both behavioral tests across the two sessions suggested an effect of novelty, with more exploration of open areas in the first session compared to the second. The results indicate that immobility in the FST may have served as a coping behavior in rats with a model of depression. Alternatively, the stressors involved in CVS may have failed to induce a model of depression. The results also reveal a need to create more reliable animal models of depression and measures of depression-like behavior.

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List of Abbreviations

ACTH: adrenocorticotropic hormone

ANOVA: analysis of variance

BDNF: brain-derived neurotrophic factor

CRH: corticotropin-releasing hormone

CVS: chronic variable stress

EPM: elevated plus maze

EZM: elevated zero maze

FST: forced swim test

GABA: gamma-aminobutyric acid

GR: glucocorticoid receptor

HPA: hypothalamic-pituitary-adrenal

MAO: monoamine oxidase

MAOI: monoamine oxidase inhibitor

MDD: major depressive disorder

MR: mineralocorticoid receptor

OFC: orbitofrontal cortex

OFT: open field test

PFC: prefrontal cortex

PVN: paraventricular nucleus

SNRI: serotonin-norepinephrine reuptake inhibitor

SPT: sucrose preference test

SSRI: selective serotonin reuptake inhibitor

TCA: tricyclic antidepressant

TRD: treatment-resistant depression

CHAPTER I: INTRODUCTION

Overview

With major depressive disorder (MDD) being one of the most common psychiatric disorders, it is vital to find treatments that work on all patients. The monoamine hypothesis of depression states that MDD is caused by low levels of monoamines like serotonin, dopamine, and norepinephrine; however, considering that people with treatment-resistant depression (TRD) do not respond to monoamine-targeting antidepressants, there must be more to the etiology of depression. Glutamate has also been theorized to play a role in depression, supported by the fact that many patients with TRD respond to ketamine: an NMDA receptor antagonist. Dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis' response to stress may also be a factor, as cortisol levels tend to be more variable in patients with MDD. This study aims to determine the efficacy of repeated small doses of ketamine in reversing the behaviors caused by a model of depression brought on by chronic variable stress (CVS).

Depression

Major depressive disorder (MDD) is characterized by symptoms such as depressed mood, deep sadness, feelings of worthlessness, hopelessness, and guilt, decreased interest in activities, sleep disturbances, and suicidal ideation (American Psychiatric Association 2013). Patients can experience difficulty functioning in their daily lives due to trouble concentrating, making decisions, and remembering. People with MDD may also feel a deep sense of emotional numbness or despair. There are physical symptoms as well, such as chronic fatigue, body aches, digestive problems, and an increased susceptibility to infections. MDD is more likely to develop

in those who have lived through traumatic, stressful experiences (National Institute of Mental Health 2023). In fact, 8.8% of adults and 19.5% of youth aged 12-17 suffer from MDD (Substance Abuse and Mental Health Services Administration 2022). The number of adults in the USA with MDD was estimated at nearly 20 million in 2019 (Greenberg et al. 2023). Depression can lead to decreased productivity, more days of work missed, and a loss of motivation to do work. The economic burden on society caused by MDD was estimated at $333.7 billion in 2023, or nearly $17,000 for every adult with MDD (Greenberg et al. 2023). This cost was created by factors like healthcare expenses, unemployment, absenteeism, mortality, and household expenses. MDD is most often treated using therapy, medication, and sometimes hospitalization, which all accrue healthcare expenses. Furthermore, the cognitive and emotional symptoms caused by depression can impact work quality and may even result in people with MDD losing their jobs.

In terms of relationships, people with MDD may be more likely to withdraw socially, lash out at friends and family, and stop engaging in activities they once enjoyed, leading to social isolation that exacerbates the depressive symptoms (Goodman et al. 2019). Problematic familial, friendly, and romantic relationships in early life and adolescence factor into the severity of MDD symptoms later on, and conversely, MDD symptoms in young adulthood predict antisocial behavior and relationship problems (Kupferberg and Hasler 2023). This results in a reciprocal risk between depression and social impairments. Depression is expected to rank as the leading cause of disability and poor health worldwide by 2030 (Guan et al. 2022). Furthermore, in 2021, suicide was the third leading cause of death in the United States in people aged 15-24, and the second leading cause of death in people aged 25-34 (Centers for Disease Control and Prevention,

2021). As such, it is important to understand the biological factors underlying the development of MDD so as to determine how to best alleviate it.

Neurological Basis of Depression

As a psychological disorder, depression arises from abnormal neuronal function and communication. Neurons are the cellular basis of the brain, consisting of three major components: dendrites, which receive signals; the cell body, which generates the signal; and axons, which transmit the signal to other neurons. A synapse, or gap, exists between the axon of the transmitting neuron and the dendrite of the receiving neuron. Certain compounds known as neurotransmitters are stored in vesicles within the axon terminals. When a signal reaches the end of an axon, the vesicles release their contents into the synapse. The neurotransmitters travel across the gap, where they bind to receptors on a dendrite of the next neuron. This allows the signal to get relayed across neurons.

After the neurotransmitters activate the next neuron in the signaling chain, they are removed from the synapse. This can occur in multiple ways. The first step in all of these methods is for the neurotransmitter to dissociate from the receptor it is bound to on the dendrite. The neurotransmitter could then diffuse into the surrounding extracellular space, or it could move back towards the axon terminal that released it. Through the reuptake process, the neurotransmitter reenters the cell. Alternatively, in enzymatic degradation, proteins that exist both in the synapse and in the presynaptic terminal can break neurotransmitters down into an inactive form. All of these processes yield the same final result: the termination of the signal in the postsynaptic neuron. An overview of signal termination is illustrated in Figure 1.

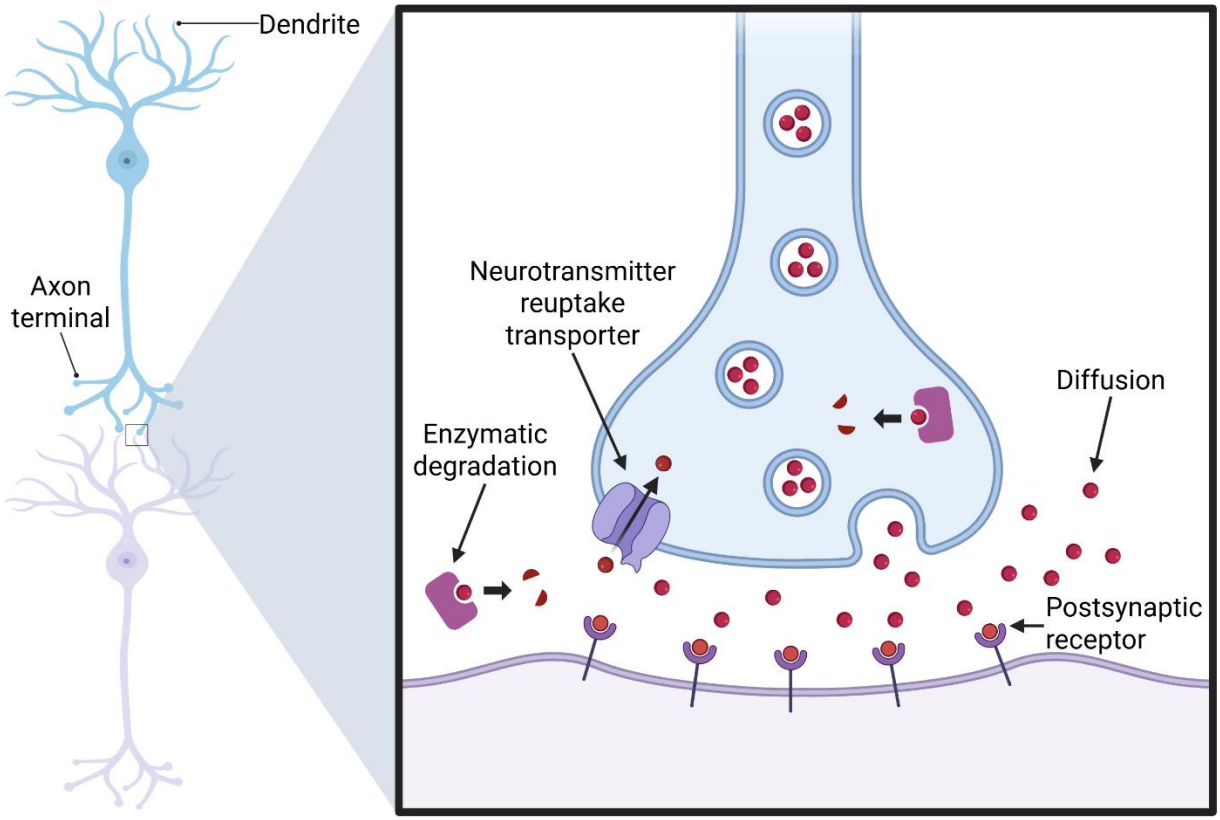

Figure 1. Synaptic transmission and signal termination. Created using BioRender.com.

Serotonin, dopamine, and norepinephrine are neurotransmitters that have been implicated in the development of depression. This concept, known as the monoamine hypothesis of depression, suggests that low levels of these neurotransmitters are the major factor in the development of MDD. Coppen (1967) first suggested that depression was caused by lower levels of monoamines. Since drugs that increase the activity of monoamines in the brain alleviate symptoms of depression, he reasoned that there must be a causal relationship between low monoamine levels and depression. The hypothesis is still widely believed today, as most antidepressants target serotonin levels, specifically the amount of serotonin that exists in the synapse for receptor binding (Albert et al. 2012).

In 1928, Mary Bernheim discovered monoamine oxidase (MAO), an enzyme that breaks down serotonin, dopamine, and norepinephrine (Green 2006). Monoamine oxidase inhibitors (MAOIs) block the function of MAO, leading to more neurotransmitter available within the axon for release into the synapse, and were some of the first antidepressants to be used in clinical settings in the 1950's (Hillhouse and Porter 2015). For example, iproniazid, an MAOI that was initially used to treat tuberculosis, was found to improve patients' mood (Albert et al. 2012). However, MAOIs also prevent the breakdown of tyramine, which is found in foods and medications, leading to elevations in blood pressure (Sub Laban and Saadabadi 2025). Some drugs had the opposite effect. Reserpine, an inhibitor of neurotransmitter packaging into vesicles, caused patients to develop depressive symptoms that were identical to patients with MDD.

Inhibition of MAO is not the only mechanism for antidepressants, however. Tricyclic antidepressants (TCAs), such as imipramine, also began to be used in the 1950's (Hillhouse and Porter 2015). Imipramine was similarly able to treat patients with depression due to its ability to block the reuptake of serotonin and norepinephrine, leaving more neurotransmitter in the synapse to interact with postsynaptic receptors. As reuptake inhibitors, once a neurotransmitter has been released into the synapse, TCAs can block the transporters in the axon terminal membrane that would normally allow the messengers to return into the cell. In the 1980's and 90's, selective serotonin reuptake inhibitors (SSRIs) and serotonin-norepinephrine reuptake inhibitors (SNRIs) were introduced, respectively. Similar to TCAs, these drugs increase the amount of serotonin – and norepinephrine in the case of SNRIs – in the synapse. Additionally, there are atypical antidepressants that do not fit into any of the other classes, and their effects are not well known.

Stress and Depression

Stressful life events have been strongly indicated as one of the causes of MDD (American Psychiatric Association 2013). A stressor is something that threatens an organism's homeostasis, whether internal or external (Tsigos et al. 2020). When a stressful situation is encountered, there are a series of responses that occur in the body across multiple systems. Almost immediately, there is a neuronal response. Once the hypothalamus – located in the brain – receives the signal that the organism is experiencing stress, it activates the sympathetic nervous system (in a response commonly referred to as "fight or flight") and triggers the release of epinephrine and norepinephrine from the sympathetic neurons, as well as from the adrenal medulla. Acting as hormones in the blood, these neurotransmitters cause physiological changes throughout the body, including increased heart rate, blood pressure, and respiratory rate, redirected blood flow from the digestive system to the muscles, and the release of stored glucose for extra energy.

At the same time, in the endocrine system, the hypothalamic-pituitary-adrenal (HPA) axis also responds to stress (Menke 2024). In response to a stressor, the paraventricular nucleus (PVN) of the hypothalamus releases corticotropin-releasing hormone (CRH). CRH then travels through the hypophyseal portal system to the pituitary gland to stimulate the anterior pituitary gland to release adrenocorticotropic hormone (ACTH). ACTH travels through the blood and stimulates the adrenal cortex to produce and release more cortisol. Activation of the HPA axis in response to acute or occasional stressors can be adaptive, as cortisol carries out several functions in the body. It increases blood glucose levels, which ensures that the muscles and brain have enough energy to escape from or deal with the stressor (Thau et al. 2025). Cortisol also attenuates inflammation and prevents excessive immune activation, preventing tissue damage

while allowing the body to focus on the stressor. It can enhance some cognitive and emotional processes in order to help an organism make quick decisions about the stressor, such as alertness, memory, and emotional processing (Stauble et al. 2013; Buchanan and Lovallo 2001). Cortisol levels are able to self-regulate using a negative feedback loop, where the hypothalamus and pituitary gland detect the increased cortisol levels in the body and stop their secretion of CRH and ACTH, respectively.

Cortisol can pass through the cell membrane and bind to intracellular receptors to cause changes in gene expression or cellular metabolism. There are two types of receptors that cortisol can bind to (Tsigos et al. 2020). The first type of receptors, type I glucocorticoid receptors (also known as mineralocorticoid receptors, or MRs), have a high affinity for cortisol, meaning these receptors are activated even at the low levels of cortisol that fluctuate throughout the day. MRs are mainly found in the hippocampus, and they regulate homeostasis, circadian rhythms, and mild stress responses. The second type of receptors, type II glucocorticoid receptors (GRs), have a lower affinity for cortisol, meaning that higher cortisol levels are required to activate these receptors. They are found in the hippocampus, amygdala, and prefrontal cortex (PFC), which are important in memory formation, emotional responses, and decision making, respectively. Type II glucocorticoid receptors help regulate acute stress responses, energy availability, suppression of inflammation, and memory enhancement.

Frequent exposure to stressful situations can cause the HPA axis to be constantly active, resulting in a higher baseline level of cortisol (Menke 2024). Furthermore, persistently high cortisol levels can cause the HPA axis to become desensitized to the presence of cortisol in the blood, causing the hypothalamus and pituitary gland to secrete even more CRH and ACTH,

respectively. Conversely to the benefits listed above, chronically elevated cortisol levels can lead to immune suppression, metabolic dysfunction, cardiovascular disease, and cognitive impairments (Thau et al. 2025; Stauble et al. 2013; Buchanan and Lovallo 2001).

High cortisol levels are correlated with a multitude of disorders, including MDD. In fact, around 60% of patients with MDD experience hypercortisolemia (high cortisol levels), with cortisol level being proportionate to the severity of the MDD symptoms (Nandam et al. 2020). High CRH levels are also associated with mood disorders, anxiety, and depression (Mikulska et al. 2021). Cortisol is not directly linked to MDD, however. For example, although women have a higher incidence rate of MDD, they do not have higher baseline cortisol levels; instead, their cortisol levels are more variable than that of a non-depressed person (Nandam et al. 2020). Furthermore, when treating MDD, there is no correlation between cortisol levels and response to treatment. Cortisol levels cannot serve as a direct indicator of the presence of MDD, but an exaggerated HPA axis response to stress could.

Stress also activates serotonergic neurons and increases serotonin release from neurons in the dorsal raphe nucleus, an area in the brainstem that targets several brain areas and controls learning, memory, and emotional expression (Bao and Swaab 2019). The serotonergic system acts directly on neurons in the PVN and can activate the HPA axis, serving as a regulator of the system. Similarly, norepinephrine neurons synapse directly on neurons in the PVN and have been shown to increase CRH release, altering the extent to which the HPA axis responds to stress. Conversely, CRH also has an impact on norepinephrine levels, as it results in increased norepinephrine release in the locus coeruleus, another brainstem structure that is involved in the physiological response to stress. However, the HPA axis can become activated without the

influence of norepinephrine in patients with MDD. Furthermore, dopamine levels are elevated by cortisol levels, and dopamine is produced by neurons in the PVN, but the role that it plays in the HPA axis is not well known. Glutamate, the main excitatory neurotransmitter in the brain, and gamma-aminobutyric acid (GABA), the main inhibitory neurotransmitter in the brain, have also been shown to influence the release of CRH in the PVN. Glutamate promotes CRH secretion, while GABA inhibits it. In patients with MDD, neurons in the PVN become less receptive to inhibition by GABA, hindering one of the main ways to deactivate the HPA axis after stress.

Magnetic resonance imaging (MRI) studies on people with MDD show moderate volume reductions in the hippocampus, which is involved in memory formation and emotional control (Palazidou 2012). The hippocampus is rich in brain-derived neurotrophic factor (BDNF) and is one of the only areas of the brain that shows new neuron growth throughout one's life, giving it a high level of neuroplasticity – the brain's ability to adapt and alter its neuronal connections (Trifu et al. 2020). High cortisol levels lower BDNF, which leads to atrophy of hippocampal neurons. As part of the negative feedback loop for the HPA axis, the atrophied hippocampus has a harder time inhibiting the hypothalamus from secreting CRH, leading to dysregulation. In fact, serum BDNF concentrations are lower in patients with MDD, indicating a link between reduced neuroplasticity and depression (Palazidou 2012). In rats, exposure to a multitude of stressors resulted in shorter dendrites and reduced synapse number in the hippocampus (McEwen et al. 2016). Rat pups that had received poor maternal care had a lower level of GRs in the hippocampus, and the DNA segment that promotes the expression of these receptors had higher rates of methylation, which inhibits regular DNA function. Fewer synaptic connections and decreased GR expression in the hippocampus both contribute to the hippocampus' inability to

send inhibitory signals to the hypothalamus.

Patients with MDD show hyperactivity of the amygdala, which is involved in the memory process and emotional learning (Trifu et al. 2020). While rats that had undergone immobilization stress had a shortening of dendrites in the hippocampus, there was an expansion of dendrites in the amygdala (McEwen et al. 2016). This was coupled with an increase in BDNF in the amygdala, promoting neurogenesis. Increased activity in the amygdala can explain depressed patients' overly negative response to negative stimuli, as there is hypoactivity of the amygdala in these patients when they are exposed to positive stimuli (Trifu et al. 2020).

MRI studies also show large reductions in PFC volume, specifically in the anterior cingulate cortex, which is involved in motivation, decision-making, and learning, and the orbitofrontal cortex (OFC), which is involved in correcting behavioral and emotional responses generated by the amygdala (Palazidou 2012). Depressed patients also show decreased blood flow and nutrient metabolism in the dorsolateral PFC, which is associated with poor psychomotor activity and anhedonia (reduced ability to experience pleasure). Studies on rats show that short-term exposure to stress increases the expression of NMDA and AMPA receptors, which are part of the glutamatergic system, in the PFC (McEwen et al. 2016). However, in rats exposed to long-term stress, there was reduced expression of NMDA and AMPA receptors on the cell membrane, as well as reduced synaptic transmission between neurons with these receptors. This was related to enzymatic degradation of receptor subunits and resulted in impaired memory function. There is also dendritic shrinking in PFC neurons after chronic stress exposure. In humans, chronic stress manifests as reduced functional connectivity within the PFC and impaired cognitive flexibility (McEwen et al. 2016).

Gender and Biological Sex Differences in Depression

While male and female incidence rates of depression are similar in childhood, they begin to diverge around puberty, resulting in female adolescents and adults having higher rates of depression than their male counterparts (DuMont et al. 2023). In fact, women are twice as likely to be diagnosed with depression than men are. This may be due to sociocultural factors affecting gender, where women are more likely to report psychological symptoms to a professional. Furthermore, depression also presents differently depending on the patient's gender, which can interfere with the diagnostic process. For example, men with depression often show symptoms like anger, aggression, substance use, and risk-taking (Mohammadi et al. 2023). Men are also over three times more likely to die by suicide related to depression. Women, on the other hand, more commonly experience classic symptoms like mood disturbance, sleep issues, and appetite changes.

In conjunction with gender differences, biological differences in the development and neurological effects of depression are also present. For example, females with MDD have reduced neuronal density in the amygdala and hippocampus compared to non-depressed controls, while males with MDD do not (Mohammadi et al. 2023). Depressed females also show more activity in the amygdala and hippocampus than non-depressed controls. In adolescents, depressive symptoms were correlated with a smaller OFC in males, but a larger OFC in females. The reasoning behind these sex differences is not completely understood, as females with MDD have the same levels of sex hormones as those without MDD, indicating that sex hormones likely do not directly cause the differences in brain structure and function seen in depression. However, sex hormones are still indicated in depression, as depression rates rise after childbirth (post-

partum depression) and menopause. This correlation suggests that depression may be more likely to develop when estrogen/progesterone levels drop.

Animal models of depression can help reveal the sex differences, as the confounding variable of gender is removed. In rats exposed to chronic stress, there was no dendritic shrinking in the hippocampus seen in females, although the heightened level of corticosterone (the rat equivalent of cortisol to humans) signified that female rats were experiencing HPA axis dysfunction (McEwen et al. 2016). This is coupled with the fact that stressed male rats show worse performance on tests of hippocampal-dependent memory compared to controls, while female rats do not. Chronically stressed female rats show dendritic expansion in the connections between the PFC and the amygdala, while stressed males do not. These differences are not as pronounced in young rats, but stressed males still show greater neurological changes than females at this stage. For example, chronically stressed juvenile females still show normal functioning of glutamatergic neurons and glutamate receptor expression in the PFC, as opposed to juvenile males, which showed decreased functioning. Inhibition of estrogen receptors in the PFC of female rats resulted in brain changes that matched that of males, indicating that estrogens protect the brain from stress-induced changes.

Despite the important role of estrogens in neurological outcomes, male animals are used five times as frequently as females are in neuroscience research (Beery 2018). This is due to the misconception that female animals display more variability in their behavior because of their estrous cycle. However, several studies have shown that females display just as much variability in their behavior as males do (Bale 2019; Beery and Zucker 2011; Becker et al. 2016). Furthermore, although women make up about 67% of patients with MDD, they only represent

about 50% of subjects in clinical trials (DuMont et al. 2023). Underrepresentation of females in both preclinical and clinical trials indicates the need for further focus on female subjects.

CVS as an Animal Model of Depression

Rats are one of the most frequently used living models in behavioral neuroscience studies (Ellenbroek and Youn 2016). Chronic variable stress (CVS) as a model of depression in rats consists of microstressors administered at frequent, unpredictable times for multiple weeks (Katz et al. 1982; Becker et al. 2021). For example, a rodent may get exposed to strobe lights or undergo a few hours of isolation. While these manipulations are intended to cause minor psychological distress or discomfort, none cause physical pain. The CVS model is well-established and has been shown to result in a multitude of neurological effects: dendritic atrophy in the PFC, increased corticosterone levels, depression-like behavior, decreased levels of BDNF in the amygdala, and fewer synapses within the amygdala (Willner 2017; Zhang et al. 2014). CVS also decreases excitatory signals triggered by serotonin in the PFC (Li et al. 2011). Furthermore, rats that had been exposed to CVS had lower expression of glutamate transporters and higher extracellular levels of glutamate in the hippocampus (Zhu et al. 2017).

