THE DOSE DEPENDENT EFFECTS OF PIMAVANSERIN ON A MODIFIED RODENT MODEL OF POST-TRAUMATIC STRESS DISORDER

by

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Dedication

to

My mother, my father, and my sister whom I love dearly

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ABSTRACT

THE DOSE DEPENDENT EFFECTS OF PIMAVANSERIN ON A MODIFIED RODENT MODEL OF POST-TRAUMATIC STRESS DISORDER

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Post-traumatic stress disorder (PTSD) is a persistent psychiatric disorder where patients develop symptoms from directly experiencing or witnessing traumatic events. The animal models are often used as preclinical studies for PTSD. Our research group previously found that the developed animal model of PTSD, which was composed of chronic and acute stress, successfully induced PTSD-like anxiety behaviors in the three behavioral measurements: elevated-plus maze, open field, and acoustic startle response. Additionally, the inverse antagonist drug Pimavanserin significantly reduced these fear-related behaviors in the animal model. In the current research, we examined the effects of each stressor, specifically the effect of social isolation, in addition to the replication of the effects of Pimavanserin on the animal model of PTSD, including the dose dependence of

Pimavanserin. As a result, higher dose injections significantly reduced rats' anxiety-level in the behavioral measures, while the smaller and no Pimavanserin injections did not change animals' behaviors. In relation to the effect of social isolation, there was no significant difference between animals in the single housed group and pair housed group. These results strongly supported the role of 5-HT2A in our PTSD-animal model. Furthermore, from the results, it is highly expected that the 5-HT2A receptors' inverse antagonists have positive therapeutic effects on PTSD patients.

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CHAPTER I:

POST-TRAUMATIC STRESS DISORDER

Definition and Diagnostic Features

Post-traumatic stress disorder (PTSD) is a severe and persistent psychiatric disorder. According to the Diagnostic statistical manual-V (DSM-V), the lifetime risk of this disorder is 8.7 % at age 75 years in the U.S. population (DSM-V, 2013). The DSM-V (2013) describes PTSD as caused by directly experiencing, witnessing, or learning of one or more traumatic events. PTSD symptoms are persistent, and they last at least a month (DSM-V, 2013). PTSD patients' symptoms are so diverse that their symptoms are mainly categorized into three clusters: persistent avoidance of the stimuli related to the traumatic memory, re-experiencing of the traumatic event, and hyperarousal (Asmundson et al., 2004). Persistent avoidance and emotional numbing occur when PTSD patients remember their traumatic memories triggered by stimuli which remind them of their traumatic events (DSM-V, 2013). This diagnostic feature can be added as a separate cluster for the symptom of PTSD, (Forbe et al., 2010). Moreover, research has shown that there is a positive correlation between avoidance and emotional numbing and other PTSD symptoms (Asmundson et al., 2004). This pattern of symptoms is unique to PTSD, and they are useful in distinguishing PTSD from similar mental conditions such as acute stress disorder. Re-experiencing the trauma is also one of the symptoms commonly seen in PTSD patients. This symptom follows emotional dysfunction, but it is distinguishable from the persistent avoidance symptoms (Hopper et al., 2007). A study with functional magnetic resonance imaging (fMRI) has found that re-experiencing symptoms activates the brain regions associated with somatic aspects of emotional states (Hopper et al., 2007). Another study has found that the hyperarousal and re-experiencing symptoms are associated with problem behaviors among veterans, including uncontrolled aggression

(Taft et al., 2007). Such persistent behavioral disorders make it difficult to recover from PTSD.

Most people show the symptoms of acute stress disorder right after they experience or witness traumatic events. However, far fewer people develop PTSD-like symptoms. There are several types of people who are more susceptible to developing PTSD symptoms from traumatic events. There are pre-trauma and post-trauma risk factors contribute to the vulnerability of PTSD. A meta-analysis from Brewin and colleagues (2000) revealed that age and gender occurred impacts the development of PTSD. The analysis also found that previous traumatic events, especially at a young age, lack of education, and high traumatic severity are major risk factors for PTSD. Risk factors are divided into three categories: existing before the traumatic events, related traumatic events, and occurring after the traumatic events (Keane et al., 2009). Biological differences also can be a risk factor that develops PTSD symptoms. For instance, Rooij and colleagues (2015) measured hippocampal sizes of PTSD patients. It was found that PTSD patients had smaller hippocampal volumes in the left hemisphere of the brain. However, hippocampal sizes were not different between healthy controls and patients who were exposed to traumatic events without developing PTSD (Rooij et al., 2015). Therefore, anatomical brain differences are potentially influential factors for developing PTSD. In summary, diverse risk factors influence whether a given trauma results in PTSD.