When studying CVS, the majority of the existing literature consists of studies done on rats, implying the existence of a consistent procedure across studies. However, there is much variability in the types of stressors used in each CVS protocol. Contrary to the modern definition of CVS emphasizing painless stressors, the original CVS protocol used electric shocks, as well as food deprivation, water deprivation, cold swim, heat stress, shaking, and day/night reversal (Katz et al. 1982). The list of stressors has expanded over time to where a study using CVS can include

any combination of the following: lights always off, lights always on, day/night reversal, crowded housing, isolation, intruder stress, wet bedding, soiled cage, cage rotation, cage shaking, predator odor, food deprivation, water deprivation, strobe lights, warm swim, cold swim, warm room, cold room, loud noise, ultrasonic noise, restraint, immobilization, hypoxia, and more (Strekalova et al. 2022). Studies tend to use anywhere from 5-14 stressors, and the administration of the stressors can be truly random or follow a set schedule. With all of these variables, CVS protocols can be difficult to replicate, indicating a need for standardization of the procedure.

Ketamine

The largest factor that implies that MDD is more diverse than the monoamine hypothesis of depression presumes is the fact that commonly prescribed antidepressants do not alleviate every case of MDD (Albert et al. 2012). Although medicines that impact monoamine levels may aid in some patients' MDD, they do not help all patients. In these instances, the patient may go through two or more different treatments and not have any relief of symptoms: this is referred to as treatment-resistant depression (TRD) (Taraku et al. 2023). In fact, over 30% of patients with MDD have TRD (Kverno and Mangano 2021). This suggests that there are different types of MDD with different causes; although the outward symptoms appear the same, the neurochemical basis is distinctly different from those with typical MDD. In some cases of depression, the glutamate and GABA systems can be affected (Albert et al. 2012). Being the most common excitatory neurotransmitter in the brain, disordered glutamate levels have widespread effects.

Ketamine, a dissociative anesthetic, is an antagonist of glutamatergic receptors – specifically NMDA receptors – and may help regulate glutamate levels (Bartoli et al. 2017).

Specifically, in patients with MDD, ketamine can increase the release of glutamate, which indirectly increases activation of AMPA receptors (McIntyre et al. 2021). Ketamine has also been shown to weakly inhibit the reuptake transporters of serotonin, norepinephrine, and dopamine, mimicking the effects of typical antidepressants. As a GABA antagonist, ketamine prevents GABA from binding to NMDA receptors, which is believed to increase BDNF levels and promote the formation of new synapses (Alnefeesi et al. 2022). Ketamine can increase levels of neuroplasticity, which is reduced in people with depression (Kang et al. 2022; Becker et al. 2021). Specifically, ketamine has been shown to enhance the activation of glutamatergic receptors that are involved in signaling cascades, which are important for synaptic neuroplasticity (Kang et al. 2022). In humans, ketamine has been shown to raise cortisol levels in healthy controls (Khalili-Mahani et al. 2015), while it temporarily decreases cortisol levels in depressed patients (Johnston et al. 2023). However, ACTH and CRH levels seem to be uncorrelated with the neurological effects of ketamine in patients with TRD (Georgiou et al. 2025). Further research is required to fully understand the relationship between ketamine and the HPA axis.

Repeated ketamine administration in rats exposed to CVS has been suggested to decrease depression-like behavior and improve cognitive functioning (Parise et al. 2013; Zhou et al. 2025). This can be for a multitude of reasons, including improving glutamate transmission, promoting the creation of new synapses in the prefrontal cortex, and reversing the loss of synapses caused by stress and depression (Duman et al. 2012). A single dose of ketamine is able to reverse dendritic atrophy and restore levels of synaptic proteins in the PFC within 24 hours (Willner 2017). Repeated ketamine doses were also shown to help raise the levels of glutamate

transporters in the hippocampus, decrease extracellular glutamate levels, and reverse depression-like behavior in rats exposed to CVS (Zhu et al. 2017). Ketamine can also return serotonin signaling to baseline levels in the PFC, reversing the deficits caused by the depression model (Li et al. 2011). In chronically stressed mice, ketamine was able to lower corticosterone levels in males, but it had no effect on corticosterone levels in females (Johnston et al. 2021).

While many antidepressants need to be taken consistently for weeks before improvement of symptoms is observed, ketamine's effects are often noticeable within hours to days and may only require a single administration to make a difference (Ahmed et al. 2023). This can be lifesaving for patients who are suicidal or in immediate danger of harming themselves. The use of ketamine as a treatment for TRD is a relatively new topic in the field of neuroscience research; however, studies have shown ketamine to be effective in decreasing symptoms of depression and suicidal ideation (Bartoli et al. 2017; Wilkinson et al. 2018). In fact, when used in conjunction with therapy, ketamine can relieve depressive symptoms long-term in up to 70% of patients with TRD (McIntyre et al. 2021).

Behavioral Tests/Corticosterone Measurements

In this study, anxiety-related behaviors will be measured using the open field test (OFT) and elevated zero maze (EZM). In the OFT, an animal is placed in an open, square box, and its locomotor activity is tracked (Simon et al. 1994). The floor is divided into zones based on distance of the animal from the wall. Typically, there is an outer zone that touches the walls of the apparatus, a middle zone, and a center zone, where the animal is most exposed. The animal is placed in a corner of the apparatus and allowed to move about freely for 5-15 minutes while its

movements are tracked. Measures can include the total distance traveled, distance traveled in each zone, time spent in each zone, and number of entries to each zone. Thigmotaxis is the tendency for an animal to remain close to the wall and is an indicator of anxiety-like behavior. This has been supported by how behavior in the OFT is changed after the administration of a drug. Anxiogenic drugs, which cause feelings of anxiety in humans, increase thigmotaxis in the OFT, while anxiolytic drugs, which relieve feelings of anxiety, decrease thigmotaxis (Simon et al. 1994).

The EZM is an adapted form of the Elevated Plus Maze (EPM) (Kulkarni et al. 2007). It consists of a circular track divided into quadrants. Two quadrants on opposite sides are surrounded by walls ("closed arms") that provide a darker, tunnel-like environment while the remaining two quadrants ("open arms") are more exposed but provide a novel environment for exploration. In the EPM, the closed arms are opposite to each other and the open arms are opposite to each other, forming a plus shape. This leaves a center square at the intersection between the open arms and closed arms that is hard to label as being part of either space. The EZM's circular design removes the center square and its ambiguity, allowing for more easily analyzable results. Animals typically have 5-10 minutes to freely explore the maze. Spending relatively more time in the closed arms, as opposed to venturing into the open arms to explore the novel environment, is an indicator of anxiety-like behavior. Similarly to the OFT, anxiogenic drugs increased the amount of time spent in the closed arms, while anxiolytic drugs decreased this behavior (Kulkarni et al. 2007).

Depression-like behaviors will be measured using the forced swim test (FST) and the sucrose preference test (SPT). The FST is a commonly used model and measure of depression-

like behavior in rodents (Molendijk and De Kloet 2015). It is a model because it is believed to induce depression-like behavior, and it is a measure because it is also believed to be able to operationalize depression-like behavior (Porsolt et al. 1978). The test is often used to test the efficacy of new antidepressants before they are approved for human trials (Molendijk and De Kloet 2022; Trunnell et al. 2024). The original FST consisted of one session a day on two consecutive days, 24 hours apart, but different models and schedules exist in the literature. In the FST, a rodent is placed in a tall, cylindrical tank with a water level high enough that the animal cannot touch the bottom without its head being submerged underwater. During the session on the first day, the rodent is put in the tank for 15 minutes to acclimate it to the apparatus. Over the next 24 hours, the animal may be given an antidepressant in one or more administrations before the session on the second day. In session two, the rodent is kept in the tank for five minutes, and its behavior is recorded. Common recorded behaviors include the amount of time spent immobile, the amount of time it took from the beginning of the session for the animal to become immobile, and the number of immobile episodes. An animal is said to express more depression-like behavior if it spends more time immobile, has a shorter latency to immobility, and has more immobile episodes compared to another experimental group. The FST is thought by some researchers to measure "despair," marked by when a rodent stops actively swimming and floats passively in the container (Porsolt et al. 1978). The theory behind the test is that the animal evaluates its situation on day two as "hopeless" after it learned on day one that it is unable to escape the container. A rodent that shows less immobility and takes longer to initially become immobile is believed to show less depression-like behavior and be more resilient to stress. If administration of an antidepressant before session two results in less immobility during that trial,

the drug is able to move onto the next stage of development (although animal trials are not required in some countries before human clinical trials) (Trunnell et al. 2024). Due to this, the FST holds a very important place in psychiatric medication research.

However, the validity of the FST has been highly disputed in the research community for several reasons. Although the FST was originally created to work as both a model and measure of depression, it is rarely ever used as a model anymore, and most research that uses the FST induces a model of depression in their rodents by some other means, like CVS, maternal separation, or brain surgery (Wang et al. 2017). Also, the test lacks two out of three types of validity (Molendijk and De Kloet 2015). Specifically, the FST does have predictive validity as a measure of depression because antidepressants work to decrease immobility, and it is reasonable to predict that an antidepressant would inhibit a depression-like behavior. However, it lacks construct validity because MDD is acquired slowly during one's life and not after one or two inescapable situations. It also lacks face validity because no symptom of human depression is directly measured; instead, immobility is assumed to translate to learned helplessness or despair. Furthermore, recent studies have suggested that immobility may actually be a measure of familiarity with the environment, or an adaptive behavior (Molendijk and De Kloet 2015; Borsini et al. 1986; Nishimura et al. 1988). In prolonged FSTs, some as long as two hours, rats that spent more time immobile were much less likely to become fatigued, while those that showed less "depression-like behavior" began to struggle to keep their heads above water (Nishimura et al. 1988). Rats that were exposed to the empty FST apparatus 24 hours before their test spent more time immobile than rats that had not been introduced to the apparatus (Borsini et al. 1986). Also, water height plays a significant role in immobile behavior, as rats spent much less time immobile

when the water was 30 cm high than when it was 4 or 15 cm high. The FST is used herein largely to replicate outcomes of prior studies of CVS.

Due to all of the discourse surrounding the FST, researchers have proposed a different way of measuring depression-like behavior in animals: the sucrose preference test (SPT). The SPT was proposed not long after the FST and specifically measures levels of anhedonia, a fundamental symptom of depression (Katz 1982). An animal who shows less (or no) preference for a sucrose solution is likely less interested in reward, and thus more depressed (Primo et al. 2023). After exposure to CVS, rats consumed up to 50% less sucrose solution (Katz 1982). Antidepressants were effective in restoring sucrose consumption, indicating that the SPT has predictive validity as a measure of depression. The test also has face validity, considering that consuming foods/drinks with high amounts of sugar is a pleasurable experience for humans and rats alike, and a decrease in this behavior could indicate anhedonia.

Blood sampling will be conducted twice throughout the experiment – once after the CVS protocol has been completed, and once after the injections have been completed – in order to measure plasma corticosterone levels.

Experimental Design

This study investigates whether CVS increases immobility and decreases latency to immobility in the FST, and if ketamine would prevent these changes. This study also investigates the effects of CVS on anxiety-like behaviors in the OFT and EZM. Finally, it explores the effect of CVS on HPA activity reflected in levels of plasma corticosterone. The experiment will follow a 2 x 2 factorial design manipulating stress (with two levels - a control group and an

experimental group that is exposed to CVS) and ketamine administration (a control group that receives a saline solution injection and an experimental group that receives a ketamine injection). The CVS protocol will be administered for three weeks, followed by baseline measurements of anxiety-like behavior using the OFT and EZM and a blood draw to measure corticosterone levels. The FST will then be performed with ketamine administered between the first and second sessions. FST sessions will continue once a week alongside weekly ketamine injections for three weeks. Finally, OFT, EZM, and corticosterone measurements will be repeated. Twenty-four hours after the final test, the animals will be sacrificed by carbon dioxide asphyxiation. A more in-depth description of the experimental timeline is shown in Table 1, and an overview is illustrated in Figure 5.

It is hypothesized that rats that undergo CVS will show more thigmotaxis in the OFT and time spent in the closed arms of the EZM compared to rats in the control groups. Rats that do not receive ketamine treatment will continue to show higher levels of thigmotaxis in the OFT and more time spent in the closed arms of the EZM, whereas rats that do receive ketamine treatment will show a decrease in anxiety-like behavior in the EZM and OFT that matches that of rats in the control groups. Rats in the CVS groups are expected to show decreased latency to immobility and more time spent immobile in the FST, with rats receiving ketamine showing a decrease in immobile behavior over time as the weekly injections are administered. Rats that undergo CVS will have higher concentrations of corticosterone when measured immediately after the end of the protocol as well as after repeated ketamine injections, based on previous studies using female rodents (Johnston et al. 2021). For rats that do not undergo CVS, no differences are expected between rats that receive ketamine injections and those that receive saline injections.

CHAPTER II: METHODS

Pilot Study

Six male and six female Long-Evans rats, weighing between 225-255 g and 175-190 g, respectively, were purchased from Inotiv (formerly Envigo). Upon arrival, rats were weighed and housed in groups of two in a temperature- and humidity-controlled vivarium. Rats were assigned identification numbers using a permanent marker on their tails. A 12-hour light/dark cycle was maintained, with food and water available *ad libitum*. Rats were allowed to acclimate for seven days before the experimental protocol began. Weights were obtained weekly.

The OFT was administered to determine baseline locomotor activity, and each rat was allowed to explore the open field for ten minutes. The following day, rats underwent the FST. Every FST session was 15 minutes long. Behavior in the FST was recorded in five-minute bins to determine changes in behavior within a session. The FST was performed again 24 hours later in order to compare results to Porsolt (1978) and other studies. The following day, the OFT was repeated to compare to behavior in the baseline session.

For the next four weeks, the FST and OFT were performed weekly, with each OFT occurring 24 hours after the FST. Animals were then sacrificed by carbon dioxide asphyxiation.

Testing Apparatuses

The OFT consisted of a wooden one-meter square apparatus enclosed by 40 cm high walls (Figure 2). Rats were placed in one corner of the apparatus and their movements were tracked for ten minutes. A camera was mounted above the apparatus, which was used for the AnyMaze software to track the rats and determine average speed, distance traveled, and time

spent in each of the three zones within the apparatus: the outer ring, middle ring, and center square. The apparatus was cleaned with 70% ethanol after each rat.

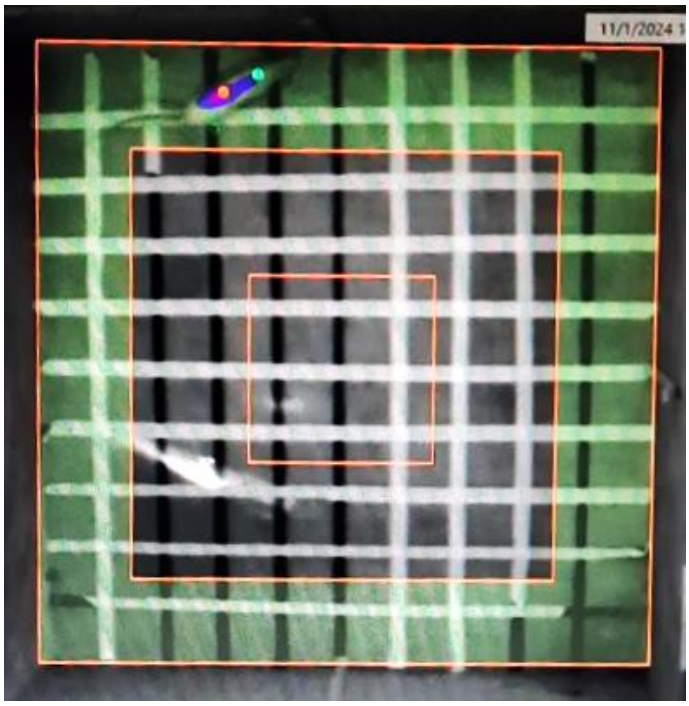

Figure 2. Open field test apparatus as seen on the AnyMaze software. Behavior was tracked for ten minutes. The orange boxes denote the borders of the outer, middle, and center zones. The rat pictured above is displaying thigmotaxis, which is typically considered an anxiety-like behavior.

The EZM consisted of a circular track about six cm wide, elevated about 50 cm off of the ground, with four quadrants (Figure 3). Two of the quadrants, opposite each other, were open, while the other two quadrants had 30 cm high walls on either side of the track, resembling a hallway. Manual measurements were taken to determine time spent in the open quadrants, time spent in the closed quadrants, and the number of entries made to both. Each rat was placed in a closed quadrant and allowed to explore the apparatus for five minutes. The apparatus was cleaned with 70% ethanol after each rat.

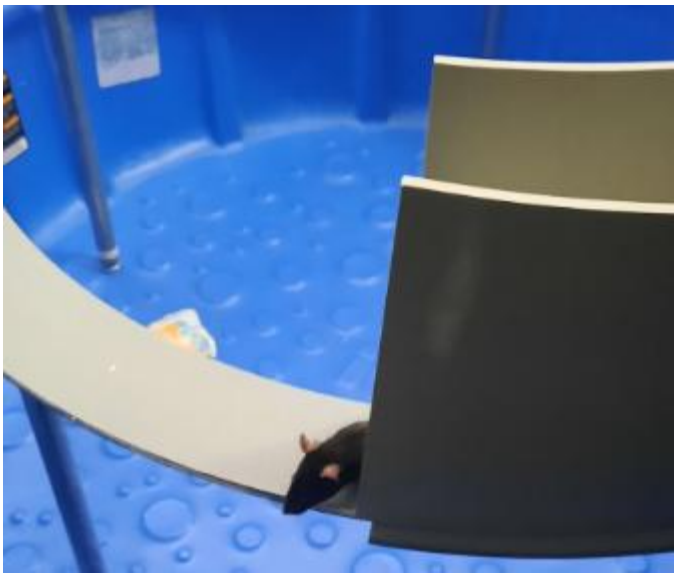

Figure 3. Elevated zero maze apparatus. Behavior was tracked for five minutes. The rat pictured above is in the process of entering an open quadrant, which is typically considered a non-anxiety-like behavior.

The FST consisted of a clear, cylindrical tank 15 cm in diameter filled with 30 cm of water 25-28°C (Figure 4). Rats were placed in the water, and manual measurements were taken to determine latency to immobility, time spent immobile, and bouts of immobility. In the pilot study, each session was 15 minutes long, but in the main experiment, they were ten minutes long. At the end of each session, the rat was taken out of the apparatus, dried with a towel, then placed in a drying cage under a heat lamp for ten minutes in order to completely dry. Fecal boli were removed after each rat, and the water was completely replaced after every two rats.

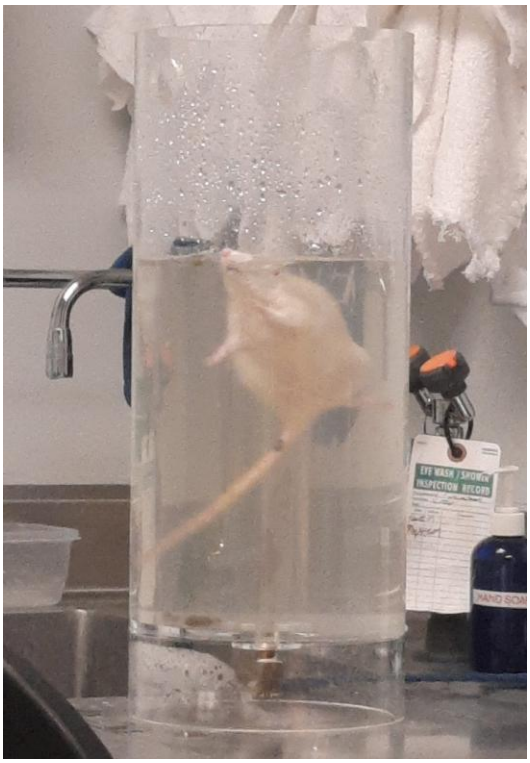

Figure 4. Forced swim test apparatus. Behavior was tracked for ten minutes. The rat pictured above is actively swimming, which is typically considered a non-depression-like behavior.

Animals

Twenty-four female Sprague Dawley rats, weighing between 160 and 175 g, were purchased from Inotiv. Long-Evans rats were the intended strain, but an error was made when ordering. Upon arrival, rats were weighed and housed in groups of three in a temperature- and humidity-controlled vivarium. Rats were assigned identification numbers using a permanent marker on their tails. Each cage was counterbalanced so as to minimize any confounding effects caused by weight. Rats were divided into four groups (n = 6): Control – Vehicle, Control – Ketamine, CVS – Vehicle, and CVS – Ketamine. A 12-hour light/dark cycle was maintained, with food and water available *ad libitum*. Rats were allowed to acclimate for 14 days before the experimental protocol began. Weights were obtained weekly.

Table 1. Experimental schedule. CS = cold swim, SL = strobe lights, USD = ultrasonic deterrent, PO = predator odor, CT = cage tilt, Imm. = immobilization, WB = wet bedding.

Sunday	Monday	Tuesday	Wednesday	Thursday	Friday	Saturday
		Rats arrived	Acclimation			
			Acclimation week 2			
			CVS begin: AM: CS PM: SL	AM: USD PM: PO, isolation	AM: CT PM: Imm.	AM: WB PM: SL
AM: CT PM: WB, isolation	AM: Imm. PM: PO	AM: USD PM: CS	AM: SL PM: CT	AM: WB PM: USD, isolation	AM: CS PM: Imm.	AM: PO PM: USD
AM: SL PM: CT, isolation	AM: WB PM: Imm.	AM: CS PM: PO	AM: CS PM: SL	AM: CT PM: PO, isolation	AM: USD PM: Imm.	AM: WB PM: SL
AM: CT PM: WB	AM: Imm. PM: PO, isolation	AM: USD PM: CS CVS end	Blood draws	OFT	OFT, SPT start	
EZM, SPT measure	FST	Injections, FST	FST	Injections, FST		
SPT start	Injections, SPT measure	FST	Injections	FST		
	Injections	FST, SPT start	Injections, SPT measure	FST		
OFT	EZM					
			Injections, OFT	Blood draws	Euthanasia	

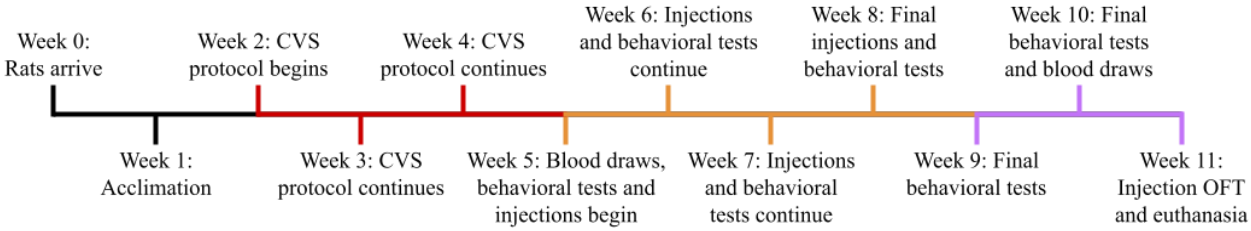

Figure 5. General timeline of protocol. The black portion refers to the acclimation period, red refers to the weeks of the CVS protocol, yellow refers to the weekly injection period, and purple refers to final testing and euthanasia.

CVS Protocol

For 21 days, 12 rats were exposed to CVS. The CVS protocol was adapted from the one used by Slater (2024) and consisted of two stressors a day: an AM stressor administered between 9:00 and 12:00, and a PM stressor administered between 14:00 and 18:00. The exact time of each stressor was varied between days, and the stressors used each day were randomized. During these three weeks, animals in the control group were housed under standard conditions and handled on a regular basis.