Biological Changes in PTSD

Traumatic events cause negative effects not only on the patients' thoughts and behaviors, but also on biological functions. The traumatic memories can induce abnormalities in a patients' behavior, and these symptoms last at least for a month (DSM-V, 2013). Such persistence symptoms are also featured in PTSD, and patients suffer from

long-lasting symptoms such as re-experiencing and avoidance of traumatic memory. This persistence is caused by anatomical changes, especially the brain. Rooij and colleagues (2015) found that smaller hippocampus volumes in PTSD patients were related to the risk factor of persistence. As the study showed, PTSD symptoms can be caused by the results of biological change from traumatic events.

Some studies have shown that PTSD patients are impaired in specific brain areas, and these impairments also cause the dysregulation of cognitive functions such as emotion and memory. Emotional and memory dysregulations in PTSD might involve the amygdala, insula, hippocampus, anterior cingulate cortex (ACC), and prefrontal cortex (Fitzgerald et al., 2018). According to the study of Fitzgerald and colleagues (2018), emotional processing of trauma results in hyper-active amygdala and insula. This activation then stimulates hippocampal engagement in learning and memory for the event. Finally, the medial prefrontal cortex (mPFC) becomes engaged and affects control of arousal. In relation to emotional memory, the amygdala and hippocampus are activated whenever people experience traumatic events (Tulmi, 2013). The amygdala is activated when people experience events that are accompanied by emotional stimuli. Emotional information enhances the consolidation of memory so that people can retrieve the memory of the events cued by emotional information (Debiec & LeDoux, 2009). The amygdala is also relevant to fear conditioning, as the lateral, basal, and central nuclei of the amygdala process fear-connected information. Research has shown there is a correlation between amygdala activation and PTSD (Debiec & LeDoux, 2009). Since the amygdala, specifically the lateral portion, is highly involved with sensory memory, PTSD patients react with hyper-responsivity when experiencing any sensory information associated with the traumatic event (Debiec & LeDoux, 2009; Shin et al., 2006).

Memory dysfunction in PTSD is likely caused by a sequence of impairments of several brain areas. There are three predominant regions in the brain that are associated with memory-related PTSD symptoms: amygdala, medial prefrontal cortex (mPFC), and hippocampus (Shin et al., 2006). As the mPFC is connected to the amygdala, it engages in the process of extinction or retention of fear conditioning (Shin et al., 2006). The hippocampus is also involved in fear conditioning and its memory and learning functions are impaired through the development of PTSD. The hippocampus is located close to the amygdala, and their interactions are related to several cognitive as well as emotional processes such as emotion memory (Heim & Nemeroff, 2009). The hippocampus has an important role for episodic and emotional memories (Burgess et al., 2002; Burguess et al., 2005). Hippocampus impairments can inhibit the re-encoding of traumatic memory so that PTSD patients cannot stop the amygdala's maladaptive response to the traumatic memories (Verfaellie & Vasterling, 2009). In sum, dysregulation of certain brain regions impairs the relations between patients' cognitive and emotional functions.

Hypothalamus pituitary-adrenal gland axis and sympathetic nervous system

Multiple studies have focused on two control pathways related to the dysregulation caused by stress: the hypothalamus pituitary-adrenal cortex (HPA) axis and the sympathetic nervous system (SNS). The HPA axis is a well-known system that responds to stressful stimuli (Lanfumey et al., 2008), and induces the circulation of hormones associated with stress. During stress exposure, the hypothalamic paraventricular nucleus (PVN) secretes corticotrophin-releasing factors (CRF) to the pituitary gland. In response the pituitary gland then sends adrenocorticotropic hormone (ACTH) to the adrenal gland. Consequently, ACTH stimulates the adrenal cortex to produce cortisol and corticosterone, hormones that cease neuronal defensive reactions (Heim & Nemeroff, 2008; Meewisse et al., 2007). As PTSD patients are exposed to one