The stressors used were immobilization, predator odor, cage tilt, strobe lights, ultrasonic deterrent, wet bedding, and cold swim. In addition to the daytime stressors, the rats were subjected to overnight isolation twice a week. During immobilization, rats were placed in open-ended polyethylene cones (DecapiCones) for 30 minutes with two binder clips used to hold each bag closed. Predator odor consisted of one Foggy Mountain Bobcat Urine Scent-Release Canister being placed above each cage upside-down for one hour. For cage tilt, six rats at a time were placed in a cage on an orbital shaker rotating at 150 RPM for 30 minutes. The strobe light stressor used a white strobe light flashing about five times per second for one hour. In ultrasonic

deterrent, rats were exposed to high frequency noises ("Mouse & Rat Ultra Sonic Deterrent," YouTube) for one hour. For the wet bedding stressor, the bedding in each cage was saturated with about 500 mL of water and left for one hour, then replaced with fresh bedding. During cold swim, each rat was placed for two minutes in a pool filled with 20°C water that was nine cm deep: shallow enough for their heads to remain above water even when not swimming. Lastly, for the overnight isolation stressor, each rat was placed in a smaller cage (42 x 27 x 15 cm) with only food, water, and bedding from 19:00-9:00 the next day.

Behavioral Testing

Following the cessation of the CVS protocol, the OFT and EZM were conducted to test for anxiety-like behaviors. The OFT was divided across two days – two and three days after the end of CVS – due to timing constrictions. Since EZM sessions were half the length of OFT sessions, the EZM was conducted for all rats five days after the end of CVS. The OFT and EZM, along with the blood draw conducted the day after CVS ended, served as baseline measurements to be compared to after the weekly injections had taken place.

Six days after the CVS protocol ended, repeated FSTs began. In the first week, the rats were first given a preliminary test between 8:00 and 10:30. Twenty-four hours after that, they were given injections (described below) and the FST was repeated three to four hours later. In this week, each rat underwent the FST twice. This was done to compare results with the existing literature, which tends to adhere to Porsolt (1978). For the following two weeks, injections were done one day before the FST from 9:00-10:00, and rats only underwent the FST once each week from 10:30-13:00. FSTs were divided into CVS and Control groups in order to stagger the tests

over multiple days and ensure that the rats all completed the FST during the same phase of their circadian rhythm. FSTs for the Control group followed the same pattern, starting two days after the CVS group.

The SPT was administered twice. First, the rats were habituated for 24 hours to the presence of two 500 mL drinking bottles (both filled with water) in their home cage. Once acclimated, water in one of the bottles was replaced by a 1% w/v sucrose solution, with a drop of blue food coloring to visually distinguish it from the other bottle. After 24 hours, the water and sucrose intake were measured in mLs. Sucrose preference was measured as the volume of sucrose solution consumed.

Once FSTs had concluded, the OFT and EZM were administered in the following days. The OFT was conducted three to five days after the last FST, depending on whether the rat was in the CVS or Control group. The EZM was conducted four to six days after the last FST. The final blood draw was collected 21 days after the last FST to allow the rats a recovery period.

To rule out a potential confounding effect of ketamine on locomotor activity in the initial behavioral tests, an investigational OFT was performed 20 days after the last FST where each rat received a ketamine/saline injection approximately three hours before they were placed in the apparatus. This OFT was conducted three weeks after the last ketamine injection to make sure that the rats did not have a tolerance for ketamine, which may have led to a decreased response to the injection.

Corticosterone Measurement

After 21 days of CVS, blood draws were performed to determine plasma corticosterone

levels as a measure of chronic stress. Blood samples were taken using the tail nick method. Animals were placed in plastic restraint tubes and approximately 100 µL of blood were collected from the tail vein. The process ranged from three to five minutes per animal from the time it entered the blood collection room to the time the sample had been completely collected. Samples were collected in heparinized tubes and kept on ice until centrifugation. After collection, the blood samples were centrifuged so that the plasma could be isolated. Supernatant was transferred to new tubes, which were stored at -80°C until testing could be performed. Corticosterone levels were measured using the Arbor Assays Corticosterone Multi-Format ELISA Kit (catalog number K014-H). Samples were read at 450 nm, and data were analyzed using a 4PL curve fit model in MyAssays.com. The process was repeated 21 days after the last FST.

Injections

During the weeks of the FSTs, rats were given intraperitoneal (i.p.) injections of either saline solution (0.9% w/v) or ketamine (10 mg/kg) (as a reference, an anesthetic dose of ketamine for a rodent is typically 85-100 mg/kg). The ketamine solution was prepared by mixing a 100 mg/mL ketamine solution with saline in a 1:10 ratio, resulting in a 10 mg/mL ketamine solution. The first injection was given 24 hours after the animal's first FST and three to four hours before its second FST. In the second and third weeks of the FST, the injection was given one day before the FST session. An in-depth view of the injection schedule, as well as all procedures previously mentioned, can be seen above in Table 1.

Euthanasia

Twenty-four hours after the final blood draw, the animals were sacrificed by carbon dioxide asphyxiation.

Statistical Analysis

The software GraphPad Prism 10 was used to conduct data analysis and create graphs. This included weights, FST, OFT, EZM, and SPT data, as well as corticosterone data. The majority of the data were analyzed with mixed model three-way analyses of variance (ANOVAs) with CVS and injection as between-subjects variables and time as a within-subjects variable. Depending on the results of each three-way ANOVA, two-way ANOVAs and Tukey's multiple comparisons tests were performed. Statistical significance was set at $p < 0.05$. For analyses that yielded a significant interaction, a Bonferroni adjustment was used to minimize the chance of type I error. Results of the pilot study and the investigational OFT are included in the Appendix.

CHAPTER III: RESULTS

*Body Weights (**Figure 6**)*

Rat weights were analyzed starting from week 2, when the CVS protocol began. This helped reduce extraneous data from weeks 0 and 1, when the rats were acclimating and there was no differential treatment between groups. Furthermore, to more clearly observe differences between groups in different stages of the experiment, only certain weeks were included in the statistical analysis of weight. These time points were week 2, week 5, week 8, and week 11, corresponding to the beginning of the CVS protocol, the end of the CVS protocol, the end of behavioral testing/injections, and the end of the recovery period after injections, respectively.

A three-way mixed model ANOVA was run with CVS and injection as between-subjects variables and stage as the within-subjects variable (Figure 6). There was a statistically significant main effect of stage on weight [$F(1.822, 36.43) = 464.3$, $\mathbf{p < 0.0001}$], as well as a significant main effect of CVS [$F(1, 20) = 5.918$, $\mathbf{p = 0.0245}$]. There was also a significant interaction between stage and CVS [$F(3, 60) = 3.271$, $\mathbf{p = 0.0272}$], indicating that the change in a rat's weight across stages, on average, varied as a function of whether or not it underwent CVS. There was no main effect of injection [$F(1, 20) = 0.1857$, $p = 0.6711$]. The interactions between stage and injection [$F(3, 60) = 2.454$, $p = 0.0719$], CVS and injection [$F(1, 20) = 0.3913$, $p = 0.5387$], and stage, CVS, and injection [$F(3, 60) = 0.5950$, $p = 0.6207$] were all not statistically significant.

Given the lack of main effect or interaction including injection, the data were consolidated to remove injection as a variable. In other words, the Control – Vehicle and Control – Ketamine groups were combined, and the CVS – Vehicle and CVS – Ketamine groups were

combined. A two-way mixed model ANOVA was then run with CVS as the between-subjects variable and stage as the within-subjects variable. Results again revealed a statistically significant main effect of stage [$F(1.931, 42.47) = 444.2$, $\mathbf{p < 0.0001}$], a significant effect of CVS [$F(1, 22) = 6.327$, $\mathbf{p = 0.0197}$], and a significant interaction between stage and CVS [$F(3, 66) = 3.123$, $\mathbf{p = 0.0317}$].

For *post-hoc* testing, the Bonferroni adjustment of $\alpha = 0.05/4 = 0.0125$ was used to compare experimental groups across stages. The only stages where there was a significant difference in weight between groups was at the end of CVS (adjusted $\mathbf{p = 0.0211}$) and at the end of behavioral testing/injections (adjusted $\mathbf{p = 0.0481}$). Specifically, the Control group weighed more, on average, than the CVS group at these time points. There were no statistically significant differences between groups when CVS began (adjusted $p = 0.1018$) or when the recovery period ended (adjusted $p > 0.9999$). To compare stages across experimental groups, the Bonferroni adjustment of $\alpha = 0.05/6 = 0.0083$ was used. There was a significant difference in weight for every combination of stages (adjusted $\mathbf{p < 0.0001}$). Specifically, rat weights in every stage were higher, on average, than the weights in previous stages.

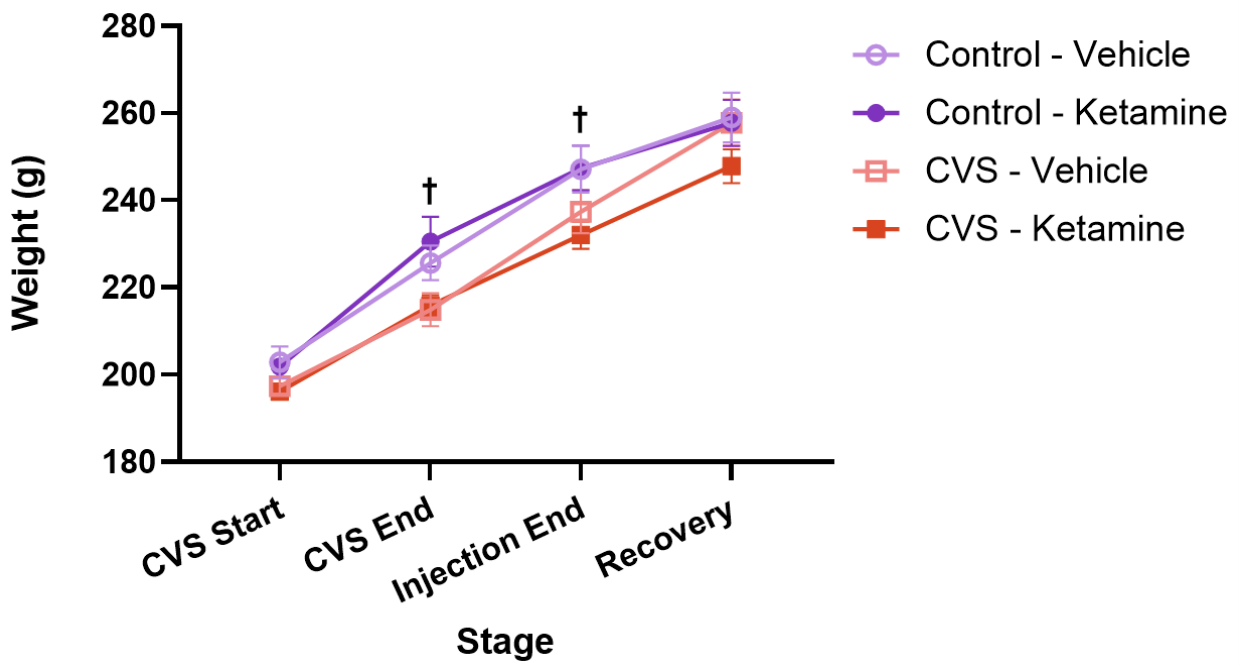

Figure 6. Weight at each stage by group. Weights were obtained weekly, and weeks corresponding to the beginning/end of different phases of the experiment were selected for analysis for better readability. Values are reported as mean ± SEM. $n = 6$ per group. There were significant main effects of CVS at the stages noted († $p < 0.05$ CVS compared to Control).

A summary of all results is shown in Table 2.

Table 2. Summary of results of three-way ANOVAs for main experiment. Significant *p*-values are bolded.

	p-values for						
Test	CVS	Injection	Time	CVS * Time	Injection * Time	CVS * Injection	CVS * Injection * Time
Body weights	**0.0245**	0.6711	**< 0.0001**	**0.0272**	0.0719	0.5387	0.6207
FST latency to immobility	0.7469	**0.0037**	**< 0.0001**	0.1176	0.2182	**0.0196**	0.0794
FST time immobile	0.3811	0.0679	0.1780	**0.0259**	0.1927	0.7729	0.0748
FST bouts of immobility	0.5211	0.4451	0.5078	0.8791	**0.0349**	0.6255	0.2836

OFT total distance	0.9100	0.7775	**0.0445**	0.2656	0.7508	0.8056	0.3435
OFT outer zone distance	0.7785	0.8447	0.1419	0.6742	0.9848	0.9059	0.8432
OFT middle zone distance	0.3978	0.6722	**0.0016**	**0.0020**	0.2226	0.7695	**0.0040**
OFT center zone distance	0.1751	0.8038	**0.0360**	**0.0396**	0.1674	0.4496	**0.0011**
OFT time in outer zone	0.1562	0.4115	**0.0023**	**0.0331**	0.3383	0.9248	0.0834
OFT time in middle zone	**0.0265**	0.8435	**0.0029**	**0.0111**	**0.0211**	0.3568	**0.0082**
OFT time in center zone	0.1362	0.3534	0.1124	**0.0010**	0.5729	0.9089	**0.0012**
EZM open arm entries	0.9130	0.9130	0.1763	0.6786	0.6786	0.6240	**0.0364**
EZM open arm time	0.1956	0.4019	**0.0007**	0.4021	0.9815	0.4175	0.5995
EZM open arm latency	0.9294	0.5540	**0.0094**	0.7918	0.5139	0.9083	0.1733
SPT volume consumed	0.4588	0.8342	0.7116	0.1668	0.4222	0.0728	0.7116
Corticosterone concentration	0.8611	0.0851	0.3255	**0.0379**	0.7006	0.1751	0.6357

*Forced Swim Test (**Figures 7-9**)*

The number of seconds between when a rat was placed in the FST and the first time it became immobile was measured as "latency to immobility." During the first session, rats still had not received their first injection, but for the second session, rats received the injection 3-4 hours before being placed in the FST. A three-way mixed model ANOVA was run with CVS and injection as between-subjects variables and session as the within-subjects variable (Figure 7). There was a statistically significant main effect of session on latency [$F(1.880, 37.60) = 18.79$, $\boldsymbol{p < 0.0001}$], as well as a significant main effect of injection [$F(1, 20) = 10.76$, $\boldsymbol{p = 0.0037}$].

Specifically, latency to immobility decreased, on average, as the sessions went on, regardless of group. Also, rats that received ketamine had lower latencies to immobility, on average, than rats that received vehicle injections, regardless of session or CVS. There was also a significant interaction between CVS and injection [$F(1, 20) = 6.444$, $\boldsymbol{p = 0.0196}$], indicating that the difference in latency to immobility across CVS groups, on average, varied as a function of whether or not the rats received weekly ketamine injections. There was no main effect of CVS [$F(1, 20) = 0.1071$, $p = 0.7469$]. The interactions between session and CVS [$F(3, 60) = 2.042$, $p = 0.1176$], session and injection [$F(3, 60) = 1.521$, $p = 0.2182$], and session, CVS, and injection [$F(3, 60) = 2.370$, $p = 0.0794$] were all not statistically significant. There was one outlier in the Control – Vehicle group during session 2. Removing this outlier and reanalyzing the data yielded no changes in which results were statistically significant, so the outlier was included in the analyses.

For *post-hoc* testing, the Bonferroni adjustment of $\alpha = 0.05/24 = 0.0021$ was used to compare experimental groups across sessions. There were no statistically significant differences between groups in the same session. To compare sessions across experimental groups, each group was analyzed separately in a repeated measures one-way ANOVA, and a Tukey's HSD test was used. For the Control – Vehicle group, there was a significant difference in latency between sessions 1 and 4 (adjusted $\boldsymbol{p = 0.0338}$). For the Control – Ketamine group, there was a significant difference between session 1 and sessions 2, 3, and 4 (adjusted $\boldsymbol{p = 0.0011}$, **0.0010**, and **0.0008**, respectively). For the CVS – Vehicle group, there were no differences between sessions. For the CVS – Ketamine group, there was a significant difference between sessions 1 and 4 (adjusted $\boldsymbol{p =}$ **0.0292**).

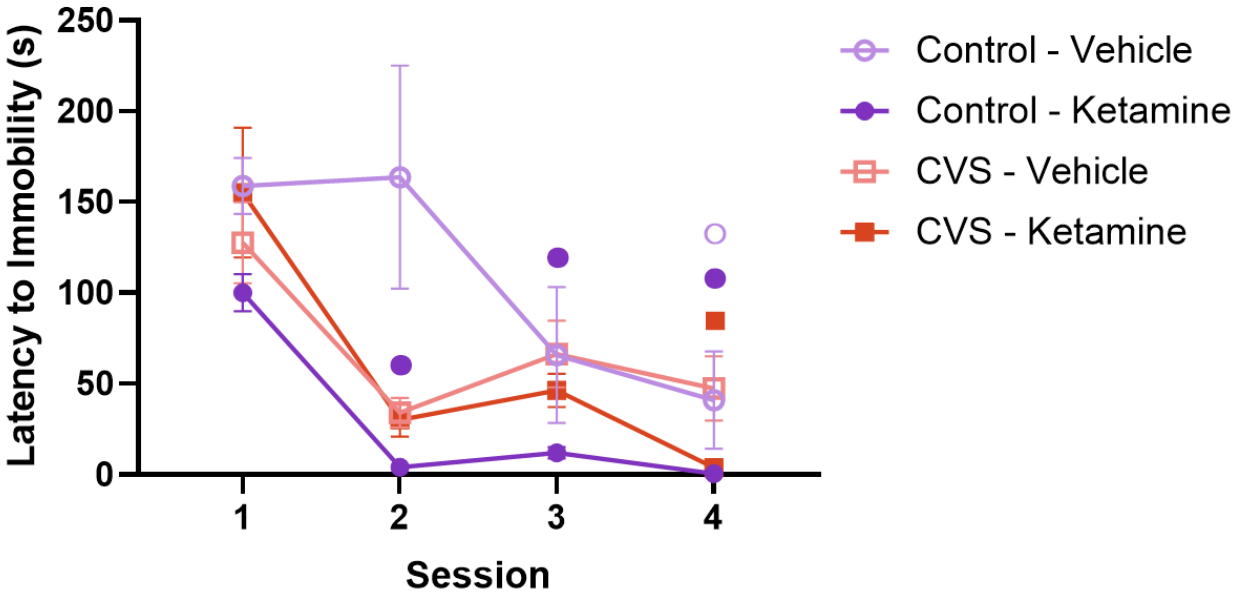

Figure 7. Latency to immobility in the FST across sessions by group. The first 2 FSTs were conducted 24 hours apart, then once a week afterwards. Values are reported as mean ± SEM. $n = 6$ per group. Rats in the Control - Vehicle group had significantly lower latency in the session noted (○ $p < 0.05$ session 4 compared to session 1). Rats in the Control - Ketamine group had significantly lower latency in the sessions noted (● $p < 0.05$ sessions 2, 3, and 4 compared to session 1). Rats in the CVS - Ketamine group had significantly lower latency in the session noted (■ $p < 0.05$ session 4 compared to session 1).

Total time spent immobile over the course of each FST was also analyzed. As with latency to immobility, a three-way mixed model ANOVA was run with CVS and injection as between-subjects variables and session as the within-subjects variable (Figure 8). There were no statistically significant main effects of session [$F(1.907, 38.14) = 1.815$, $p = 0.1780$], CVS [$F(1, 20) = 3.724$, $p = 0.0679$], or injection [$F(1, 20) = 0.8022$, $p = 0.3811$] on total time immobile. There was a significant interaction between session and CVS [$F(3, 60) = 3.311$, $\mathbf{p = 0.0259}$], indicating that the change in the amount of time a rat spent immobile across sessions, on average, varied as a function of whether or not it was exposed to CVS. The interactions between session

and injection [$F(3, 60) = 1.627$, $p = 0.1927$], CVS and injection [$F(1, 20) = 0.08561$, $p = 0.7729$], and session, CVS, and injection [$F(3, 60) = 2.421$, $p = 0.0748$] were all not statistically significant.

Given the lack of main effect or interaction including injection, the data were consolidated to remove injection as a variable. In other words, the Control – Vehicle and Control – Ketamine groups were combined, and the CVS – Vehicle and CVS – Ketamine groups were combined. A two-way mixed model ANOVA was then run with CVS as the between-subjects variable and session as the within-subjects variable. Results again revealed a statistically significant interaction between session and CVS [$F(3, 66) = 3.029$, $\mathbf{p = 0.0355}$].

For *post-hoc* testing, the Bonferroni adjustment of $\alpha = 0.05/4 = 0.0125$ was used to compare experimental groups across sessions. There were no statistically significant differences between the Control group and CVS group in any session. To compare sessions across experimental groups, the Bonferroni adjustment of $\alpha = 0.05/6 = 0.0083$ was used. Again, there were no statistically significant differences between sessions in either the Control group or the CVS group.

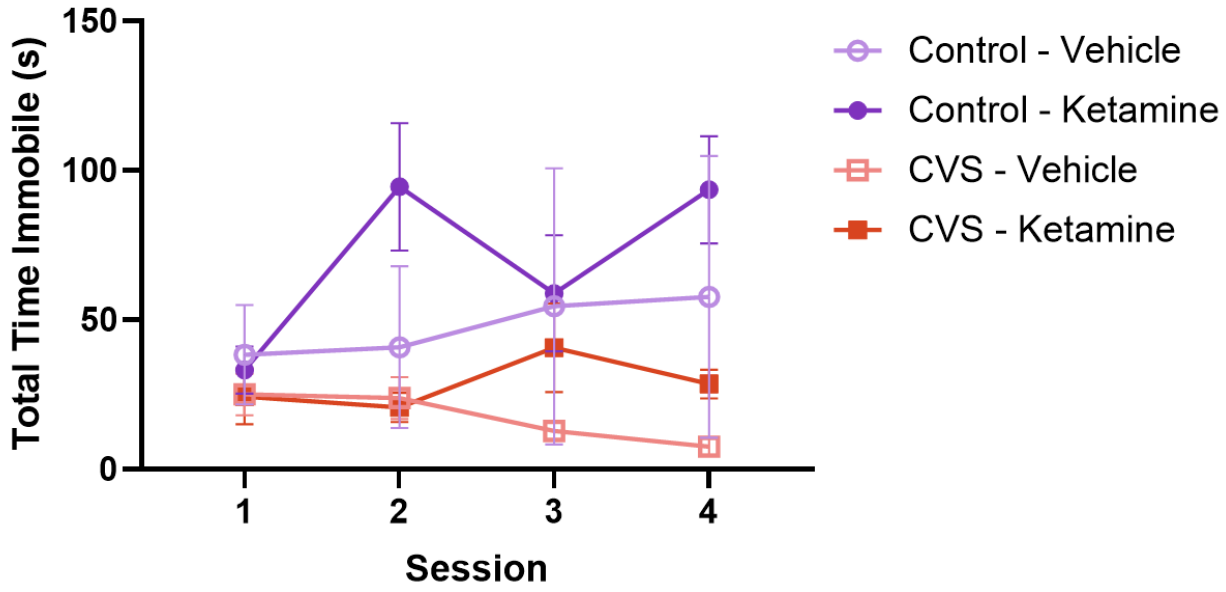

Figure 8. Total time immobile in the FST across sessions by group. The first 2 FSTs were conducted 24 hours apart, then once a week afterwards. Values are reported as mean ± SEM. $n = 6$ per group. There was a significant interaction between session and CVS, but there were no significant pairwise comparisons.

The number of bouts of immobility over the course of each FST was analyzed. As with the other FST analyses, a three-way mixed model ANOVA was run with CVS and injection as between-subjects variables and session as the within-subjects variable (Figure 9). There were no statistically significant main effects of session [$F(2.227, 44.55) = 0.7174$, $p = 0.5078$], CVS [$F(1, 20) = 0.4266$, $p = 0.5211$], or injection [$F(1, 20) = 0.6069$, $p = 0.4451$] on bouts of immobility. There was a significant interaction between session and injection [$F(3, 60) = 3.059$, $\mathbf{p = 0.0349}$], indicating that the change in the number of times a rat went immobile across sessions, on average, varied as a function of whether or not it received ketamine injections. The interactions between session and CVS [$F(3, 60) = 0.2244$, $p = 0.8791$], CVS and injection [$F(1, 20) = 0.2458$, $p = 0.6255$], and session, CVS, and injection [$F(3, 60) = 1.297$, $p = 0.2836$] were all not

statistically significant.

Given the lack of main effect or interaction including CVS, the data were consolidated to remove CVS as a variable. In other words, the Control – Vehicle and CVS – Vehicle groups were combined, and the Control – Ketamine and CVS – Ketamine groups were combined. A two-way mixed model ANOVA was then run with injection as the between-subjects variable and session as the within-subjects variable. Results again revealed a statistically significant interaction between session and injection [$F(3, 66) = 3.127$, $\boldsymbol{p = 0.0315}$].

For *post-hoc* testing, the Bonferroni adjustment of $\alpha = 0.05/4 = 0.0125$ was used to compare experimental groups across sessions. There were no statistically significant differences between the Vehicle group and Ketamine group in any session. To compare sessions across experimental groups, the Bonferroni adjustment of $\alpha = 0.05/6 = 0.0083$ was used. Again, there were no statistically significant differences between sessions in either the Vehicle group or the Ketamine group.

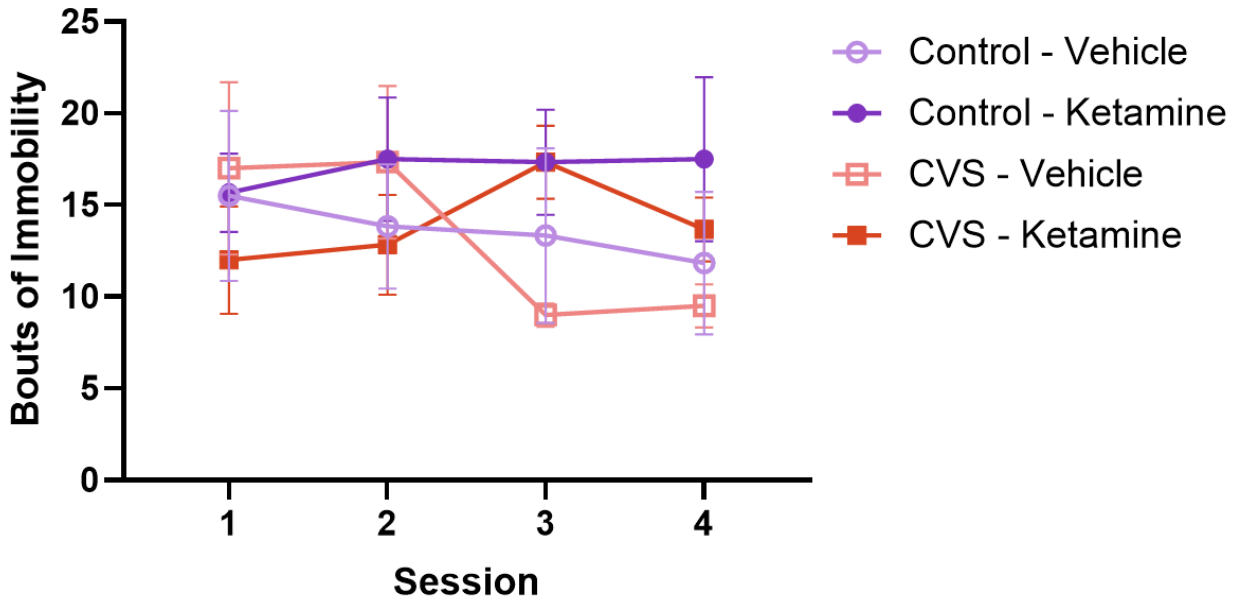

Figure 9. Total bouts of immobility in the FST across sessions by group. The first 2 FSTs were conducted 24 hours apart, then once a week afterwards. Values are reported as mean ± SEM. $n = 6$ per group. There was a significant interaction between session and injection, but there were no significant pairwise comparisons.

*Open Field Test (**Figures 10-11**)*

Total Distance

The OFT was conducted three times over the course of the experiment: once after the CVS protocol had ended, once after the ketamine injections had ended, and once more during the recovery stage in order to determine the locomotor effects of ketamine given 3-4 hours before a behavioral test, as was done in the second FST session. For repeated measures analysis, only the first two sessions were used; the last session was analyzed separately since it was not part of the primary experimental design. To analyze total distance traveled, a three-way mixed model ANOVA was run with CVS and injection as between-subjects variables and session as the within-subjects variable (Figure 10). There was a significant main effect of session [$F(1, 20) =$

4.595, ***p* = 0.0445**]. There were no significant main effects of CVS [$F(1, 20) = 0.01310$, $p = 0.9100$] or injection [$F(1, 20) = 0.08201$, $p = 0.7775$]. There were also no significant interactions between session and CVS [$F(1, 20) = 1.312$, $p = 0.2656$], session and injection [$F(1, 20) = 0.1037$, $p = 0.7508$], CVS and injection [$F(1, 20) = 0.06222$, $p = 0.8056$], or session, CVS, and injection [$F(1, 20) = 0.9415$, $p = 0.3435$].

To describe the nature of the main effect of session, cell means were used. Rats traveled farther, on average, in the first session than the second session.

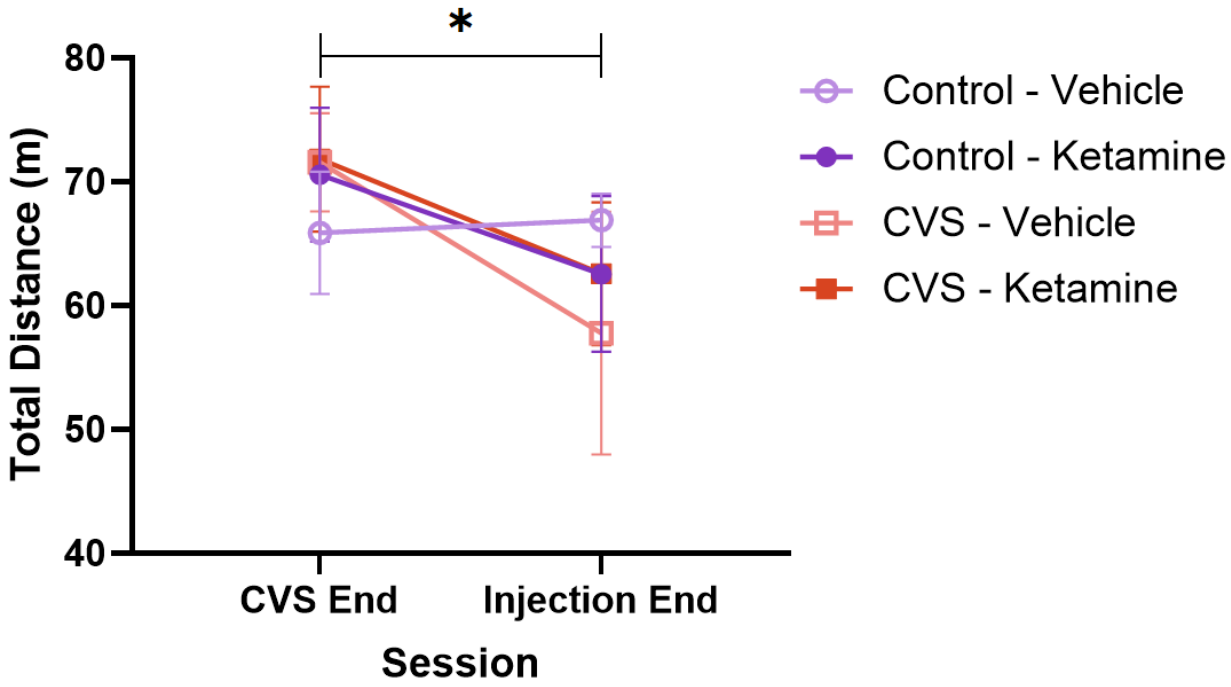

Figure 10. Total distance traveled in the OFT across sessions by group. The first OFT was conducted after CVS ended, and the second was conducted after weekly injections ended. Values are reported as mean ± SEM. $n = 6$ per group. Rats travelled a significantly shorter distance in the second session (* $p <$ 0.05 CVS End session compared to Injection End session).

For each analysis of the outer, middle, and center zones, a three-way mixed model

ANOVA was run with CVS and injection as between-subjects variables and session as the within-subjects variable.

Outer Zone Distance

For distance traveled in the outer zone, there were no significant main effects of session [$F(1, 20) = 2.338$, $p = 0.1419$], CVS [$F(1, 20) = 0.08132$, $p = 0.7785$], or injection [$F(1, 20) = 0.03937$, $p = 0.8447$] (Figure 11A). There were also no significant interactions between session and CVS [$F(1, 20) = 0.1820$, $p = 0.6742$], session and injection [$F(1, 20) = 0.0003732$, $p = 0.9848$], CVS and injection [$F(1, 20) = 0.01433$, $p = 0.9059$], or session, CVS, and injection [$F(1, 20) = 0.04015$, $p = 0.8432$].

Outer Zone Time

For time spent in the outer zone, there was a significant main effect of session [$F(1, 20) = 12.18$, $\boldsymbol{p = 0.0023}$] (Figure 11B). Specifically, rats spent less time in the outer zone, on average, in the first session than the second session. There were no significant main effects of CVS [$F(1, 20) = 2.171$, $p = 0.1562$] or injection [$F(1, 20) = 0.7035$, $p = 0.4115$]. There was a significant interaction between session and CVS [$F(1, 20) = 5.236$, $\boldsymbol{p = 0.0331}$], but no significant interactions between session and injection [$F(1, 20) = 0.9623$, $p = 0.3383$], CVS and injection [$F(1, 20) = 0.009147$, $p = 0.9248$], or session, CVS, and injection [$F(1, 20) = 3.320$, $p = 0.0834$].

Given the lack of main effect or interaction including injection, the data were consolidated to remove injection as a variable. In other words, the Control – Vehicle and Control – Ketamine groups were combined, and the CVS – Vehicle and CVS – Ketamine groups were

combined. A two-way mixed model ANOVA was then run with CVS as the between-subjects variable and session as the within-subjects variable. Results again revealed a statistically significant interaction between session and injection [$F(1, 22) = 4.744$, $\mathbf{p = 0.0404}$].

For *post-hoc* testing, the Bonferroni adjustment of $\alpha = 0.05/2 = 0.025$ was used to compare experimental groups across sessions. There was a significant difference between Control and CVS groups in the Injection End session (adjusted $\mathbf{p = 0.0320}$). Specifically, rats in the CVS group spent more time, on average, in the outer zone than the Control group did in the second session. To compare sessions across experimental groups, the Bonferroni adjustment of $\alpha = 0.05/2 = 0.025$ was used. For the CVS group, there was a significant difference between the CVS End and the Injection End sessions (adjusted $\mathbf{p = 0.0016}$). Specifically, rats that received CVS spent less time in the outer zone, on average, after the CVS stage than after the injection stage. There was no significant difference between sessions for the Control group.

Middle Zone Distance

For distance traveled in the middle zone, there was a significant main effect of session [$F(1, 20) = 13.38$, $\mathbf{p = 0.0016}$] (Figure 11C). Specifically, rats traveled farther in the middle zone, on average, in the first session than the second session. There were no significant main effects of CVS [$F(1, 20) = 0.7467$, $p = 0.3978$] or injection [$F(1, 20) = 0.1844$, $p = 0.6722$]. There was a significant interaction between session and CVS [$F(1, 20) = 12.67$, $\mathbf{p = 0.0020}$], but no significant interactions between session and injection [$F(1, 20) = 1.584$, $p = 0.2226$] or CVS and injection [$F(1, 20) = 0.08819$, $p = 0.7695$]. There was a significant three-way interaction between session, CVS, and injection [$F(1, 20) = 10.56$, $\mathbf{p = 0.0040}$], indicating that the interaction

between CVS and injection varied across sessions. To describe the nature of the three-way interaction, the data were consolidated all 3 ways, and two-way ANOVAs were conducted.

First, the data were consolidated to remove injection as a variable. In other words, the Control – Vehicle and Control – Ketamine groups were combined, and the CVS – Vehicle and CVS – Ketamine groups were combined. The two-way ANOVA revealed a significant main effect of session [$F(1, 22) = 9.158$, $\boldsymbol{p = 0.0062}$], no main effect of CVS [$F(1, 22) = 0.8103$, $p = 0.3778$], and a significant interaction between session and CVS [$F(1, 22) = 8.668$, $\boldsymbol{p = 0.0075}$]. For *post-hoc* testing, the Bonferroni adjustment of $\alpha = 0.05/2 = 0.025$ was used to compare experimental groups across sessions. There were no significant differences between Control and CVS groups in the CVS End or Injection End sessions. To compare sessions across experimental groups, the Bonferroni adjustment of $\alpha = 0.05/2 = 0.025$ was used. For the CVS group, there was a significant difference between the CVS End and the Injection End sessions (adjusted $\boldsymbol{p = 0.0007}$). Specifically, rats that received CVS traveled farther in the middle zone, on average, after the CVS stage than after the injection stage. There was no significant difference between sessions for the Control group.

The middle zone data were then consolidated to remove CVS as a variable. The two-way ANOVA revealed a significant main effect of session [$F(1, 22) = 6.810$, $\boldsymbol{p = 0.0160}$]. There was no significant main effect of injection [$F(1, 22) = 0.1947$, $p = 0.6633$], and there was no significant interaction between session and injection [$F(1, 22) = 0.8064$, $p = 0.3789$]. To describe the nature of the main effect of session, cell means were used. Rats traveled farther in the middle zone, on average, in the first session than the second session.

Lastly, the middle zone data were consolidated to remove session as a variable, and a

two-way ANOVA was conducted with both CVS and injection as between-subjects variables. There were no significant effects of CVS [$F(1, 20) = 0.7081$, $p = 0.4100$], injection [$F(1, 20) = 0.007078$, $p = 0.9338$], or interaction between CVS and injection [$F(1, 20) = 0.2836$, $p = 0.6002$].

Middle Zone Time

For time spent in the middle zone, there was a significant main effect of session [$F(1, 20) = 11.53$, $\mathbf{p = 0.0029}$] (Figure 11D). Specifically, rats spent more time in the middle zone, on average, in the first session than the second session. There was also a main effect of CVS [$F(1, 20) = 5.738$, $\mathbf{p = 0.0265}$], but no significant main effect of injection [$F(1, 20) = 0.04001$, $p = 0.8435$]. Specifically, rats that received CVS spent less time in the middle zone, on average, than rats that did not receive CVS. There was a significant interaction between session and CVS [$F(1, 20) = 7.829$, $\mathbf{p = 0.0111}$] and session and injection [$F(1, 20) = 6.265$, $\mathbf{p = 0.0211}$], but no significant interaction between CVS and injection [$F(1, 20) = 0.8897$, $p = 0.3568$]. There was a significant three-way interaction between session, CVS, and injection [$F(1, 20) = 8.621$, $\mathbf{p = 0.0082}$], indicating that the interaction between CVS and injection varied across sessions. To describe the nature of the three-way interaction, the data were consolidated all 3 ways, and two-way ANOVAs were conducted.

First, the data were consolidated to remove injection as a variable. The two-way ANOVA revealed a significant main effect of session [$F(1, 22) = 7.269$, $\mathbf{p = 0.0132}$] and CVS [$F(1, 22) = 6.032$, $\mathbf{p = 0.0224}$], as well as a significant interaction between session and CVS [$F(1, 22) = 4.937$, $\mathbf{p = 0.0369}$]. For *post-hoc* testing, the Bonferroni adjustment of $\alpha = 0.05/2 = 0.025$ was

used to compare experimental groups across sessions. There was a significant difference between Control and CVS groups in the Injection End session (adjusted $\boldsymbol{p = 0.0037}$). Specifically, rats in the CVS group spent less time, on average, in the middle zone than the Control group did in the second session. To compare sessions across experimental groups, the Bonferroni adjustment of $\alpha = 0.05/2 = 0.025$ was used. For the CVS group, there was a significant difference between the CVS End session and the Injection End session (adjusted $\boldsymbol{p = 0.0043}$. Specifically, rats that received CVS spent more time in the middle zone, on average, after the CVS stage than after the injection stage.

The middle zone data were then consolidated to remove CVS as a variable. The two-way ANOVA revealed a significant main effect of session [$F(1, 22) = 6.957$, $\boldsymbol{p = 0.0150}$]. There was no significant main effect of injection [$F(1, 22) = 0.03306$, $p = 0.8574$], nor was there a significant interaction between session and injection [$F(1, 22) = 3.781$, $p = 0.0647$]. To describe the nature of the main effect of session, cell means were used. Rats spent more time in the middle zone, on average, in the first session than the second session.

Lastly, the middle zone data were consolidated to remove session as a variable, and a two-way ANOVA was conducted with both CVS and injection as between-subjects variables. There were no significant effects of CVS [$F(1, 20) = 3.356$, $p = 0.0819$], injection [$F(1, 20) = 0.2736$, $p = 0.6067$], or interaction between CVS and injection [$F(1, 20) = 1.523$, $p = 0.2314$].

Center Zone Distance

For distance traveled in the center zone, there was a significant main effect of session [$F(1, 20) = 5.054$, $\boldsymbol{p = 0.0360}$] (Figure 11E). Specifically, rats traveled farther in the center zone,

on average, in the first session than the second session. There were no significant main effects of CVS [$F(1, 20) = 1.976$, $p = 0.1751$] or injection [$F(1, 20) = 0.06341$, $p = 0.8038$]. There was a significant interaction between session and CVS [$F(1, 20) = 4.846$, $\mathbf{p = 0.0396}$], but no significant interactions between session and injection [$F(1, 20) = 2.053$, $p = 0.1674$] or CVS and injection [$F(1, 20) = 0.5947$, $p = 0.4496$]. There was a significant three-way interaction between session, CVS, and injection [$F(1, 20) = 14.48$, $\mathbf{p = 0.0011}$], indicating that the interaction between CVS and injection varied across sessions. To describe the nature of the three-way interaction, the data were consolidated all 3 ways, and two-way ANOVAs were conducted.

First, the data were consolidated to remove injection as a variable. The two-way ANOVA revealed no significant effects of session [$F(1, 22) = 3.044$, $p = 0.0950$], CVS [$F(1, 22) = 2.105$, $p = 0.1610$], or interaction between session and CVS [$F(1, 22) = 2.918$, $p = 0.1016$].

The center zone data were then consolidated to remove CVS as a variable. The two-way ANOVA revealed no significant effects of session [$F(1, 22) = 2.828$, $p = 0.1068$], injection [$F(1, 22) = 0.06181$, $p = 0.8060$], or interaction between session and injection [$F(1, 22) = 1.148$, $p = 0.2955$].

Lastly, the center zone data were consolidated to remove session as a variable, and a two-way ANOVA was conducted with both CVS and injection as between-subjects variables. There were no significant effects of CVS [$F(1, 20) = 2.792$, $p = 0.1103$], injection [$F(1, 20) = 0.3125$, $p = 0.5824$], or interaction between CVS and injection [$F(1, 20) = 1.030$, $p = 0.3223$].

Center Zone Time

For time spent in the center zone, there were no significant main effects of session [$F(1,$

20) = 2.757, $p = 0.1124$], CVS [$F(1, 20) = 2.410$, $p = 0.1362$], or injection [$F(1, 20) = 0.9026$, $p = 0.3534$] (Figure 11F). There was a significant interaction between session and CVS [$F(1, 20) = 14.86$, $\boldsymbol{p = 0.0010}$], but no significant interactions between session and injection [$F(1, 20) = 0.3286$, $p = 0.5729$] or CVS and injection [$F(1, 20) = 0.01342$, $p = 0.9089$]. There was a significant three-way interaction between session, CVS, and injection [$F(1, 20) = 14.25$, $\boldsymbol{p = 0.0012}$], indicating that the interaction between CVS and injection varied across sessions. To describe the nature of the three-way interaction, the data were consolidated all 3 ways, and two-way ANOVAs were conducted.

First, the data were consolidated to remove injection as a variable. The two-way ANOVA revealed no main effect of session [$F(1, 22) = 1.754$, $p = 0.1990$] or CVS [$F(1, 22) = 2.535$, $p = 0.1256$], and a significant interaction between session and CVS [$F(1, 22) = 9.452$, $\boldsymbol{p = 0.0055}$]. For *post-hoc* testing, the Bonferroni adjustment of $\alpha = 0.05/2 = 0.025$ was used to compare experimental groups across sessions. There was a significant difference between Control and CVS groups in the Injection End session (adjusted $\boldsymbol{p = 0.0038}$). Specifically, rats in the CVS group spent less time, on average, in the center zone than the Control group did in the second session. To compare sessions across experimental groups, the Bonferroni adjustment of $\alpha = 0.05/2 = 0.025$ was used. For the CVS group, there was a significant difference between the CVS End session and the Injection End session (adjusted $\boldsymbol{p = 0.0102}$). Specifically, rats that received CVS spent less time in the middle zone, on average, after the CVS stage than after the injection stage.

The center zone data were then consolidated to remove CVS as a variable. The two-way ANOVA revealed no significant effects of session [$F(1, 22) = 1.235$, $p = 0.2784$], injection [$F(1,$

22) = 0.8856, p = 0.3569], or the interaction between session and injection [F(1, 22) = 0.1472, p = 0.7049].

Lastly, the center zone data were consolidated to remove session as a variable, and a two-way ANOVA was conducted with both CVS and injection as between-subjects variables. There were no significant effects of CVS [F(1, 20) = 2.798, p = 0.1100], injection [F(1, 20) = 3.697, p = 0.0689], or interaction between CVS and injection [F(1, 20) = 0.6701, p = 0.4227].