or more traumatic events, both human and animal research have shown there is a connection between PTSD and the dysregulations of the HPA axis (Ana et al., 2006; Meewisse et al, 2007). Cortisol is often used as the measurement of the HPA-axis functions in human populations (Pan et al., 2018). It is produced in the adrenal gland in response to the secretion of ACTH from the pituitary within the HPA axis. An extensive meta-analysis (Pan et al., 2018) showed that there is a negative correlation between PTSD and salivary cortisol level. Salivary cortisol in PTSD patients is lower than in healthy people. However, dysfunction of the HPA axis is not limited to PTSD. Low cortisol levels are also associated with major depression disorder (MDD), which also suggests dysregulation of the HPA axis (Morris et al., 2012). Thus, it cannot be concluded that HPA axis impairment is specific to PTSD as the HPA axis can be related to other emotional disorders involving stress.

The HPA axis also interacts with the autonomic nervous system which regulates cardiovascular activity and various other physiological functions. Research has shown that autonomic nervous system abnormalities are commonly found in PTSD patients, leading to accelerated heart rate and elevated blood pressure (Heim & Nemeroff, 2009). Similar to the HPA-axis, the sympathetic nervous system (SNS) is also activated in response to stress. The SNS is a division of the autonomic nervous system, and the hypothalamus mediates both the HPA-axis and SNS activation in response to stress. SNS activation results in the release of norepinephrine (NE), and epinephrine, affecting our arousal and memories (Lipov & Kaelzenberg, 2012; Southwick et al., 1999). Concentrations of NE is significantly higher among PTSD patients compared to people without any psychiatric disorder (Geracioti et al., 2001). The reduction of NE activation has been shown to lessen hyperarousal symptoms of PTSD (Taylor et al., 2006).

The 5HT2A Receptor

The serotonergic system has known involvement with anxiety disorders. Serotonin (5-HT) is produced in the dorsal and medial raphe' nuclei of the brainstem. Serotonin is predominantly supplied to the forebrain, including the amygdala, hippocampus, and PFC (Heim & Nemeroff, 2009). Serotonergic functions are different from each other depending on areas of the brain and types of serotonergic receptors. This neurotransmitter has a wide variety of functions within the central nervous system (CNS), including sleep, memory, and learning (Lanfumey et al., 2008). Serotonin function has also been correlated with stress (Chaouloff et al., 1999). Stress induces increased 5-HT release and activates several kinds of serotonergic receptors. For instance, 5-HT2A receptors help mediate anxiogenic effects, and stimulation of 5-HT1A receptors are related to adaptive responses to aversive events (Charney, 2004). Heim and Numeroff (2009) found evidence that 5-HT release is induced by stress impacts on the HPA axis. Another study has also shown that the hippocampus has essential roles in the interaction between 5-HT and the HPA axis (Laufumey et al., 2008). Thus, stress can affect bodily functions through serotonergic systems activation.

Fifteen types of serotonergic receptors divided into three major families have been found. They each have their own functions that differ from other 5HT receptors. Lanfumey and colleagues (2008) reviewed the diverse effects of different 5-HT receptors. For instance, 5-HT2C receptors are relevant to food intake, anxiety, and depression. They also found that 5-HT2A receptors have an effect on schizophrenia, the cycle of sleep and waking, and blood flow. The 5-HT2A receptors also have an excitatory effect on neurons. 5HT2A receptors have a higher density in layer V of the neocortex, and they are also distributed in broad regions in the brain such as the prefrontal cortex, hippocampus, and amygdala (Bombardi, 2018). There are multiple functions of 5-HT2A receptors, which include cognitive, perceptual, and emotional regulation. In the

amygdala, 5-HT2A receptors activate CRF neurons and increase serotonin synthesis in response to stress and fear (Moulédous & Guiard, 2018). The functions of 5HT2A receptors are also related to the regulation and processing of anxiety and fear memory. The 5-HT2A receptors in the medial prefrontal cortex (mPFC) are positively correlated with threat-related amygdala reactivity (Zhang & Stackman, 2015). An abundance of research focuses on 5-HT2A receptors within the basolateral amygdala (BLA) and medial amygdala (MeA), and their interactions with GABAergic interneurons (Moulédous & Guiard, 2018). In relation to fearful memories, the 5-HT2A receptors within the hippocampus have an important role in the extinction of the fear memory. In a study conducted by Jiang and colleagues (2020), hippocampal 5-HT2A receptor activation enhanced contextual fear memory. To further support these findings, Zhang and colleagues (2013) found that the activation of 5-HT2A receptors affected the consolidation and extinction of fear memory. As these results have shown, 5-HT2A receptors are strongly correlated with anxiety and fear memory, which is processed by several regions in the brain, especially the amygdala and hippocampus.