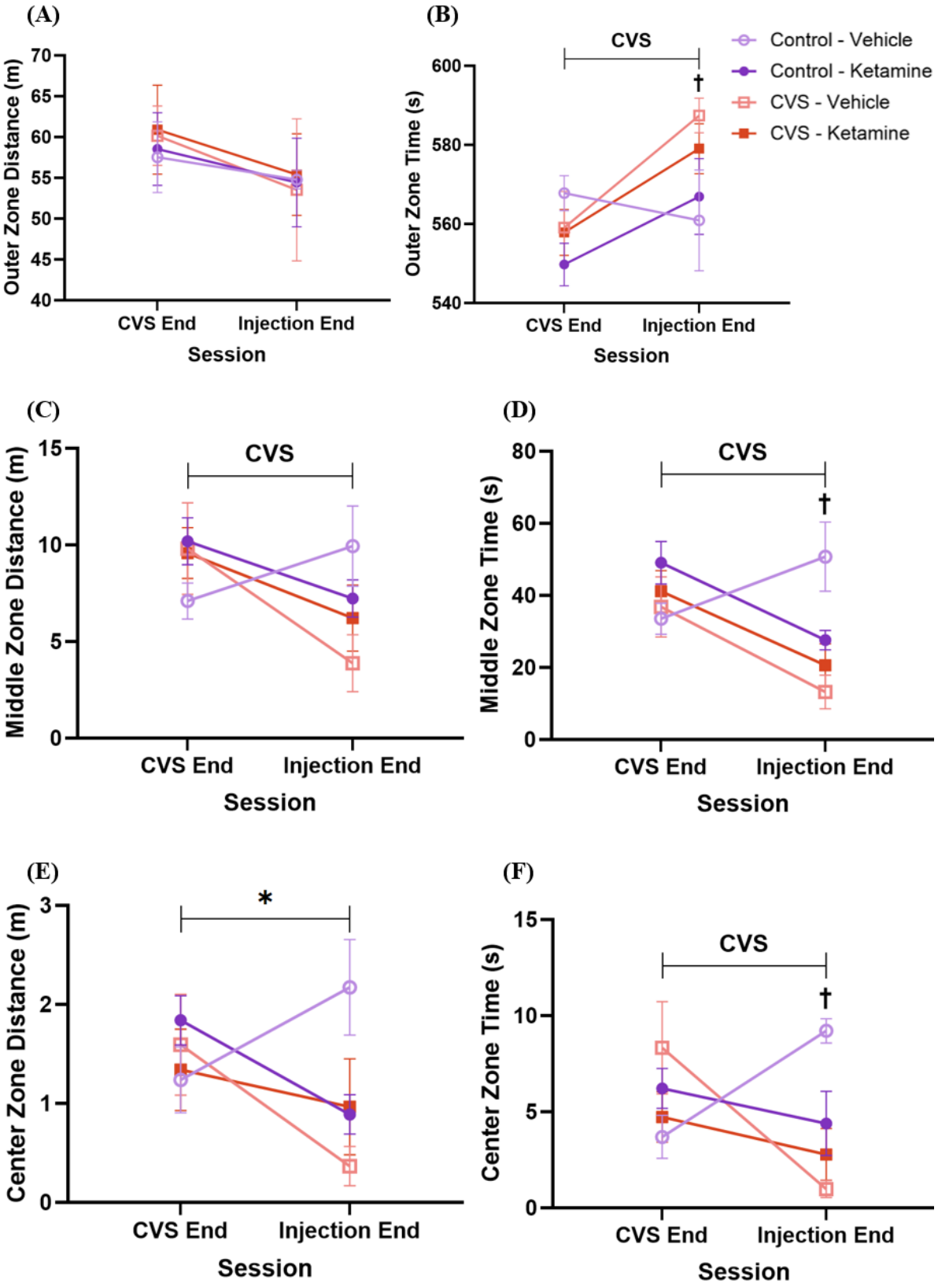
(A)
Outer Zone Distance (m)
70
65
60
55
50
45
40
CVS End
Injection End
Session
(B)
CVS
†
Outer Zone Time (s)
600
580
560
540
CVS End
Injection End
Session
Control - Vehicle
Control - Ketamine
CVS - Vehicle
CVS - Ketamine
(C)
CVS
Middle Zone Distance (m)
15
10
5
0
CVS End
Injection End
Session
(D)
CVS
†
Middle Zone Time (s)
80
60
40
20
0
CVS End
Injection End
Session
(E)
*
Center Zone Distance (m)
3
2
1
0
CVS End
Injection End
Session
(F)
CVS
†
Center Zone Time (s)
15
10
5
0
CVS End
Injection End
Session

Figure 11. All OFT data across sessions by group. The first OFT was conducted after CVS ended, and the second was conducted after weekly injections ended. Values are reported as mean ± SEM. $n = 6$ per group. **(A)** Distance traveled in the outer zone. There were no significant differences found. **(B)** Time spent in the outer zone. Rats in the CVS groups spent significantly more time in the outer zone in the second session (CVS $p < 0.05$ CVS End session compared to Injection End session). There was a significant main effect of CVS at the session noted († $p < 0.05$ CVS compared to Control). **(C)** Distance traveled in the middle zone. Rats in the CVS groups travelled a significantly shorter distance in the middle zone in the second session (CVS $p < 0.05$ CVS End session compared to Injection End session). **(D)** Time spent in the middle zone. Rats in the CVS groups spent significantly less time in the middle zone in the second session (CVS $p < 0.05$ CVS End session compared to Injection End session). There was a significant main effect of CVS at the session noted († $p < 0.05$ CVS compared to Control). **(E)** Distance traveled in center zone. Rats travelled a significantly shorter distance in the center zone in the second session (* $p < 0.05$ CVS End session compared to Injection End session). **(F)** Time spent in center zone. Rats in the CVS groups spent significantly less time in the center zone in the second session (CVS $p < 0.05$ CVS End session compared to Injection End session). There was a significant main effect of CVS at the session noted († $p < 0.05$ CVS compared to Control).

*Elevated Zero Maze (**Figure 12**)*

Entries Into Open Arms

For entries into the open arms, a three-way mixed model ANOVA was run with CVS and injection as between-subjects variables and session as the within-subjects variable (Figure 12A). There were no statistically significant main effects of session [$F(1, 20) = 1.965$, $p = 0.1763$], CVS [$F(1, 20) = 0.01224$, $p = 0.9130$], or injection [$F(1, 20) = 0.01224$, $p = 0.9130$]. There were no significant interactions between session and CVS [$F(1, 20) = 0.1768$, $p = 0.6786$], session and injection [$F(1, 20) = 0.1768$, $p = 0.6786$], or CVS and injection [$F(1, 20) = 0.2479$, $p = 0.6240$]. There was a significant three-way interaction between session, CVS, and injection [$F(1, 20) = 5.029$, $\mathbf{p = 0.0364}$], indicating that the interaction between CVS and injection varied across sessions. To describe the nature of the three-way interaction, the data were consolidated all 3 ways, and two-way ANOVAs were conducted.

First, the data were consolidated to remove injection as a variable. The two-way ANOVA

revealed no significant effects of session [$F(1, 22) = 1.715$, $p = 0.2039$], CVS [$F(1, 22) = 0.01330$, $p = 0.9092$], or the interaction between session and CVS [$F(1, 22) = 0.1543$, $p = 0.6982$].

The data were then consolidated to remove CVS as a variable. The two-way ANOVA revealed no significant effects of session [$F(1, 22) = 1.715$, $p = 0.2039$], injection [$F(1, 22) = 0.01330$, $p = 0.9092$], or the interaction between session and injection [$F(1, 22) = 0.1543$, $p = 0.6982$], identically to the two-way ANOVA of session and CVS.

Lastly, the data were consolidated to remove session as a variable, and a two-way ANOVA was conducted with both CVS and injection as between-subjects variables. There were no significant effects of CVS [$F(1, 20) = 0.01224$, $p = 0.9130$], injection [$F(1, 20) = 0.01224$, $p = 0.9130$], or interaction between CVS and injection [$F(1, 20) = 0.2479$, $p = 0.6240$]. The lack of any significant effects in any of the two-way ANOVAs indicates that there is a high amount of variability in the data, and no conclusions can be made.

Time Spent in Open Arms

For time spent in the open arms, a three-way mixed model ANOVA was run with CVS and injection as between-subjects variables and session as the within-subjects variable (Figure 12B). There was a significant main effect of session [$F(1, 20) = 16.24$, $\mathbf{p = 0.0007}$], but no significant main effects of CVS [$F(1, 20) = 1.792$, $p = 0.1956$] or injection [$F(1, 20) = 0.7333$, $p = 0.4019$]. There were no significant interactions between session and CVS [$F(1, 20) = 0.7330$, $p = 0.4021$], session and injection [$F(1, 20) = 0.0005496$, $p = 0.9815$], CVS and injection [$F(1, 20) = 0.6854$, $p = 0.4175$], or session, CVS, and injection [$F(1, 20) = 0.2847$, $p = 0.5995$]. To

describe the nature of the main effect of session, cell means were used. Rats spent more time in the open arms, on average, in the first session than the second session.

Latency to Open Arms

For latency to enter the open arms, a rat who never entered an open arm during a session was given a value of 300 seconds, the length of the session, in order to prevent missing values. A three-way mixed model ANOVA was run with CVS and injection as between-subjects variables and session as the within-subjects variable (Figure 12C). As with time spent in open arms, there was a significant main effect of session [$F(1, 20) = 8.243$, $\boldsymbol{p = 0.0094}$], but no significant main effects of CVS [$F(1, 20) = 0.008043$, $p = 0.9294$] or injection [$F(1, 20) = 0.3623$, $p = 0.5540$]. There were no significant interactions between session and CVS [$F(1, 20) = 0.07159$, $p = 0.7918$], session and injection [$F(1, 20) = 0.4417$, $p = 0.5139$], CVS and injection [$F(1, 20) = 0.01360$, $p = 0.9083$], or session, CVS, and injection [$F(1, 20) = 1.994$, $p = 0.1733$]. To describe the nature of the main effect of session, cell means were used. Rats had a shorter latency to enter the open arms, on average, in the first session than the second session.

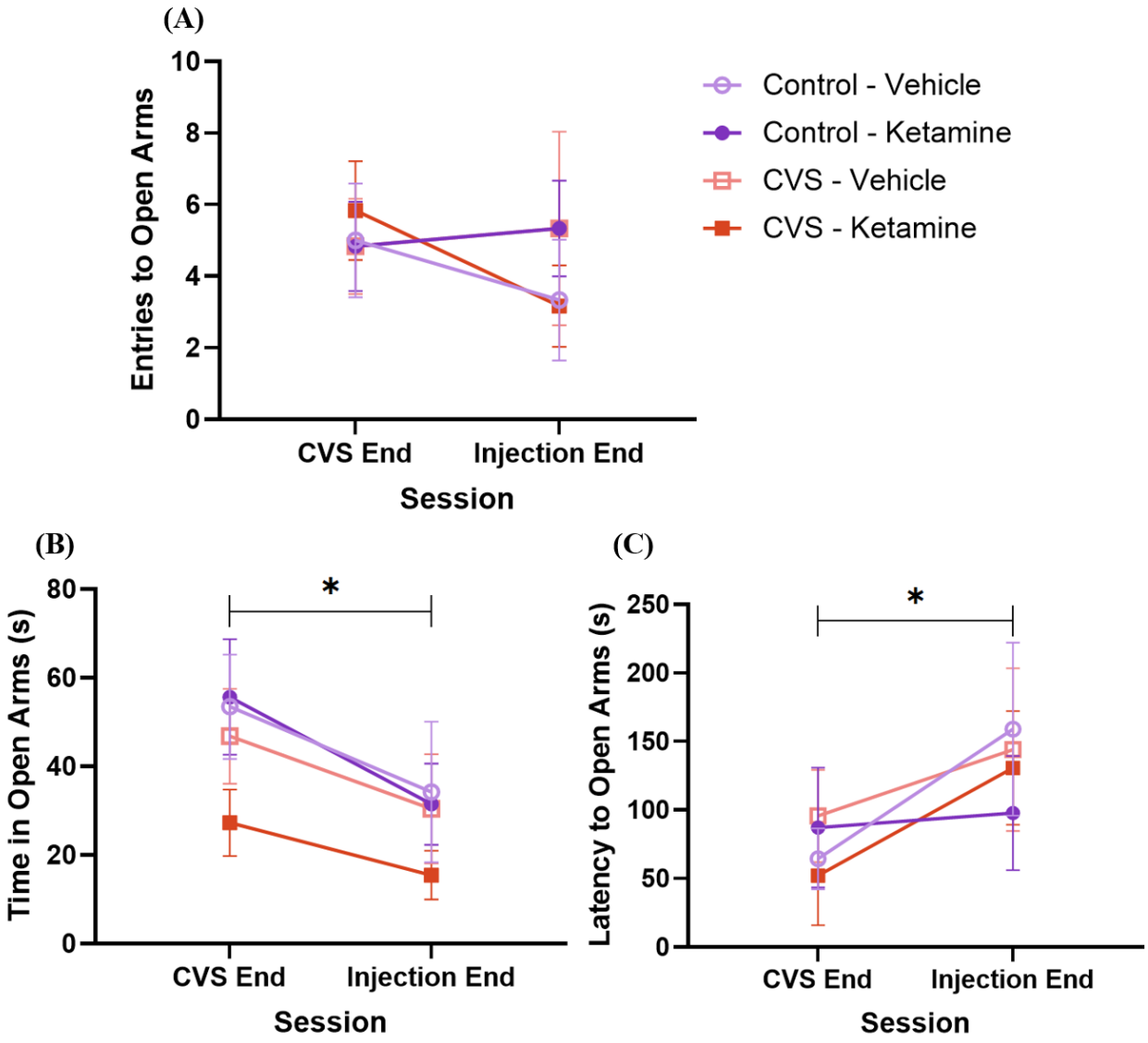

Figure 12. All EZM data across sessions by group. The first EZM was conducted after CVS ended, and the second was conducted after weekly injections ended. Values are reported as mean ± SEM. $n = 6$ per group. **(A)** Total number of entries into the open arms of the EZM across sessions by group. There was a significant three-way interaction between session, CVS, and injection, but there were no significant pairwise comparisons. **(B)** Time spent in the open arms of the EZM across sessions by group. Rats spent significantly less time in the open arms in the second session (* $p < 0.05$ CVS End session compared to Injection End session). **(C)** Latency to enter the open arms of the EZM across sessions by group. Rats had a significantly longer latency to enter the open arms in the second session (* $p < 0.05$ CVS End session compared to Injection End session).

*Sucrose Preference Test (**Figure 13**)*

The first session of the SPT was discarded because it was run under different conditions. Since rats had access to both bottles for longer, there was a ceiling effect where most cages consumed all of their sucrose solution, therefore differences could not be gauged. Each data point analyzed was obtained from a cage of three animals, yielding an n of 2 per group. For total sucrose consumed, a three-way mixed model ANOVA was run with CVS and injection as between-subjects variables and session as the within-subjects variable (Figure 13). There were no statistically significant main effects of session [$F(1, 4) = 0.1576$, $p = 0.7116$], CVS [$F(1, 4) = 0.6708$, $p = 0.4588$], or injection [$F(1, 4) = 0.04990$, $p = 0.8342$]. There were also no significant interactions between session and CVS [$F(1, 4) = 2.847$, $p = 0.1668$], session and injection [$F(1, 4) = 0.7980$, $p = 0.4222$], CVS and injection [$F(1, 4) = 5.856$, $p = 0.0728$], or session, CVS, and injection [$F(1, 4) = 0.1576$, $p = 0.7116$].

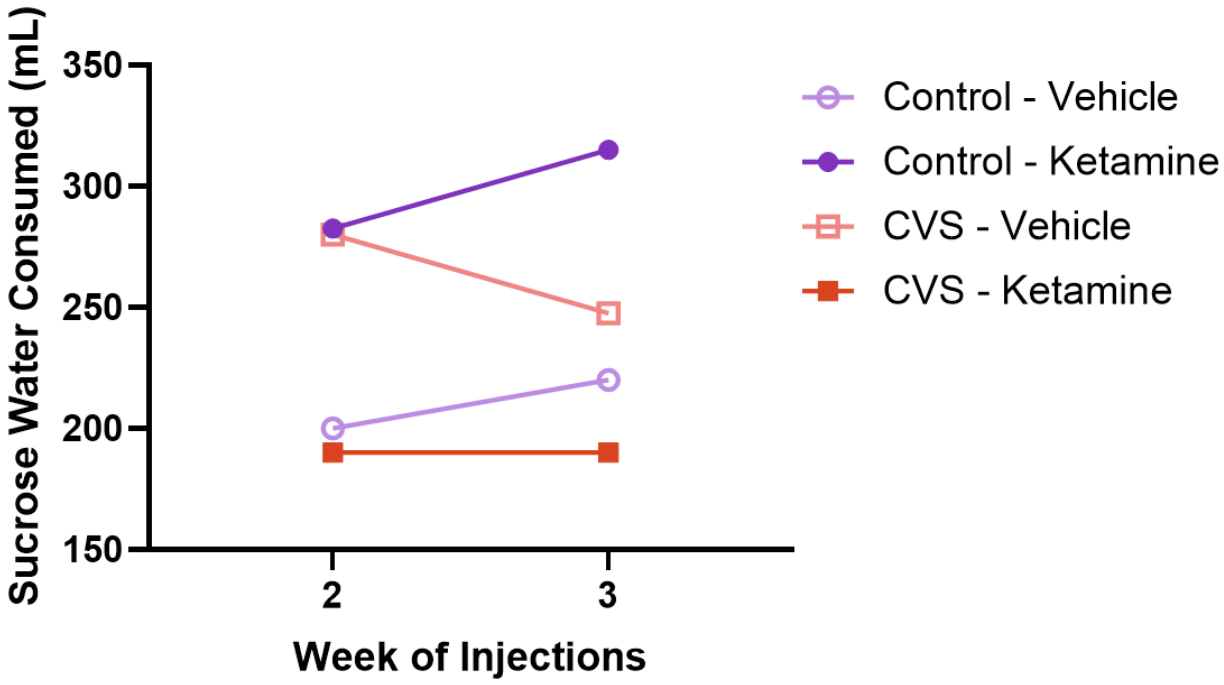

Figure 13. Volume of sucrose water consumed per cage in the SPT across session by group. The 2 SPT sessions analyzed occurred during the second and third weeks of injections. Values were reported as mean, and $n = 2$ per group. No significant main effects or interactions were found.

*CORT Assay (**Figure 14**)*

Plasma corticosterone samples were tested in triplicate. Absorbance values that fell outside of the range of the standard curve were excluded. For average corticosterone concentration, a mixed effects analysis was run with CVS and injection as between-subjects variables and stage as the within-subjects variable (Figure 14). There were no statistically significant main effects of stage [$F(1, 17) = 1.025$, $p = 0.3255$], CVS [$F(1, 19) = 0.03144$, $p = 0.8611$], or injection [$F(1, 19) = 3.301$, $p = 0.0851$]. There was a significant interaction between stage and CVS [$F(1, 17) = 5.069$, $\mathbf{p = 0.0379}$]. There were no significant interactions between stage and injection [$F(1, 17) = 0.1529$, $p = 0.7006$], CVS and injection [$F(1, 19) = 1.984$, $p = 0.1751$], or stage, CVS, and injection [$F(1, 17) = 0.2327$, $p = 0.6357$].

Given the lack of main effect or interaction including injection, the data were consolidated to remove injection as a variable. In other words, the Control – Vehicle and Control – Ketamine groups were combined, and the CVS – Vehicle and CVS – Ketamine groups were combined. A two-way mixed model ANOVA was then run with CVS as the between-subjects variable and stage as the within-subjects variable. Results again revealed a statistically significant interaction between stage and CVS [$F(1, 19) = 6.015$, $\boldsymbol{p = 0.0240}$].

For *post-hoc* testing, the Bonferroni adjustment of $\alpha = 0.05/2 = 0.025$ was used to compare experimental groups across stages. There were no statistically significant differences between the Control group and CVS group at either stage. To compare stages across experimental groups, the Bonferroni adjustment of $\alpha = 0.05/2 = 0.025$ was used. For the CVS group, there was a significant difference between the CVS End stage and the Injection End stage (adjusted $\boldsymbol{p = 0.0269}$). Specifically, rats in the CVS group had a higher corticosterone concentration, on average, in the second test than the first one. There was no difference between the two stages for the Control group.

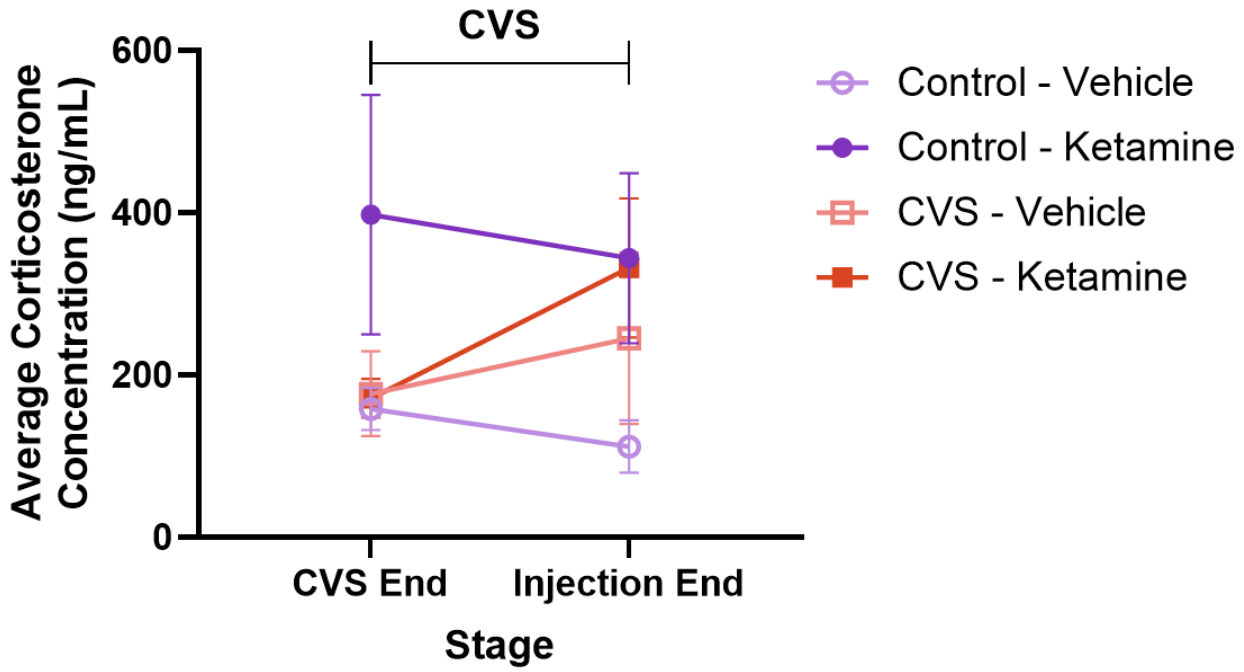

Figure 14. Average plasma corticosterone concentration across time by group. The first blood sample was taken after CVS ended, and the second was conducted after weekly injections ended. Values are reported as mean ± SEM. $n = 6$ per group. Rats in the CVS group had significantly higher corticosterone levels after weekly injections than after CVS (CVS $p < 0.05$ CVS End session compared to Injection End session).

CHAPTER IV: DISCUSSION

Overview

The purpose of this research was to test the hypothesis that ketamine is able to reverse the behavioral effects caused by chronic variable stress (CVS). This was done by manipulating exposure to CVS (with two levels: control and CVS) and injection type (with two levels: vehicle and ketamine). Two weeks after arrival, rats in the CVS groups were exposed to three weeks of CVS. The stressors included immobilization, predator odor, cage tilt, strobe lights, ultrasonic deterrent, wet bedding, cold swim, and overnight isolation. After the CVS protocol was completed, all rats underwent repeated behavioral tests, including the forced swim test (FST), open field test (OFT), elevated zero maze (EZM), and sucrose preference test (SPT), as well as blood draws to determine corticosterone levels.

Findings

For weekly body weights, rats in both CVS groups weighed less than the rats in the Control groups at the end of CVS and at the end of injections. This suggests that the CVS protocol was stressful enough to have a short-term effect on the growth of the rats that were exposed to it. Previous studies have also shown that CVS results in slower weight gain (reviewed in Willner 2017). The lack of differences between groups by the end of the experiment suggests that the rats were able to recover after having been exposed to the stressors. This CVS protocol only had temporary effects on weight. It should be noted that behavioral testing was done within the time frame where there was still a significant difference in weight between CVS and Control groups. The effects of CVS on body weight were transient, however, as the groups no longer

differed in weight six weeks after cessation of CVS.

During the FST, there was a significant effect of session on latency to immobility for every group except the CVS – Vehicle group. Rats in the Control – Ketamine group had a lower latency to immobility for every session that they received ketamine compared to the first session, where they did not receive ketamine. Rats in the Control – Vehicle and CVS – Ketamine groups had a lower latency to immobility in session 4 compared to session 1. Traditionally, a low latency to immobility in the FST is considered depression-like behavior (Porsolt et al. 1978). If that were to be applied to this study, that would suggest that rats that received CVS and saline injections were the only ones to not show depression-like behaviors. An alternative explanation is that repeated exposure to the FST resulted in the acquisition of an adaptive behavior that rats in the CVS – Vehicle group could not learn. These results, in conjunction with previous studies, suggest that immobility in the FST may have been a coping behavior as opposed to a depression-like behavior (Nishimura et al. 1988; Taghzouti et al. 1999). Independent of its antidepressant effects, ketamine has been shown to improve memory consolidation in rats (Parise et al. 2013; Zhou et al. 2025), which could explain why rats in the CVS – Ketamine group were able to learn the passive coping strategy of immobility. This may also explain why rats in the Control – Ketamine group acquired a low latency to immobility by the second session, while rats in the Control – Vehicle group acquired it in the fourth session. While there was a significant interaction between CVS and session for total time immobile, there were no significant differences across groups or sessions in the pairwise comparisons. Although ketamine at higher doses may affect locomotor functioning, the lack of an effect of ketamine on the total time spent immobile during the FST suggests that this was not the case.

Altogether, the body weight and FST data suggest that the CVS protocol, while stressful, was unable to produce a model of depression. This result disagrees with many other studies that suggest that CVS can be used as a depression model (Willner 2017; Wang et al. 2017; Zhang et al. 2014; Li et al. 2011; Zhu et al. 2017). The most likely explanation for this is that different CVS protocols use different stressors given in different schedules, which makes behavior after CVS variable and hard to compare to other studies (Becker et al. 2021). It is possible that the stressors used in this study were not stressful enough, or they may not have been given frequently enough. This may also explain why weight differences between CVS and Control groups were temporary. In other studies, the duration of the CVS protocol ranges from three days to ten weeks, with most lasting between three to six weeks (Strekalova et al. 2022). Animals are typically exposed to three stressors a day, allowing for less recovery time between stressors. These stressors include constant lighting, isolation, cage shaking, predator odor, and more. The study that was used as inspiration for this study's CVS protocol (Slater 2024) found no differences between CVS and Control groups, save for a transient difference in weight. This may have been due to delayed behavioral testing after the cessation of CVS, allowing the animals to recover from the CVS before any data were collected.

During the OFT, time significantly decreased total distance traveled and distance traveled in the center zone. These findings suggest that the novelty of the OFT to the rats played a role in their exploratory behavior. Repeated testing in the OFT has been shown to reduce center zone activity and locomotor activity as a whole (Chen et al. 2023). This indicates that the OFT may only yield valid results the first time, and that repetitions of the OFT do not provide a reliable measure of anxiety-like behavior. There was a significant interaction between CVS and time on

distance traveled in the middle zone and time spent in each zone. Rats that received CVS traveled a shorter distance in the middle zone and spent more time in the outer zone in the second session compared to the first. The amount of time that rats in the CVS groups spent in the middle and center zones was significantly below the Control groups in the second session, but not the first. Given that time was a fixed value (600 seconds), it makes sense that an increased amount of time spent in the outer zone would be paired with decreased time spent in the middle and center zones. Distance traveled, however, is variable from animal to animal. Given that rats in the CVS groups spent more time in the outer zone in the second session, but traveled the same distance, this indicates that they spent more time immobile during the OFT than rats in the Control groups did in the second session. Spending more time in the outer zone, also known as thigmotaxis, is an anxiety-like behavior (Simon et al. 1994). The effect of habituation to the apparatus may have been pronounced in rats that received CVS. Alternatively, CVS may have had a delayed effect on behavior, where thigmotaxis was only observed three weeks after the CVS protocol ended.

During the EZM, there was a significant main effect of time, where the latency of the rats to enter the open arms increased and the amount of time they spent in the open arms decreased in the second session. There were three-way interactions between CVS, ketamine, and time in the number of entries to the open arms, but the lack of significant pairwise comparisons implies that the three-way interaction was not actually relevant. Decreased exploration over time, regardless of group, suggests that there was an effect of novelty in a rat's behavior during the EZM, similar to the OFT. This effect has been shown in a mouse study using the EZM (Tucker and McCabe 2017). As with the OFT, these results suggest that tests that measure exploratory behavior may only be used once to prevent the confound of habituation. Furthermore, while the OFT and EZM

were both used to measure anxiety-like behavior, the results of each test do not match. The results were similar in many ways, such as all rats (except for those in the Control – Vehicle group) spending more time in the safe zone in the second test. However, the tests differed in their results for the CVS groups. For example, while rats in the CVS groups had a significant decrease in the amount of time they spent in the middle and center zones over time, as well as being significantly different from the Control groups in the second session, this difference between groups was not observed in the EZM. This may be because the EZM was only conducted for five minutes per animal, while the OFT was conducted for ten minutes per animal.

The testing conditions of the SPT resulted in a low *n* that made it difficult to draw any conclusions about the effects of CVS, ketamine, or time on sucrose consumption. However, some qualitative observations will be made. Most notably, sucrose consumption appears to increase in the Control groups in the second session, while it remained stable or decreased in the CVS groups. This suggests a delayed effect of CVS, as mentioned above in the OFT. It also suggests an inability of ketamine to restore sucrose consumption in stressed rats.

There was an increase in corticosterone levels in the CVS groups between the end of CVS and the end of injections. While there were no differences between CVS and Control groups at either stage, rats in the CVS groups had a significant rise in their plasma corticosterone levels over time. These results were contrary to what was expected, as corticosterone was expected to be at its highest at the end of CVS and decrease in the recovery stage. It is worth noting that samples from several animals in the CVS groups had to be excluded because they were above the range of sensitivity for the standard curve, so the means calculated from the remaining values may be inappropriately low. Other studies have found CVS to produce higher

corticosterone levels (Wang et al. 2017; Johnston et al. 2021; McEwen et al. 2016). As with the OFT, this result suggests a delayed effect of CVS. The injection phase – along with re-exposure to the blood collection room – may have served as another stressor that the rats in the CVS groups were more sensitive to, causing their corticosterone levels to rise. This is likely what occurred, given that exposure to prolonged stress causes a dysregulation of the negative feedback loop, leading to the ineffective shutdown of cortisol/corticosterone secretion (Menke 2024). Memories can serve as a trigger of the HPA axis, since the hippocampus has an input to the PVN of the hypothalamus (Menke 2024; Trifu et al. 2020). Furthermore, the weekly ketamine injections were unable to prevent the increase in corticosterone. Another study on mice has shown that ketamine can decrease the corticosterone response to stressors in males, but not females (Johnston et al. 2021). This further accentuates the need for more studies on female animals to determine the best method of lowering corticosterone levels after CVS.

Effectiveness of CVS Protocol

In other studies, rats that have been exposed to CVS display decreased sucrose consumption in the SPT, less time spent in the open arms of the EZM, and increased time spent immobile during the FST (Becker et al. 2021). None of these behaviors were observed in this study (a trend was observed in the SPT, but this was not statistically analyzable due to the low *n*). CVS stressors that are not stressful enough have been shown to increase anxiety-like behavior and activity during behavioral tests (Strekalova et al. 2022). This can impact the interpretation of results. For example, adequate stressors during CVS can lead to depression-like behavior (e.g. decreased latency to immobility in the FST), while inadequate stressors may lead to the opposite

behavioral effect.

There are a few reasons why exposure to CVS could have resulted in a higher latency to immobility in the FST. One possible reason is that the CVS protocol administered in this study was unable to create a model of depression in the rats. Another possible reason is that CVS did create a depression model, but the FSTs were not truly measuring depression-like behavior. Previous studies have suggested that behavior during the FST is actually a measure of memory formation and recall (Molendijk and De Kloet 2022). The CVS protocol in this study may have impacted the abilities of the rats in the CVS – Vehicle group to remember the FSTs administered in previous weeks, resulting in them spending more time actively trying to escape before eventually becoming immobile. Lastly, since the stressors in the CVS protocol were relatively mild by design, the CVS protocol could have made the rats more resilient to stressors, giving them a defense against acquiring learned helplessness. For example, mild stressors have been shown to decrease immobility in the FST in some cases, but increase immobility in others (Bogdanova et al. 2013). This ties back to the variability of CVS protocols, where stressors that are too mild can actually have the reverse effect than what was intended.

Exposure to CVS raises corticosterone levels in rats and mice (McEwen et al. 2016; Johnston et al. 2021), which was observed in this study. This indicates that CVS caused a dysregulation of the HPA axis in the stressed animals. However, due to the inability to perform histological studies on the brains of the rats, no definitive statements can be made about potential changes to their PFCs, amygdalae, or hippocampi. If it is to be believed that rats in the CVS – Vehicle group had trouble remembering previous encounters with the FST, this may indicate hippocampal atrophy in this group, although female rats should have been protected from this

effect of CVS due to their estrogen levels (McEwen et al. 2016). Chronic activation of the HPA axis may have lowered the rats' BDNF levels, leading to the atrophy. The impaired memory function may also indicate changes in the glutamatergic system in the PFC, specifically reduced expression of NMDA and AMPA receptors leading to less synaptic transmission. Furthermore, if the trend observed in the SPT was significant, this would indicate anhedonia in both CVS groups. CVS may have increased BDNF levels in the amygdala, promoting neurogenesis and flattening the response to positive stimuli (McEwen et al. 2016; Trifu et al. 2020).

Validity of Behavioral Tests

Given the inconsistencies between results, it is important to discuss the validity of the behavioral tests. Most notably, the FST has faced heavy criticism for not truly measuring what it claims to measure. Many studies in the past have suggested that the FST measures a rodent's ability to form memories in stressful situations, and not learned helplessness or despair (Nishimura et al. 1988; Taghzouti et al. 1999; Molendijk and De Kloet 2022). Furthermore, many studies do not collect data on the first day of the FST, which prevents within-group comparisons on the effect of repeated FST exposure. In order to analyze the results of the FST in as comprehensive of a way as possible, it is important to record behavior in all sessions.

In this study, analysis of the OFT and EZM yielded different results, although they were both meant to observe anxiety-like behavior. This may reflect a need to alter the EZM protocol, including the duration of the sessions and the parameters recorded. For example, measuring the latency to the open arms may yield more easily comparable results than measuring the number of individual entries to the open arms.

Limitations

One limitation of this research was the small sample size ($N = 24$; $n = 6$ per group). Given the constraint of time and available resources, this sample size could not have been made larger. For example, when administering behavioral tests, it is important to test all subjects during the same phase of their circadian rhythm in order to prevent any effects of sleepiness/wakefulness or hormone fluctuations. With the FST and OFT both taking ten minutes per animal, testing all subjects would take about four hours, not including the time needed to transfer the rats in and out of their cages, as well as keep the apparatus clean. This is the main reason why most behavioral tests were divided between two days.

Although the intention was to use Long-Evans rats for this study, Sprague Dawleys were purchased by mistake. Long-Evans rats, specifically, are often used in behavioral studies due to their increased responsiveness to the environment, larger variety of possible behaviors, and increased responsiveness to stress compared to Sprague-Dawley rats (Turner and Burne 2014). It is possible that, had Long-Evans rats been used, the CVS protocol would have been adequate to produce the model of depression. Furthermore, the lack of male rats in this study prevents the ability of direct comparison to studies that use males.

Furthermore, animal researchers are encouraged to follow the "three R's" outlined by Russell and Burch (1959). This guideline ensures that experimenters, to the extent their study will allow, have *refined* their protocol to cause the animal subjects the minimum amount of harm, *reduced* the number of animals they use, and *replaced* the species with the least sentient lifeform possible, or even a non-living model.

Due to the small sample size, potential significant differences may have been missed

during statistical analysis. For example, for the SPT, the group size was only $n = 2$ due to the inability to measure each individual rat's consumption of sucrose solution. This incredibly small sample size made it effectively impossible to find any significant differences between groups or draw any significant conclusions. Although a larger sample size in the initial experiments could increase confidence in the results and prevent repeat studies that would use more animals in the long run (Button et al. 2013), typical behavioral studies use 6-12 animals per group (Johnston et al. 2021; Zhu et al. 2017; Porsolt et al. 1978), making a sample size of 6 not unusual.

Future Directions

Future studies can be done to find which mild stressors are best for inducing depression-like behavior in the CVS model. Once the most effective CVS protocol has been found, further experiments on CVS schedules can be done to find the length and frequency that it must be administered to produce depression-like behaviors. Furthermore, memory impairments after CVS can be tested using the Morris Water Maze or Novel Object Recognition Test to determine if CVS leads to similar cognitive deficits as observed in humans with MDD, and if ketamine restores memory function after CVS. Given the complexity of the systems involved in MDD, coupled with the fact that it is an affective disorder that involves complex cognitive processes that may not be adequately modeled in rodents, further studies need to be done to determine the differences in ketamine's neurological effects between humans and rodents, especially within the HPA axis.

Conclusion

To conclude, the hypothesis that exposure to CVS would result in a model of depression was not supported. Rather, this study established that CVS, while stressful, did not lead to any depression-like behaviors in the FST. There is a need to establish which stressors are most effective at producing behavioral changes by the end of the CVS protocol. Because a model of depression was not created, the effects of ketamine could not be easily determined. This study is unique because most studies on depression models in rodents focus on males, while this study focused on females.

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REFERENCES

Ahmed GK, Elserogy YM, Elfadl GMA, Ghada Abdelsalam K, Ali MA. 2023. Antidepressant and anti-suicidal effects of ketamine in treatment-resistant depression associated with psychiatric and personality comorbidities: A double-blind randomized trial. Journal of Affective Disorders. 325:127–134. doi:10.1016/j.jad.2023.01.005. https://linkinghub.elsevier.com/retrieve/pii/S0165032723000149.

Albert PR, Benkelfat C, Descarries L. 2012. The neurobiology of depression—revisiting the serotonin hypothesis. I. Cellular and molecular mechanisms. Phil Trans R Soc B. 367(1601):2378–2381. doi:10.1098/rstb.2012.0190. https://royalsocietypublishing.org/doi/10.1098/rstb.2012.0190.

Alnefeesi Y, Chen-Li D, Krane E, Jawad MY, Rodrigues NB, Ceban F, Di Vincenzo JD, Meshkat S, Ho RCM, Gill H, et al. 2022. Real-world effectiveness of ketamine in treatment-resistant depression: A systematic review & meta-analysis. Journal of Psychiatric Research. 151:693–709. doi:10.1016/j.jpsychires.2022.04.037. https://linkinghub.elsevier.com/retrieve/pii/S0022395622002400.

American Psychiatric Association, American Psychiatric Association, editors. 2013. Diagnostic and statistical manual of mental disorders: DSM-5. 5th ed. Washington, D.C: American Psychiatric Association.

Bale TL. 2019. Sex matters. Neuropsychopharmacol. 44(1):1–3. doi:10.1038/s41386-018-0239-x. https://www.nature.com/articles/s41386-018-0239-x.

Bao A-M, Swaab DF. 2019. The human hypothalamus in mood disorders: The HPA axis in the center. IBRO Reports. 6:45–53. doi:10.1016/j.ibror.2018.11.008.

https://linkinghub.elsevier.com/retrieve/pii/S2451830117300432.

Bartoli F, Riboldi I, Crocamo C, Di Brita C, Clerici M, Carrà G. 2017. Ketamine as a rapid-acting agent for suicidal ideation: A meta-analysis. Neuroscience & Biobehavioral Reviews. 77:232–236. doi:10.1016/j.neubiorev.2017.03.010. https://linkinghub.elsevier.com/retrieve/pii/S0149763416308430.

Becker JB, Prendergast BJ, Liang JW. 2016. Female rats are not more variable than male rats: a meta-analysis of neuroscience studies. Biol Sex Differ. 7(1):34. doi:10.1186/s13293-016-0087-5. https://bsd.biomedcentral.com/articles/10.1186/s13293-016-0087-5.

Becker M, Pinhasov A, Ornoy A. 2021. Animal models of depression: What can they teach us about the human disease? Diagnostics. 11(1):123. doi:10.3390/diagnostics11010123. https://www.mdpi.com/2075-4418/11/1/123.

Beery AK. 2018. Inclusion of females does not increase variability in rodent research studies. Current Opinion in Behavioral Sciences. 23:143–149. doi:10.1016/j.cobeha.2018.06.016. https://linkinghub.elsevier.com/retrieve/pii/S235215461730205X.

Beery AK, Zucker I. 2011. Sex bias in neuroscience and biomedical research. Neuroscience & Biobehavioral Reviews. 35(3):565–572. doi:10.1016/j.neubiorev.2010.07.002. https://linkinghub.elsevier.com/retrieve/pii/S0149763410001156.

Bogdanova OV, Kanekar S, D'Anci KE, Renshaw PF. 2013. Factors influencing behavior in the forced swim test. Physiology & Behavior. 118:227–239. doi:10.1016/j.physbeh.2013.05.012. https://linkinghub.elsevier.com/retrieve/pii/S0031938413001534.

Borsini F, Volterra G, Meli A. 1986. Does the behavioral "despair" test measure "despair"?

Physiology & Behavior. 38(3):385–386. doi:10.1016/0031-9384(86)90110-1. https://linkinghub.elsevier.com/retrieve/pii/0031938486901101.

Buchanan TW, Lovallo WR. 2001. Enhanced memory for emotional material following stress-level cortisol treatment in humans. Psychoneuroendocrinology. 26(3):307–317. doi:10.1016/S0306-4530(00)00058-5. https://linkinghub.elsevier.com/retrieve/pii/S0306453000000585.

Button KS, Ioannidis JPA, Mokrysz C, Nosek BA, Flint J, Robinson ESJ, Munafò MR. 2013. Power failure: why small sample size undermines the reliability of neuroscience. Nat Rev Neurosci. 14(5):365–376. doi:10.1038/nrn3475. https://www.nature.com/articles/nrn3475.

Centers for Disease Control and Prevention. 2021. Web-based injury statistics query and reporting system (WISQARS). National Centers for Injury Prevention and Control, Centers for Disease Control and Prevention. https://www.cdc.gov/injury/wisqars/index.html.

Chen W, Wang Z, Ma C, Ma X, Meng W, Yin F, Yang Y. 2023. Tactile cues are important to environmental novelty during repeated open field tests. Behavioural Processes. 204:104796. doi:10.1016/j.beproc.2022.104796. https://linkinghub.elsevier.com/retrieve/pii/S0376635722002133.

Coppen A. 1967. The biochemistry of affective disorders. Br J Psychiatry. 113(504):1237–1264. doi:10.1192/bjp.113.504.1237. https://www.cambridge.org/core/product/identifier/S0007125000085925/type/journal_article.

Duman RS, Li N, Liu R-J, Duric V, Aghajanian G. 2012. Signaling pathways underlying the rapid antidepressant actions of ketamine. Neuropharmacology. 62(1):35–41. doi:10.1016/j.neuropharm.2011.08.044. https://linkinghub.elsevier.com/retrieve/pii/S0028390811003777.

DuMont M, Agostinis A, Singh K, Swan E, Buttle Y, Tropea D. 2023. Sex representation in neurodegenerative and psychiatric disorders' preclinical and clinical studies. Neurobiology of Disease. 184:106214. doi:10.1016/j.nbd.2023.106214. https://linkinghub.elsevier.com/retrieve/pii/S0969996123002292.

Ellenbroek B, Youn J. 2016. Rodent models in neuroscience research: is it a rat race? Dis Model Mech. 9(10):1079–1087. doi:10.1242/dmm.026120. https://doi.org/10.1242/dmm.026120.

Georgiou P, Farmer CA, Medeiros GC, Yuan P, Johnston J, Kadriu B, Gould TD, Zarate CA. 2025. Associations between hypothalamic-pituitary-adrenal (HPA) axis hormone levels, major depression features and antidepressant effects of ketamine. Journal of Affective Disorders. 373:126–132. doi:10.1016/j.jad.2024.12.036. https://linkinghub.elsevier.com/retrieve/pii/S0165032724020226.

Goodman RJ, Samek DR, Wilson S, Iacono WG, McGue M. 2019. Close relationships and depression: A developmental cascade approach. Dev Psychopathol. 31(04):1451–1465. doi:10.1017/S0954579418001037. https://www.cambridge.org/core/product/identifier/S0954579418001037/type/journal_article.

Green AR. 2006. Neuropharmacology of 5-hydroxytryptamine. British J Pharmacology. 147(S1).

doi:10.1038/sj.bjp.0706427. https://bpspubs.onlinelibrary.wiley.com/doi/10.1038/sj.bjp.0706427.

Greenberg P, Chitnis A, Louie D, Suthoff E, Chen S-Y, Maitland J, Gagnon-Sanschagrin P, Fournier A-A, Kessler RC. 2023. The economic burden of adults with major depressive disorder in the United States (2019). Adv Ther. 40(10):4460–4479. doi:10.1007/s12325-023-02622-x. https://link.springer.com/10.1007/s12325-023-02622-x.

Guan N, Guariglia A, Moore P, Xu F, Al-Janabi H. 2022. Financial stress and depression in adults: A systematic review. Magalhaes PVDS, editor. PLoS ONE. 17(2):e0264041. doi:10.1371/journal.pone.0264041. https://dx.plos.org/10.1371/journal.pone.0264041.

Hillhouse TM, Porter JH. 2015. A brief history of the development of antidepressant drugs: From monoamines to glutamate. Experimental and Clinical Psychopharmacology. 23(1):1–21. doi:10.1037/a0038550. https://doi.apa.org/doi/10.1037/a0038550.

Johnston CJ, Fitzgerald PJ, Gewarges JS, Watson BO, Spencer-Segal JL. 2021. Ketamine decreases HPA axis reactivity to a novel stressor in male but not female mice. doi:10.1101/2021.06.29.450387. http://biorxiv.org/lookup/doi/10.1101/2021.06.29.450387.

Johnston JN, Greenwald MS, Henter ID, Kraus C, Mkrtchian A, Clark NG, Park LT, Gold P, Zarate CA, Kadriu B. 2023. Inflammation, stress and depression: An exploration of ketamine's therapeutic profile. Drug Discov Today. 28(4):103518. doi:10.1016/j.drudis.2023.103518. https://doi.org/10.1016/j.drudis.2023.103518.

Kang MJY, Hawken E, Vazquez GH. 2022. The mechanisms behind rapid antidepressant effects of ketamine: A systematic review with a focus on molecular neuroplasticity. Front

Psychiatry. 13:860882. doi:10.3389/fpsyt.2022.860882. https://www.frontiersin.org/articles/10.3389/fpsyt.2022.860882/full.