5HT2A Receptors in Psychiatric Disorder

The 5-HT2A receptor has been associated with various psychiatric disorders. Lysergic acid diethylamide (LSD) is a hallucinogenic drug that acts on serotonergic receptors, particularly the 5HT2A receptor. This action increases the release of dopamine, a transmitter shown to be highly involved in schizophrenia. Research has shown that LSD strongly alters functional brain connectivity (Preller et al., 2018).

LSD can induce auditory and visual hallucinations, as well as panic reactions. These hallucinations are primarily due to the binding of LSD on 5-HT2A receptors (Roth et al., 1999). Additionally, LSD has been shown to induce dream-like effects through the activation of the 5-HT2A receptors (Krahenmann et al., 2017). These dream-like effects

are associated with loss of self-boundaries and cognitive control. On the other hand, M100907, which is a high selective 5-HT2A antagonist, has been known as a selective 5-HT2A antagonist. Several studies have found that M100907 attenuates the release of dopamine (Minabe et al., 2001; Pehek et al., 2000). The prefrontal cortex has a high density of 5-HT2A receptors and its blockages in this region have been known to reduce DA secretions (Pehek et al., 2006). Specifically, Pehek and colleagues (2006) reported that DA secretions from the medial prefrontal cortex of rats' brains decreased after M100907 treatment.

Blockages of 5-HT2A receptors affect mental disorders such as schizophrenia. Talvik-Lotfi and colleagues (2000) investigated the effect of M100907 on schizophrenic patients, and their results revealed very high occupancy of M100907 binding to 5-HT2A receptors. Although this study was limited due to the minimal amount of patients with schizophrenia, several studies have shown an effect of M100907 against aspects of this disorder. (Ansah et al., 2011). Further, blockages of 5-HT2A receptors also have antidepressant effects, and antagonists of the receptor can reduce depressive-like behaviors (Pandey et al., 2010).

PTSD research has also focused on a possible role for the 5HT2A receptor. In animal models, anxiety-like behavior was associated with 5-HT1A and 5-HT2A receptors (Xiang et al., 2018). Moreover, in the human population, Mellman and colleagues (2009) found a unique variation in genes for the 5HT2A receptor in PTSD patients.

Behavioral Treatments for PTSD

There are multiple behavioral and pharmacological treatments for PTSD. The main behavioral treatments are cognitive-behavioral therapy (CBT) and cognitive processing therapy (CPT). Through CBT, people can learn healthy coping skills to alleviate their suffering, while CPT helps to mediate harmful cognitive strategies caused

by traumatic events (APA, 2020). Kar (2011) reviewed the efficiency of CBT on PTSD patients and found CBT to be an effective treatment for patients with chronic and acute PTSD. CBT has both short-term and long-term effects on PTSD patients. In addition, several studies have shown the effectiveness of CPT treatment for patients with PTSD (Schulz et al., 2006). In the study by Schulz and colleagues (2006), the PTSD Symptom Scale (PSS) was used to measure the effectiveness of behavioral therapy on patients suffering from PTSD. The helpful biological effects of these behavioral therapies have also been observed. For example, CBT reduced atypical hyper-activity of the amygdala in patients with anxiety disorders (McClure et al., 2006).

Pharmacological Treatment for PTSD

Selective serotonin reuptake inhibitors (SSRI) are the most common treatments used for major depression disorder (MDD) and anxiety-related disorders. SSRIs increase serotonin activity by inhibiting serotonin reuptake within synapses. Through the acute increase of 5-HT within the synapse, SSRIs affect cognitive functions. For instance, the intake of SSRIs enhances cognitive performances of patients with certain neurological disorders, such as Alzheimer's disease (Chow et al., 2007). However, the treatment mechanisms of SSRIs are not completely understood. For instance, a study found that escitalopram, a commonly prescribed SSRI, impaired verbal memory (Soczynska et al., 2014). SSRIs have been used as the first-line therapy for PTSD. In another study, the SSRI paroxetine has shown to enhance emotional tasks in PTSD patients by suppressing irregular activity in the PFC (Pehek et al., 2006). Although many studies focus on the antidepressant effects of SSRIs, other studies have shown adverse side effects of SSRIs. Since serotonin stimulates many different receptors with many different effects, there can be many side effects, such as interference with memory processes. This raises the

possibility that an approach specifically targeted on a particular receptor might have a more desirable therapeutic profile.

Pimavanserin (Pima)

Pimavanserin (Pima) is an approved FDA drug for the treatment of hallucinations and delusions in Parkinson's disease. It is a selective inverse agonist of the 5-HT2A receptor. That means it inactivates both the spontaneous and stimulated activity of the receptor. Parkinson's disease is caused by a depletion of dopamine. Therefore, standard antipsychotic drugs cannot be used in this disease because they all interfere with dopamine receptors. However, there may also be a serotonergic aspect of Parkinson's disease. .Multiple studies have shown the reduction of serotonin metabolites in relation to Parkinson's disease (Mayeux et al., 1984; Scatton et al., 1983). McFarland and colleagues (2011) examined the effect of Pima on rodent models of Parkinson's disease. In that study, Pima injection attenuated psychosis-related behavior in rats, such as head tremors and shakes. Additionally, the study revealed that Pima injection had few side-effects in the rats. In particular, they found that Pima injection did not impair motor skills in rodents. This preclinical work eventually led to the successful use of Pima in human Parkinson patients (Cumming et al., 2014).

CHAPTER II:

ANIMAL MODEL OF PTSD

The Rationale of Animal Research

Studies of PTSD have been conducted using animals as well as human subjects. There are numerous case studies about PTSD patients who developed the symptoms after they experienced various traumatic events. As the studies of PTSD have shown, the types of traumatic events vary widely. In addition, the symptoms vary depending on individual risk factors. Since these studies collected data from patients who had different risk factors such as age, gender and differing traumatic memories, it is difficult to identify the common development mechanisms of PTSD. Further, case studies sometimes lack reliability and statistical power needed to determine the actual effects of traumatic events and risk factors. Additionally, ethical concerns may prevent testing novel experimental treatments on human PTSD patients. The studies which use animals for their subjects are able to control and standardize their experimental designs. For instance, it is possible to use the same traumatic events and situations among all subjects by using animal models (Borghans & Homebrg, 2015). Furthermore, researchers can use animal models to keep individual risk factors consistent between subjects. Therefore, preclinical studies are necessary to understand the underlying mechanisms of PTSD.

Sex Difference

The animal model also can be used to examine the epidemiology of PTSD such as sex differences. According to Breslau (2009), there is a significantly higher risk for the female human population to develop the symptoms of PTSD after they experience traumatic events. In addition, men and women may experience somewhat different symptoms and durations of PTSD (Breslau, 2009). There are numerous reasons that may explain these differences. For instance, the patients who developed PTSD symptoms

often had different types of traumatic events depending on their gender (DSM-V, 2013). Therefore, the effects of sex differences as a risk factor of PTSD are complicated and require further research. Animal models are useful for conducting research to investigate the sex difference by controlling subjects' environment and risk factors. Cohen and Yehuda (2011) reviewed the gender differences in animal models of PTSD and they found that the corticosterone levels were higher after trauma in female rats compared to those of male rats. Further, the results of the animal study revealed differences in resilience in responding to traumatic stress (Pooley et al., 2018).