Katz RJ. 1982. Animal model of depression: Pharmacological sensitivity of a hedonic deficit. Pharmacology Biochemistry and Behavior. 16(6):965–968. doi:10.1016/0091-3057(82)90053-3. https://linkinghub.elsevier.com/retrieve/pii/0091305782900533.

Khalili-Mahani N, Martini CH, Olofsen E, Dahan A, Niesters M. 2015. Effect of subanaesthetic ketamine on plasma and saliva cortisol secretion. British Journal of Anaesthesia. 115(1):68–75. doi:10.1093/bja/aev135. https://linkinghub.elsevier.com/retrieve/pii/S0007091217312424.

Kulkarni, S.K., Singh, K., Bishnoi, M. 2007. Elevated zero-maze: A paradigm to evaluate anti-anxiety effects of drugs. Methods and Findings in Experimental and Clinical Pharmacology. 29(5):343. doi:10.1358/mf.2007.29.5.1117557. http://access.portico.org/stable?au=pjbf78vzp41.

Kupferberg A, Hasler G. 2023. The social cost of depression: Investigating the impact of impaired social emotion regulation, social cognition, and interpersonal behavior on social functioning. Journal of Affective Disorders Reports. 14:100631. doi:10.1016/j.jadr.2023.100631. https://linkinghub.elsevier.com/retrieve/pii/S2666915323001695.

Kverno KS, Mangano E. 2021. Treatment-resistant depression: Approaches to treatment. J Psychosoc Nurs Ment Health Serv. 59(9):7–11. doi:10.3928/02793695-20210816-01. https://journals.healio.com/doi/10.3928/02793695-20210816-01.

Li N, Liu R-J, Dwyer JM, Banasr M, Lee B, Son H, Li X-Y, Aghajanian G, Duman RS. 2011.

Glutamate N-methyl-D-aspartate receptor antagonists rapidly reverse behavioral and synaptic deficits caused by chronic stress exposure. Biological Psychiatry. 69(8):754–761. doi:10.1016/j.biopsych.2010.12.015. https://linkinghub.elsevier.com/retrieve/pii/S0006322310013065.

McEwen BS, Nasca C, Gray JD. 2016. Stress effects on neuronal structure: Hippocampus, amygdala, and prefrontal cortex. Neuropsychopharmacol. 41(1):3–23. doi:10.1038/npp.2015.171. https://www.nature.com/articles/npp2015171.

McIntyre RS, Rosenblat JD, Nemeroff CB, Sanacora G, Murrough JW, Berk M, Brietzke E, Dodd S, Gorwood P, Ho R, et al. 2021. Synthesizing the evidence for ketamine and esketamine in treatment-resistant depression: An international expert opinion on the available evidence and implementation. AJP. 178(5):383–399. doi:10.1176/appi.ajp.2020.20081251. https://psychiatryonline.org/doi/10.1176/appi.ajp.2020.20081251.

Menke A. 2024. The HPA axis as target for depression. CN. 22(5):904–915. doi:10.2174/1570159X21666230811141557. https://www.eurekaselect.com/219684/article.

Mikulska J, Juszczyk G, Gawrońska-Grzywacz M, Herbet M. 2021. HPA axis in the pathomechanism of depression and schizophrenia: New therapeutic strategies based on its participation. Brain Sciences. 11(10):1298. doi:10.3390/brainsci11101298. https://www.mdpi.com/2076-3425/11/10/1298.

Mohammadi S, Seyedmirzaei H, Salehi MA, Jahanshahi A, Zakavi SS, Dehghani Firouzabadi F, Yousem DM. 2023. Brain-based sex differences in depression: A systematic review of

neuroimaging studies. Brain Imaging and Behavior. 17(5):541–569. doi:10.1007/s11682-023-00772-8. https://link.springer.com/10.1007/s11682-023-00772-8.

Molendijk ML, De Kloet ER. 2015. Immobility in the forced swim test is adaptive and does not reflect depression. Psychoneuroendocrinology. 62:389–391. doi:10.1016/j.psyneuen.2015.08.028. https://linkinghub.elsevier.com/retrieve/pii/S0306453015009014.

Molendijk ML, De Kloet ER. 2022. Forced swim stressor: Trends in usage and mechanistic consideration. Eur J of Neuroscience. 55(9–10):2813–2831. doi:10.1111/ejn.15139. https://onlinelibrary.wiley.com/doi/10.1111/ejn.15139.

Mouse & rat ultra sonic deterrent. Mice ultrasonic repellent. 12 hours. 2018. YouTube. https://www.youtube.com/watch?v=ZMpX3vtDICY.

Nandam LS, Brazel M, Zhou M, Jhaveri DJ. 2020. Cortisol and major depressive disorder—translating findings from humans to animal models and back. Front Psychiatry. 10:974. doi:10.3389/fpsyt.2019.00974. https://www.frontiersin.org/article/10.3389/fpsyt.2019.00974/full.

National Institute of Mental Health (NIMH). 2023. Major depression. https://www.nimh.nih.gov/health/statistics/major-depression.

Nishimura H, Tsuda A, Oguchi M, Ida Y, Tanaka M. 1988. Is immobility of rats in the forced swim test “behavioral despair?” Physiology & Behavior. 42(1):93–95. doi:10.1016/0031-9384(88)90266-1. https://linkinghub.elsevier.com/retrieve/pii/0031938488902661.

Palazidou E. 2012. The neurobiology of depression. British Medical Bulletin. 101(1):127–145. doi:10.1093/bmb/lds004. https://academic.oup.com/bmb/article-

lookup/doi/10.1093/bmb/lds004.

Parise EM, Alcantara LF, Warren BL, Wright KN, Hadad R, Sial OK, Kroeck KG, Iñiguez SD, Bolaños-Guzmán CA. 2013. Repeated ketamine exposure induces an enduring resilient phenotype in adolescent and adult rats. Biological Psychiatry. 74(10):750–759. doi:10.1016/j.biopsych.2013.04.027. https://linkinghub.elsevier.com/retrieve/pii/S000632231300406X.

Porsolt RD, Anton G, Blavet N, Jalfre M. 1978. Behavioural despair in rats: A new model sensitive to antidepressant treatments. European Journal of Pharmacology. 47(4):379–391. doi:10.1016/0014-2999(78)90118-8. https://linkinghub.elsevier.com/retrieve/pii/0014299978901188.

Primo MJ, Fonseca-Rodrigues D, Almeida A, Teixeira PM, Pinto-Ribeiro F. 2023. Sucrose preference test: A systematic review of protocols for the assessment of anhedonia in rodents. European Neuropsychopharmacology. 77:80–92. doi:10.1016/j.euroneuro.2023.08.496. https://linkinghub.elsevier.com/retrieve/pii/S0924977X23006594.

Russell WMS, Burch RL. 1959. The principles of humane experimental technique. doi:10.5694/j.1326-5377.1960.tb73127.x. https://onlinelibrary.wiley.com/doi/abs/10.5694/j.1326-5377.1960.tb73127.x.

Simon P, Dupuis R, Costentin J. 1994. Thigmotaxis as an index of anxiety in mice. Influence of dopaminergic transmissions. Behavioural Brain Research. 61(1):59–64. doi:10.1016/0166-4328(94)90008-6. https://linkinghub.elsevier.com/retrieve/pii/0166432894900086.

Slater M. 2024. Sex differences for developing anxiety or depression related behaviors after early life exposure to chronic variable stress (CVS) in Long-Evans rats. [Undergraduate Honor Thesis in Neuroscience, Drew University]. Drew University online repository. https://research.ebsco.com/linkprocessor/plink?id=270a3358-4c3e-394b-b17b-cf84702fe2b8.

Stauble MR, Thompson LA, Morgan G. 2013. Increases in cortisol are positively associated with gains in encoding and maintenance working memory performance in young men. Stress. 16(4):402–410. doi:10.3109/10253890.2013.780236. https://www.tandfonline.com/doi/full/10.3109/10253890.2013.780236.

Strekalova T, Liu Y, Kiselev D, Khairuddin S, Chiu JLY, Lam J, Chan Y-S, Pavlov D, Proshin A, Lesch K-P, et al. 2022. Chronic mild stress paradigm as a rat model of depression: facts, artifacts, and future perspectives. Psychopharmacology. 239(3):663–693. doi:10.1007/s00213-021-05982-w. https://link.springer.com/10.1007/s00213-021-05982-w.

Sub Laban T, Saadabadi A. 2025. Monoamine oxidase inhibitors (MAOI). In: StatPearls. Treasure Island (FL): StatPearls Publishing. http://www.ncbi.nlm.nih.gov/books/NBK539848/.

Substance Abuse and Mental Health Services Administration. 2022. National survey on drug use and health (NSDUH). https://www.samhsa.gov/data/report/2022-nsduh-detailed-tables.

Taghzouti K, Lamarque S, Kharouby M, Simon H. 1999. Interindividual differences in active and passive behaviors in the forced-swimming test: Implications for animal models of psychopathology. Biological Psychiatry. 45(6):750–758. doi:10.1016/S0006-

3223(98)00156-5. https://linkinghub.elsevier.com/retrieve/pii/S0006322398001565.

Taraku B, Woods RP, Boucher M, Espinoza R, Jog M, Al-Sharif N, Narr KL, Zavaliangos-Petropulu A. 2023. Changes in white matter microstructure following serial ketamine infusions in treatment resistant depression. Human Brain Mapping. 44(6):2395–2406. doi:10.1002/hbm.26217. https://onlinelibrary.wiley.com/doi/10.1002/hbm.26217.

Thau L, Gandhi J, Sharma S. 2025. Physiology, cortisol. In: StatPearls. Treasure Island (FL): StatPearls Publishing. http://www.ncbi.nlm.nih.gov/books/NBK538239/.

Trifu SC, Trifu AC, Aluaş E, Tătaru MA, Costea RV. 2020. Brain changes in depression. Rom J Morphol Embryol. 61(2):361–370. doi:10.47162/RJME.61.2.06. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7864313/.

Tsigos C, Kyrou I, Kassi E, Chrousos GP. 2020. Stress: Endocrine physiology and pathophysiology. In: Feingold KR, Ahmed SF, Anawalt B, Blackman MR, Boyce A, Chrousos G, Corpas E, de Herder WW, Dhatariya K, Dungan K, et al., editors. Endotext. South Dartmouth (MA): MDText.com, Inc. http://www.ncbi.nlm.nih.gov/books/NBK278995/.

Tucker LB, McCabe JT. 2017. Behavior of male and female C57BL/6J mice is more consistent with repeated trials in the elevated zero maze than in the elevated plus maze. Front Behav Neurosci. 11. doi:10.3389/fnbeh.2017.00013. http://journal.frontiersin.org/article/10.3389/fnbeh.2017.00013/full.

Wang Q, Timberlake MA, Prall K, Dwivedi Y. 2017. The recent progress in animal models of depression. Progress in Neuro-Psychopharmacology and Biological Psychiatry. 77:99–109. doi:10.1016/j.pnpbp.2017.04.008.

https://linkinghub.elsevier.com/retrieve/pii/S0278584617301215.

Wilkinson ST, Ballard ED, Bloch MH, Mathew SJ, Murrough JW, Feder A, Sos P, Wang G, Zarate CA, Sanacora G. 2018. The effect of a single dose of intravenous ketamine on suicidal ideation: A systematic review and individual participant data meta-analysis. AJP. 175(2):150–158. doi:10.1176/appi.ajp.2017.17040472. https://psychiatryonline.org/doi/10.1176/appi.ajp.2017.17040472.

Willner P. 2017. The chronic mild stress (CMS) model of depression: History, evaluation and usage. Neurobiology of Stress. 6:78–93. doi:10.1016/j.ynstr.2016.08.002. https://linkinghub.elsevier.com/retrieve/pii/S2352289516300170.

Zhang L, Luo J, Zhang M, Yao W, Ma X, Yu SY. 2014. Effects of curcumin on chronic, unpredictable, mild, stress-induced depressive-like behaviour and structural plasticity in the lateral amygdala of rats. Int J Neuropsychopharm. 17(05):793–806. doi:10.1017/S1461145713001661. https://academic.oup.com/ijnp/article-lookup/doi/10.1017/S1461145713001661.

Zhou X, Zhang L, Gao W, Li H, Guo Q, Dai J, Gao F, Wang L. 2025. Esketamine alleviates cognitive impairment signs induced by modified electroconvulsive therapy in a depression rat model via the KLF4/p38 MAPK pathway. Journal of Affective Disorders. 376:302–312. doi:10.1016/j.jad.2025.02.012. https://linkinghub.elsevier.com/retrieve/pii/S0165032725002009.

Zhu X, Ye G, Wang Z, Luo J, Hao X. 2017. Sub-anesthetic doses of ketamine exert antidepressant-like effects and upregulate the expression of glutamate transporters in the hippocampus of rats. Neuroscience Letters. 639:132–137.

doi:10.1016/j.neulet.2016.12.070.

https://linkinghub.elsevier.com/retrieve/pii/S0304394016310187.

APPENDIX

Pilot Study

Forced Swim Test (**Figures 15-17**)

To determine whether or not differences in behavior exist between sexes in the FST, total time immobile was analyzed between sexes and across sessions. A two-way mixed model ANOVA was run with sex as the between-subjects variable and session as the within-subjects variable (Figure 15). There was a statistically significant main effect of session on total time spent immobile [$F(2.995, 29.95) = 3.811$, $\mathbf{p = 0.0200}$]. There was no significant main effect of sex [$F(1, 10) = 3.412$, $p = 0.0945$]. There was also no significant interaction between session and sex [$F(5, 50) = 0.5682$, $p = 0.7239$].

A multiple comparisons test using the Tukey adjustment revealed no significant differences between individual sessions. Given the lack of main effect or interaction including sex, the data were consolidated to remove sex as a variable. A one-way repeated measures ANOVA was then run with session as the within-subjects variable. Results again revealed a significant effect of session [$F(3.143, 34.57) = 3.966$, $\mathbf{p = 0.0145}$]. To describe the nature of the main effect, a Tukey HSD test was used. The only sessions that were significantly different were sessions 1 and 3 (adjusted $\mathbf{p = 0.0310}$) and sessions 2 and 3 (adjusted $\mathbf{p = 0.0062}$). Specifically, rats spent less time immobile, on average, during sessions 1 and 2 than session 3.

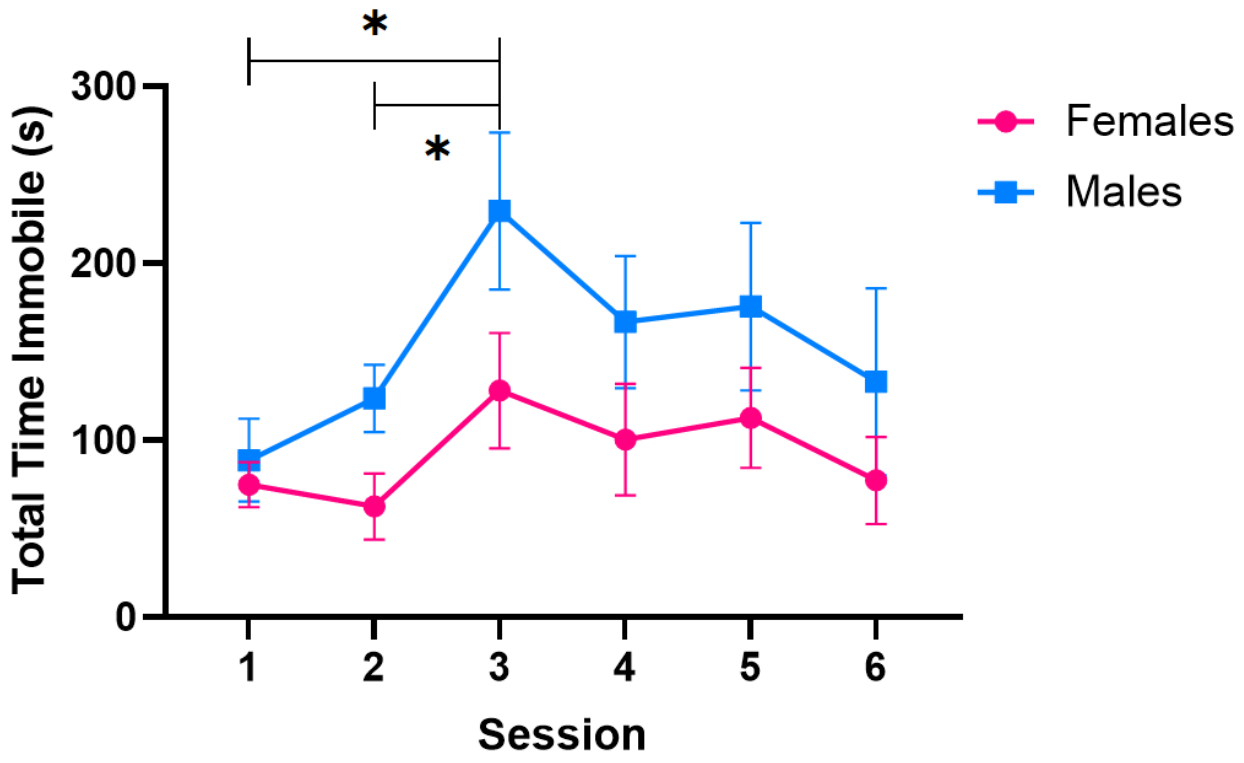

Figure 15. Total time immobile in the FST across sessions by sex during pilot study. The FST was conducted twice, 24 hours apart, then once a week afterwards. Values are reported as mean ± SEM. $n = 6$ per group. Rats were significantly more immobile during session 3 than the previous sessions (* $p < 0.05$ session 3 compared to sessions 1 and 2).

In the pilot study, the 15-minute FSTs were broken into three five-minute bins in order to find potential differences in time spent immobile in each third of each session. Due to the limitations of GraphPad Prism, males and females were only analyzed together for the first two FST sessions. A three-way mixed model ANOVA was run with sex as the between-subjects variable and session and five-minute bin as within-subjects variables (Figure 16). The main effects of sex [$F(1, 10) = 2.499$, $p = 0.1450$], session [$F(1, 10) = 0.8718$, $p = 0.3724$], and five-minute bin [$F(1.250, 12.50) = 1.568$, $p = 0.2398$] were all not statistically significant. There were also no significant interactions between sex and five-minute bin [$F(2, 20) = 0.04005$, $p =$

0.9608], sex and session [$F(1, 10) = 3.802$, $p = 0.0798$], five-minute bin and session [$F(1.882, 18.82) = 2.041$, $p = 0.1595$], or sex, session, and five-minute bin [$F(2, 20) = 0.4417$, $p = 0.6490$]. Neither a rat's sex, the repetition of the FST, how far along the FST was, or a combination of these variables had a significant impact on how much time an animal spent immobile in the first two sessions of the FST.

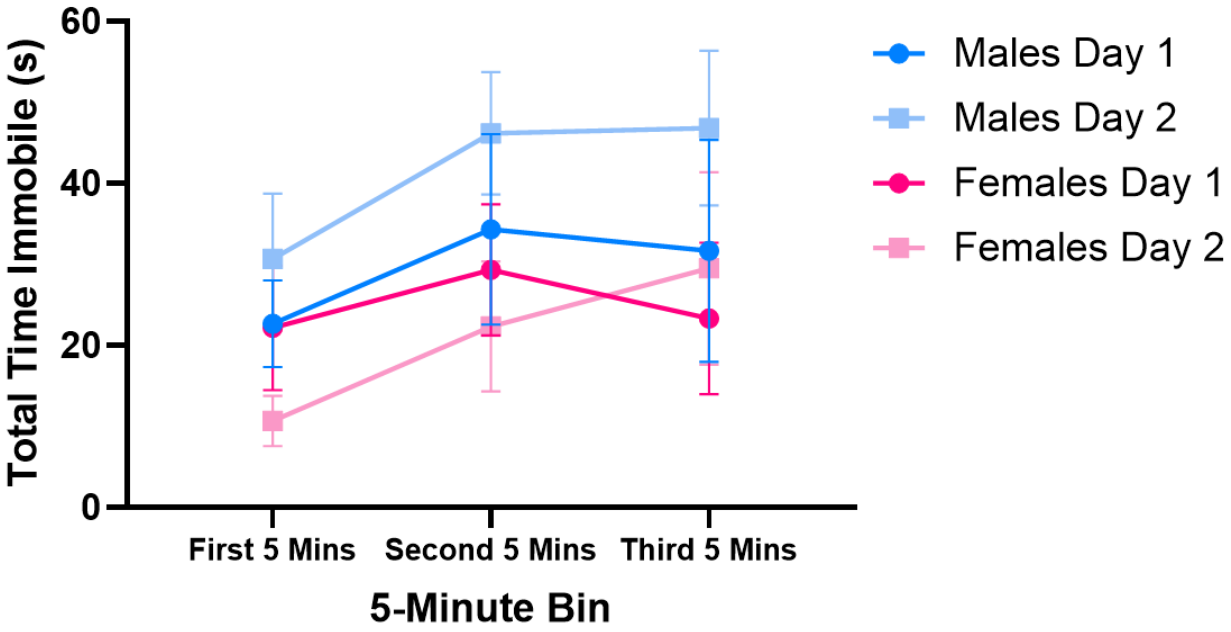

Figure 16. Total time immobile across two FST sessions and five-minute bins by sex during pilot study. The first two FSTs were conducted 24 hours apart, and each session lasted 15 minutes. Immobility during the FST was measured in five-minute intervals to determine potential changes in behavior within each session. Values are reported as mean ± SEM. $n = 6$ per group. There were no differences by sex, session, or five-minute bin.

For analyses of five-minute bins across all sessions, males and females were analyzed separately. A two-way repeated measures ANOVA was run with session and five-minute bins as the within-subjects variables (Figure 17).

For males, there was a statistically significant main effect of five-minute bin [$F(1.434,$

7.171) = 5.666, ***p* = 0.0402**], indicating that the amount of time a male rat was immobile during the FST varied as a function of whether it was in the first, middle, or last third of the session (Figure 17A). There was no significant main effect of session [$F(2.242, 11.21) = 2.290$, $p = 0.1433$], nor was there a significant interaction between 5-minute bin and session [$F(2.196, 10.98) = 1.737$, $p = 0.2206$]. A multiple comparisons test using the Tukey adjustment was done to describe the nature of the effect of five-minute bin, which revealed significant differences between the first and last five-minute bins during session 3 (adjusted ***p* = 0.0127**), as well as the first and middle five-minute bins during session 4 (adjusted ***p* = 0.0262**). No other significant differences between sessions were found.

For females, there was also a significant main effect of five-minute bin [$F(1.214, 6.071) = 7.199$, ***p* = 0.0327**], indicating that the amount of time a female rat was immobile during the FST varied as a function of whether it was in the first, middle, or last third of the session (Figure 17B). There was no significant main effect of session [$F(2.566, 12.83) = 1.878$, $p = 0.1877$], nor was there a significant interaction between five-minute bin and session [$F(2.242, 11.21) = 2.202$, $p = 0.1531$]. A multiple comparisons test using the Tukey adjustment revealed no significant differences between five-minute bins in each session. Given the lack of main effect or interaction including session, the data were consolidated to remove session as a variable. A one-way repeated measures ANOVA was then run with five-minute bin as the within-subjects variable. Results again revealed a significant effect of five-minute bin [$F(1.214, 6.071) = 7.199$, ***p* = 0.0327**]. To describe the nature of the main effect, a Tukey HSD test was used. There was a significant difference between the first and middle five-minute bins when the session variable was removed (adjusted ***p* = 0.0477**). Specifically, female rats spent more time immobile, on

average, in the middle five minutes of a 15-minute FST than the first five minutes.