Methods to Induce PTSD-Like Behavior in Animals

There are several ways to condition animals in models of PTSD. Borghans and Homberg (2015) overviewed stress models in animals in relation to PTSD. Physical, social, and psychological stressors have been used. Each stressor has been developed through many preclinical studies using animals. In the previous University of Houston Clear Lake (UHCL) study, Campbell (2019) found that the combination of physical and social stressors was enough to induce PTSD-like anxiety behaviors in rats. More specifically, several studies have shown that predator exposure and social defeat stress are enough to cause PTSD-like symptoms in animals (Borghans & Homberg, 2015). The previous UHCL study used a combined stress model, which was composed of acute stresses and chronic stress, to condition rats to PTSD-like symptoms (Campbell, 2019). After rats were habituated to handling and the housing, they were socially isolated. Rats were put into individual cages for the duration of the experiment. On each of the two acute stress days, rats were restrained in a small cage and exposed to predator odor. For measurements of the level of anxiety, three behavioral tests were used: open field behavior, elevated plus-maze, and acoustic startle response test. The results revealed that the combination of stressors was sufficient for developing anxiety and avoidant behavior.

Physical Stressor

This type of stressor has been used to expose animals to aversive stimuli, such as near-death experiences or accidents, as traumatic events. The single prolonged stress (SPS) model is one of the most common stressors used to induce PTSD-like symptoms in animals. The stressors are usually composed of two hours of restraint and 20 minutes of forced group swim. In this model, rodents are exposed to these stress events until they lose consciousness (Liberzon and Young, 1997). Numerous studies have indicated that rodents later showed PTSD-like behaviors, such as enhanced fear learning, hyperarousal, and cognitive dysfunction (Lisieski et al., 2018). In addition, restraint stress by itself is the other method that has been used as a physical stressor for animal models of PTSD. This stressor involves immobilization in a tight space. This is sometimes enough to cause deficits in the HPA axis similar to those observed in PTSD patients (Liberzon et al., 1997). In the previous study, one-hour restraint combined with predator odor was used to simulate traumatic events. As a result of this exposure, stressed rats showed significantly higher anxiety behaviors than control animals.

Social Stressor

This stressor does not include direct aversive stimuli, pain, or seeming threats to survival. Since rats are social animals similar to humans, both chronic social isolation and maternal separation are somewhat comparable to social stressors in humans. A social stressor is not necessarily utilized by itself to condition animals to the PTSD model. This stressor can be used through combining with other acute stressors, such as physical and psychological stressors. For instance, early life stress is one of the most common methods used in PTSD animal models. The exposure to traumatic events at an early age is a risk factor for PTSD effects such as abnormalities in the HPA axis (Heim & Nemeroff, 2001). The changes in the HPA axis from maternal separation were long-lasting when compared

to the effects of adult traumas (Zoladz et al., 2012). Studies have shown that maternal separation at an early age increases the complexity of PTSD symptoms in the human population (Cloitre et al., 2009). Studies using the psychosocial stress animal model, which was composed of social isolation and predator odor stress, showed that the model increased anxiety behaviors and caused cognitive deficits resembling those seen in PTSD patients (Zoladz & Diamond, 2016).

Psychological Stressor

Physical and social stressors are enough to cause neurological and behavioral abnormalities in animals. However, these stressors do not take into account the various psychological vulnerabilities of PTSD patients (Whitaker et al., 2014). The use of psychological stressors deals with this issue by inducing animals' fear through ecologically natural stressors such as predator odor. Studies have also shown that the exposure of predator scent stress (PSS) induces abnormal responses in both animals' behaviors and physiology, including the HPA axis abnormalities (Albrechet-Souza & Gilpin, 2019). In the previous UHCL experiment, predator odor (wildcat urine) was used as the predator odor. This stressor was combined with chronic social tress (single housing), and a physical stressor (tight restraint), and the results demonstrated there were sufficiently induced biological and behavioral differences from control rats (Campbell, 2019).

Measurements

Elevated Plus Maze (EPM)

The elevated plus maze (EPM) has been widely used as a measurement for rodents' anxiety (Komada et al., 2008). The raised EPM apparatus consists of four arms radiating from a small central square. Two arms are open and the other two arms are enclosed by high walls. Testing begins after the rat is placed in the middle of the plus

maze, facing an open arm. The time spent in the open or closed arms and entries to each arm is usually recorded for the analysis. The EPM has a strong predictive validity, based on the effects of known anxiolytic and anxiogenic drugs. These predictably decrease or increase anxiety scores in the EPM (Komada et al., 2008). The rats with a higher anxiety level will be expected to decrease the entries to open arms and spend more time in the closed arm, which conflicts with rodents' innate motivation to explore novel environments (Walf & Frye, 2007).

Open Field

This behavioral assessment has also been used to measure rodents' locomotor activities and anxiety-related emotional behaviors (Seibenhener & Wooten, 2015). The open field (OF) is made of a large black box with four walls enclosing an open space. For testing, rats are placed into the box, and their behaviors are recorded for analysis. The apparatus is divided into 2 areas: the inner zone and the outer zone near the walls. When rats feel more emotionally insecure, they tend to spend more in the outer zone to avoid open and unknown environments (Seibenhener & Wooten, 2015). Further, rats' locomotor activities also decrease if they feel more anxiety about the environment. Similar to the EPM, rodents' locomotor activities and ethological behavior are collected and used for the analysis, as well as the measurement of the effect of drug effects on rodents' anxiety behavior. OF is a means of ethological assessment of rodents' behaviors. Choleris and colleagues (2001) revealed that anxiolytic drugs increased some behaviors, such as rearing and grooming, which are generally suppressed by anxiety. Several studies on animal models of PTSD utilized OF to evaluate rodents' anxiety behaviors. For instance, a study has found that the increase of noradrenergic activity in the amygdala is associated with the hyperarousal symptoms in animal models of PTSD (Ronzoni et al.,

2016). In this experiment, rats conditioned to the PTSD model by inescapable foot-shock (IFS) decreased locomotor activity and rearing behavior in the open field maze.

Acoustic Startle Response

This behavioral measurement has been used to measure responses to sudden sounds in rodents. Heightened startle responses are also observed in the human population with PTSD (Morgan et al., 1996). During the testing, animals' startle responses to a burst of white noises are observed as unconditioned behaviors that are heightened by anxiety. Anxiety levels are evaluated through the force of the rodent's jumping or flinching repose. Testing is conducted in a sound-attenuated chamber. The chamber has a speaker, and rodents are exposed to randomly repeated bursts of different decibel (dB) level noises. The force of rodents' startle to each noise is measured through a load cell under the holding box in which animals are placed. The load cell detects the peak pressure from the rodent's startle reflex. Normal animals are gradually habituated to loud noise, reducing startle reaction force (Valsamis & Schmid, 2011). In contrast, startle responses are persistently higher in anxious animals than normal control animals (Garrick et al., 2001). Furthermore, the force of the startle response also correlates with vulnerabilities to the development of PTSD (Rasmussen et al., 2008).

Hypothesis

The main purposes of the present experiment were to validate the UHCL animal model of PTSD (Campbell, 2019) and confirm the effect of Pimavanserin on multiple measures of anxiety in this PTSD model. Three kinds of stressors were used in the experiment: chronic social isolation and acute restraint stress with exposure to predator odor. It was hypothesized that these combined stressors would increase scores of anxiety and avoidant behavior in the acoustic startle test, the elevated plus-maze test and the open field. It was also hypothesized that chronic social isolation alone would not produce

comparable effects. Finally, it was hypothesized that Pimavanserin would dose-dependently reduce the PTSD-like effects of the combined stressors.

CHAPTER III:

METHOD

Animal

48 female Lewis rats were used in the experiment. All rats were housed in the climate-controlled housing facility, and they were provided enough water and food as their needs. Light conditions in the housing were a 12-hour light/as-hour light condition, and red-light conditions began at 6:00 p.m. and ended at 6:00 a.m. All procedures of these experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Houston-Clear Lake.

Habituation

Once the animals arrived, they were group-housed for four days to get used to the environment in the housing room. On day five, two rats were paired and put into each cage. In addition, gentle handling started on the same day to get them used to human hands. Each rat was handled for five minutes a day for four days.

Stress Exposure

Social Isolation and Early life Stress

After seven days of habituation to the lab housing room and handling, animals in the stress group were isolated from each other and put into a 47 x 41 x 21 cm clear plastic cage. White panels were inserted into the space between cages to prevent rats from seeing the other rats in the next cage. This social isolation was continued throughout the experiment.