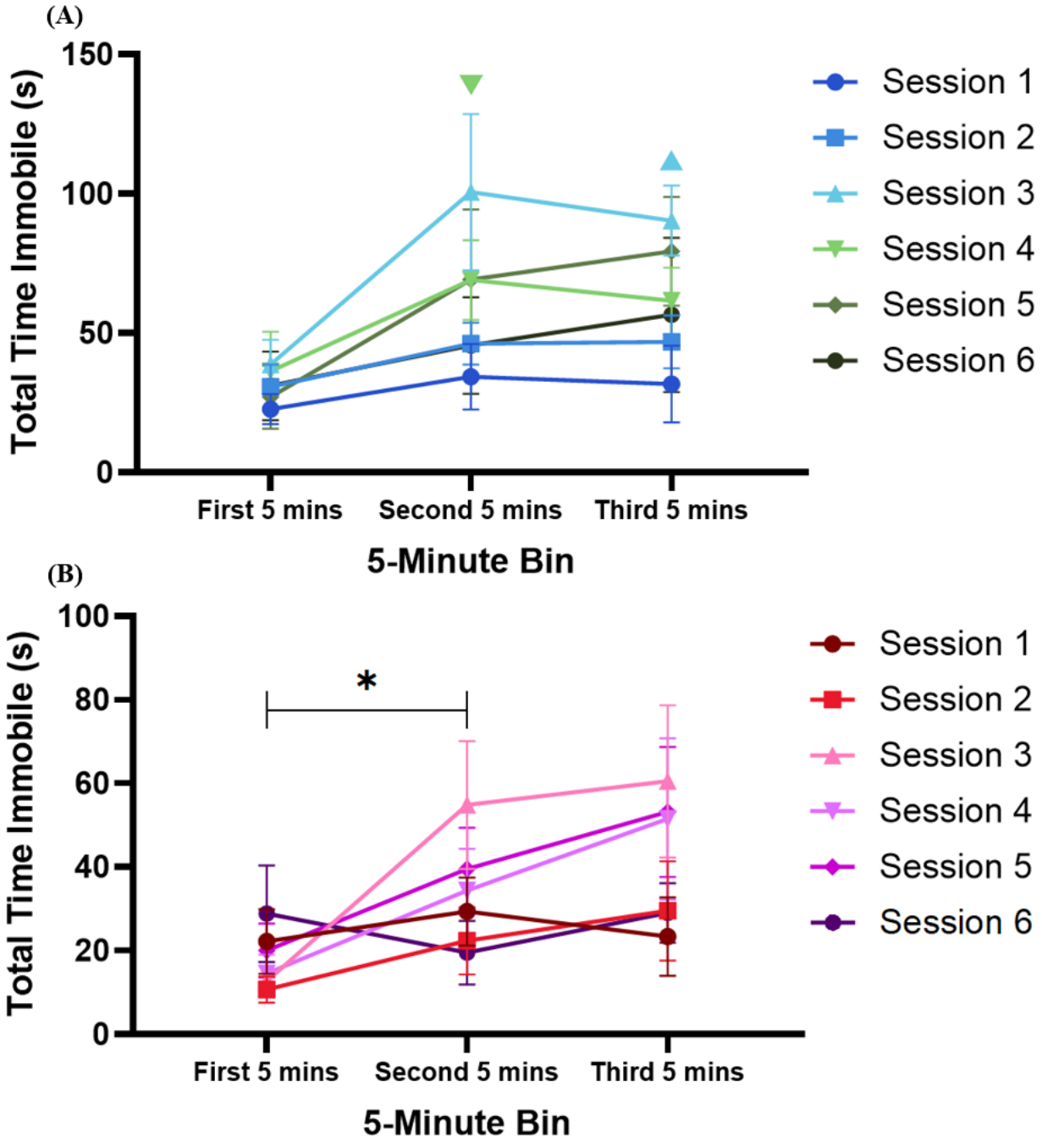

Figure 17. Total time immobile in the FST across sessions and five-minute bins during pilot study, separated by sex. The first two FSTs were conducted 24 hours apart, then once a week afterwards.

Immobility during the FST was measured in 5-minute intervals to determine potential changes in behavior within each session. Values are reported as mean ± SEM. $n = 6$ per group. **(A)** Total time immobile in male rats. There were no differences by session. There was a significant main effect of five-minute bin at session 3 (▲ $p < 0.05$ third five minutes compared to first five minutes), as well as at session 4 (▼ $p < 0.05$ second five minutes compared to first five minutes). **(B)** Total time immobile (s) in female rats. There were no differences by session. Rats were significantly more active in the first five minutes than the middle five minutes, regardless of session (* $p < 0.05$ first five-minute bin compared to second five-minute bin).

Open Field Test (**Figures 18-19**)

In the pilot study, the OFT was conducted five times to determine differences in behavior with repeated exposure to the apparatus. To determine whether or not differences in behavior exist between sexes in the OFT, total distance traveled was analyzed between sexes and across sessions. A two-way mixed model ANOVA was run with sex as the between-subjects variable and session as the within-subjects variable (Figure 18). There was a statistically significant main effect of session on total distance [$F(2.419, 24.19) = 16.77$, $\mathbf{p < 0.0001}$]. There was no significant main effect of sex [$F(1, 10) = 3.837$, $p = 0.0786$]. There was a significant interaction between session and sex [$F(4, 40) = 3.249$, $\mathbf{p = 0.0213}$].

For *post-hoc* testing, the Bonferroni adjustment of $\alpha = 0.05/5 = 0.01$ was used to compare sexes across sessions. The only session where there was a significant difference in distance traveled between sexes was in session 3 (adjusted $\mathbf{p = 0.0149}$). Specifically, females traveled farther, on average, than males did during this session. To compare sessions across sexes, the Bonferroni adjustment of $\alpha = 0.05/10 = 0.005$ was used. For females, there were statistically significant differences between sessions 1 and 4 (adjusted $\mathbf{p = 0.0056}$) and sessions 3 and 4 (adjusted $\mathbf{p = 0.0298}$). Specifically, females traveled farther, on average, in sessions 1 and 3 than in session 4. For males, there were statistically significant differences between sessions 1

and 3 (adjusted ***p* = 0.0064**) and sessions 1 and 5 (adjusted ***p* = 0.0411**). Specifically, males traveled farther, on average, in session 1 than in sessions 3 and 5.

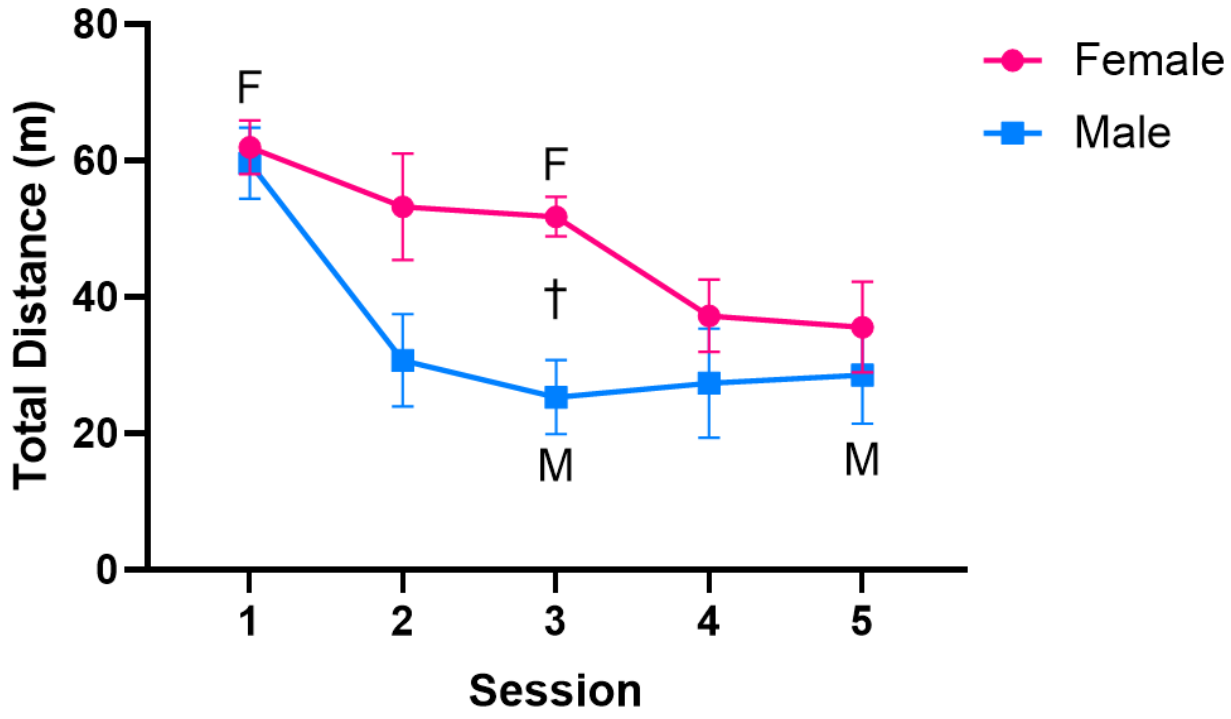

Figure 18. Total distance traveled in the OFT across sessions by sex. The first OFT was administered before the first FST, then readministered 72 hours later after two FSTs. Every OFT afterwards was administered weekly. Values are reported as mean ± SEM. $n = 6$ per group. Female rats traveled significantly farther in sessions 1 and 3 than in session 4 (F $p < 0.05$). Males traveled significantly farther in session 1 than in sessions 3 and 5 (M $p < 0.05$). Females traveled significantly farther than males in session 3 († $p < 0.05$).

Distance traveled in the outer, middle, and center zones were analyzed separately. For each analysis, a two-way mixed model ANOVA was run with sex as the between-subjects variable and session as the within-subjects variable.

For the outer zone, there was no significant main effect of sex [$F(1, 10) = 3.548$, $p = 0.0890$], but there was a main effect of session [$F(2.336, 23.36) = 17.29$, ***p* < 0.0001**] (Figure

19A). There was also a significant interaction between session and sex [$F(4, 40) = 3.985$, $\mathbf{p = 0.0082}$]. For the middle zone, there was no significant main effect of either session [$F(2.241, 22.41) = 2.633$, $p = 0.0888$] or sex [$F(1, 10) = 2.589$, $p = 0.1387$] (Figure 19C). There was no significant interaction between session and sex [$F(4, 40) = 2.393$, $p = 0.0666$]. The same results were seen for the center zone, with no significant effects of session [$F(2.416, 24.16) = 1.235$, $p = 0.3141$], sex [$F(1, 10) = 2.074$, $p = 0.1804$], or the interaction between session and sex [$F(4, 40) = 1.179$, $p = 0.3348$] (Figure 19E).

For *post-hoc* testing on the outer zone data, the Bonferroni adjustment of $\alpha = 0.05/5 = 0.01$ was used to compare sexes across sessions. The only session where there was a significant difference in distance traveled between sexes was in session 3 (adjusted $\mathbf{p = 0.0228}$). Specifically, females traveled farther, on average, than males did during this session. To compare sessions across sexes, the Bonferroni adjustment of $\alpha = 0.05/10 = 0.005$ was used. For females, there were statistically significant differences between sessions 1 and 4 (adjusted $\mathbf{p = 0.0266}$) and sessions 2 and 4 (adjusted $\mathbf{p = 0.0383}$). Specifically, females traveled farther, on average, in sessions 1 and 2 than in session 4. For males, there were statistically significant differences between sessions 1 and 3 (adjusted $\mathbf{p = 0.0052}$), sessions 1 and 4 (adjusted $\mathbf{p = 0.0273}$), and sessions 1 and 5 (adjusted $\mathbf{p = 0.0241}$). Specifically, males traveled farther, on average, in session 1 than in sessions 3, 4, and 5.

For the middle zone, there was an outlier in the male group for session 4. With this value excluded, there were significant main effects of session [$F(2.746, 26.77) = 4.555$, $\mathbf{p = 0.0122}$] and sex [$F(1, 10) = 5.044$, $\mathbf{p = 0.0485}$], but no significant interaction [$F(4, 39) = 1.935$, $p = 0.1239$]. To describe the nature of the main effects, a Tukey HSD test was used. There were no

differences between sessions for either females or males. When comparing sexes in the same session, there were significant differences in session 1 (adjusted ***p* = 0.0439**) and session 3 (adjusted ***p* = 0.0252**). Specifically, female rats traveled farther in the middle zone, on average, than males did during sessions 1 and 3. Removing the same outlier from the center zone did not create any significant effects, so the outlier was left in for that analysis.

For the amount of time spent in each zone, there were no significant main effects or interactions for the outer, middle, or center zones. However, removing the same outlier as above from each analysis created significant results in each zone, so the outlier was excluded for all three analyses. A mixed-effects analysis was conducted for all three zones, with sex as the between-subjects variable and session as the within-subjects variable.

For the outer zone, there was a significant main effect of session [$F(2.091, 20.39) = 4.567$, ***p* = 0.0217**], but no significant effect of sex [$F(1, 10) = 1.904$, $p = 0.1977$] (Figure 19B). There was also no significant interaction between session and sex [$F(4, 39) = 0.6323$, $p = 0.6425$]. To describe the nature of the main effect of session, a Tukey HSD test was used. Using multiple comparisons, there were no significant differences between sessions.

For the middle zone, there was also a significant main effect of session [$F(1.938, 18.89) = 3.704$, ***p* = 0.0451**], but no significant effect of sex [$F(1, 10) = 0.8843$, $p = 0.3692$] (Figure 19D). There was also no significant interaction between session and sex [$F(4, 39) = 0.7899$, $p = 0.5389$]. To describe the nature of the main effect of session, a Tukey HSD test was used. Using multiple comparisons, there was a significant difference between sessions 1 and 4 (adjusted ***p* = 0.0439**). Specifically, rats spent more time in the middle zone, on average, during session 1 than session 4. There were no other significant differences between sessions.

For the center zone, there was a significant main effect of sex [$F(1, 10) = 5.650$, $\boldsymbol{p = 0.0388}$], but no significant effect of session [$F(1.502, 14.64) = 2.017$, $p = 0.1741$] (Figure 19F). There was also no significant interaction between session and sex [$F(4, 39) = 0.9332$, $p = 0.4548$]. To describe the nature of the main effect of sex, cell means were used. Females spent more time in the center zone, on average, than males did.

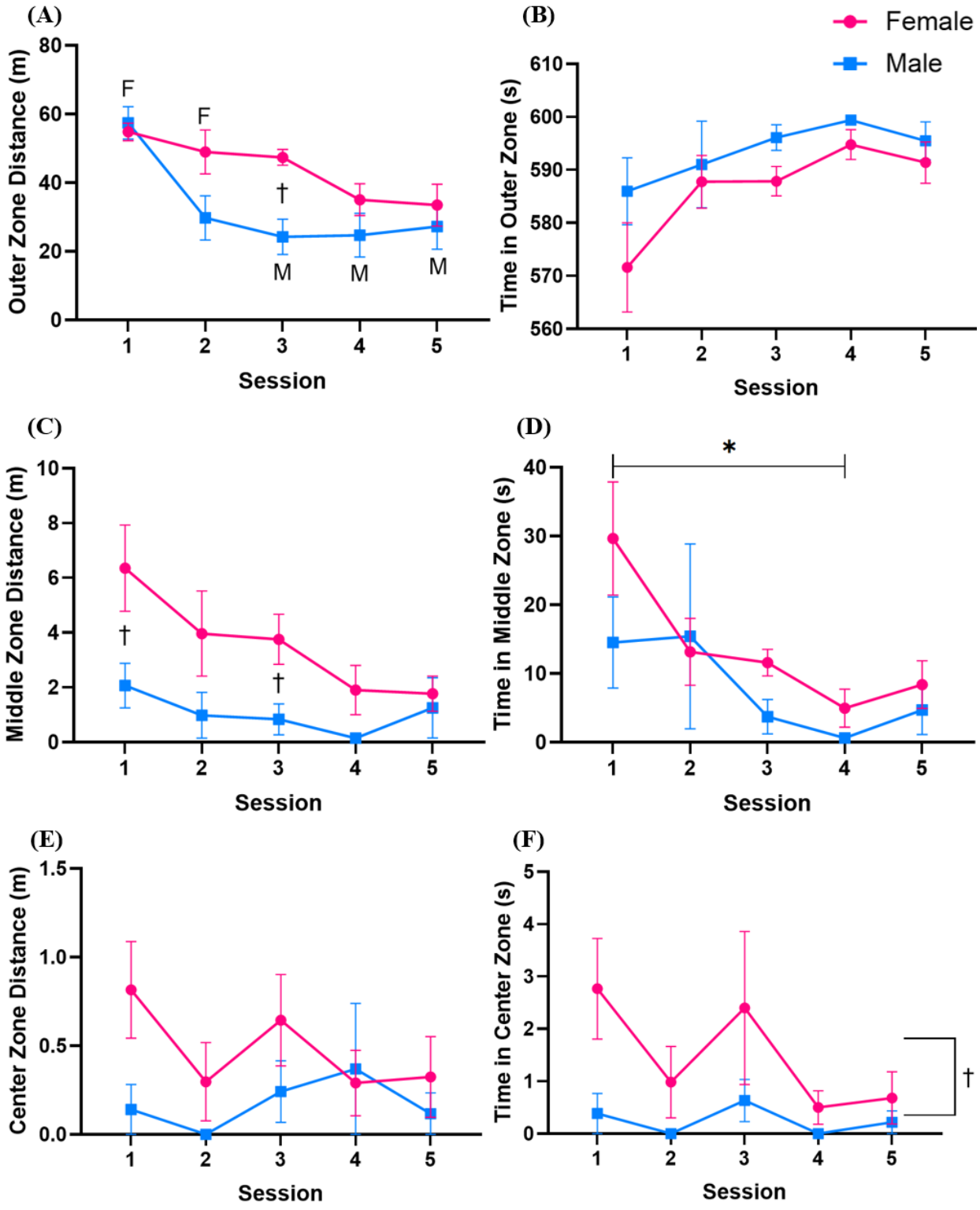

Figure 19. All OFT data across sessions by sex during pilot study. The first OFT was administered

before the first FST, then readministered 72 hours later after two FSTs. Every OFT afterwards was administered weekly. Values are reported as mean ± SEM. $n = 6$ per group. **(A)** Distance traveled in the outer zone. Female rats traveled significantly farther in sessions 1 and 2 than in session 4 (F $p < 0.05$). Males traveled significantly farther in session 1 than in sessions 3, 4, and 5 (M $p < 0.05$). Females traveled significantly farther than males in session 3 († $p < 0.05$). **(B)** Time spent in the outer zone. There was a significant effect of session, but no significant pairwise comparisons. **(C)** Distance traveled in the middle zone. Females traveled significantly farther than males in sessions 1 and 3 († $p < 0.05$). **(D)** Time spent in the middle zone. Rats spent significantly more time in the middle zone during session 1 than session 4 (* $p < 0.05$). **(E)** Distance traveled in the center zone. There were no significant differences of session or sex. **(F)** Time spent in the center zone. Females spent significantly more time in the center zone than males did († $p < 0.05$).

Main Experiment

Investigational Open Field Test (**Figure 20**)

To rule out a potential confounding effect of ketamine on locomotor activity in the initial behavioral tests, another OFT was performed 20 days after the last FST where each rat received a ketamine/saline injection approximately three hours before they were placed in the apparatus, and the total distance traveled was analyzed. A two-way ANOVA was conducted with both CVS and injection as between-subjects variables (Figure 21). There were no significant effects of CVS [$F(1, 20) = 1.126$, $p = 0.3013$], injection [$F(1, 20) = 0.01113$, $p = 0.9170$], or interaction between CVS and injection [$F(1, 20) = 0.3533$, $p = 0.5589$].

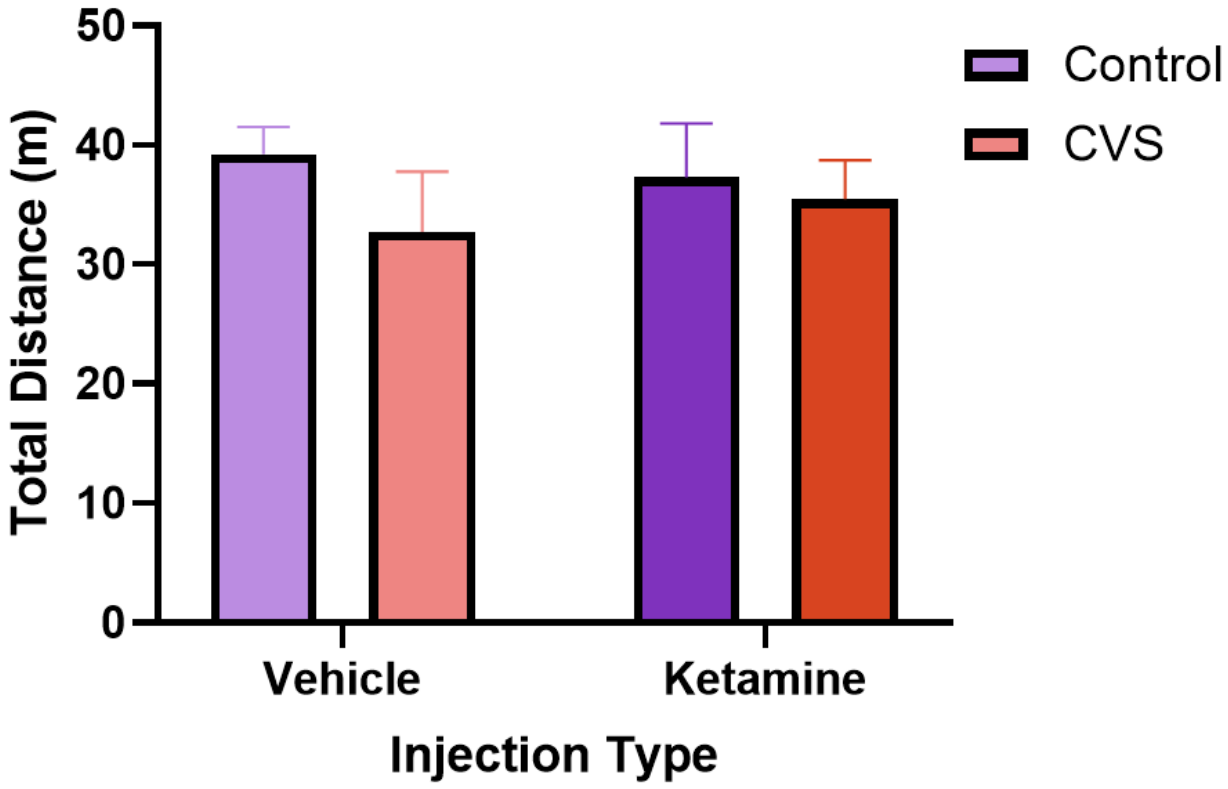

Figure 20. Total distance traveled in the investigational OFT by group. This OFT was conducted three weeks after the last injection, and injections were given three hours before the session. Values are reported as mean ± SEM. *n* = 6 per group. No significant main effects or interactions were found.