Acute Stress and Sham Stress Procedure

12 and 19 days after the social isolation started, rats in the stress group were exposed to physical (restraints for one-hour) and psychological stressor (cat odor). Animals in the stress group had both acute stress (physical and psychological) and social

stress (isolation). On the other hand, rats in the isolation only group had social isolation, but they were placed into a single cage without stressor (sham stress) on stress exposure days instead of stress exposure. Both stress exposure and sham stress procedures were conducted on the same day. First, the animals were weighed and given an injection of the saline solution before the stress exposure. They were then placed into a single cage. Rats in the stress group were also restrained from their movements for one hour. The other rats were just placed into a single cage with the restrainer and spent one hour without restraints. They were also exposed to either wildcat urine (PMart, Sandy Point, ME) or saline in a petri dish with the restraints. Additionally, a strip of cat collar either worn by a cat (stress procedure) for approximately one month or never worn (sham stress procedure) was put into the petri dish. The petri dish was attached to the restrainer directly in front of the rats' faces to prevent them from avoiding the smell. As a conditioned stimulus, 0.2 second sound bursts with 2 kHz at 70 dB occurred at 30 seconds intervals during the last 30 seconds of the stress/ sham stress exposure.

Injections

Rats in the experimental group received injection of either saline or Pimavanserin on both testing days. 1 mg/kg or 0.3 mg/kg Pimavanserin, which is a selective inverse agonist of the 5-HT2A receptor, was dissolved in 1 mg/kg saline and injected in animals. Injections were managed one hour before the testing.

Effect of pimavanserin on anxiety measures following stressors.				
Days 1-3 Handling	Day 4 Start single caging (continues throughout)	Day 17 Stressors (restraint stress & predator odor)	Day 27 Repeat stressors	Days 34-36 Habituation to Startle apparatus
Days 37-38 Pimavanserin 0, 0.3 or 1.0 mg/kg s.c. 1 hr. later: Behavioral Testing Startle, Plus-maze, Open Field		Day 45 Blood Collection Plasma Preparation for Corticosterone Assay		

Table 1. Timeline of the experiment

Testing

On day 37 and 38, three behavioral tests were conducted: Elevated Plus Maze (EPM), Open Field, and Acoustic Startle Response (ACSR) tests. These tests were run over two days, and tests were counterbalanced across groups. These behavioral testing measured different aspects of PTSD-like anxiety behaviors. All measurements were started at 6:00 p.m. during the red lighting circle. All rooms and apparatus were cleaned one hour before the testing started. One-hour before the testing started, rats received injections of either Pimavanserin or saline. Since the EPM and OF were measured on the same group' animals on the same day, rats had a one-hour cool-down period between tests.

Elevated Plus Maze

The EPM consists of four arms: two arms were open and the other two arms were enclosed by 40 cm tall walls. The apparatus was elevated 50 cm elevated from the floor. One hour before the testing, the room and apparatus were cleaned with 10% bleach water. Testing and recording began once after the rat was placed in the middle of the plus-maze facing an open arm. Rats freely explored the maze for five minutes, and their behaviors were recorded through a video camera. The camera was linked to a computer with AnyMaze software (Stoelting Co., Wood Dale, IL) that counted the time spent in the open/ closed arms and entries to each arm. When a rat fell off from the maze, the observer placed them back in the center square. Once the testing finished, rats were removed and put back into their cages. The maze was cleaned every time the testing was completed.

Open Field

A black open field maze was 76 cm x 76 cm with walls. The inner zone was defined as the area of the central 46 cm x 46 cm square, and the outer zone was 15 cm

along with the walls. In the testing days, all rats were placed on the same corner in the maze and left for 6 minutes. During the last three minutes of testing, the intermittent tone (two kHz at three-second intervals) was played as the cue of the fear-conditioned memory. The tone was the same sound that was used during the stress exposure procedure. Rats' activities were recorded through a video camera suspended over the maze, and it was connected to the computer to save the data. Once the testing was completed, rats were returned to their cages, and the maze was cleaned with 10% bleach water. Data scoring was conducted by using Anymaze software. Rats' behaviors, especially, the exploration time and distance traveled in the inner zone and the entries into the inner zone were used for the analysis. In addition, freezing behaviors during the last three minutes and rearing were counted for the analysis.

Acoustic Startle Response (ACSR)

A sound-attenuated startle chamber (Columbus Instruments, Columbus, Ohio) was used to measure the fourth of the rat's startle responses. A holding box with a cover was set in the chamber, and rats were put into the box during the testing. A load cell under the holding box measured the intensity of rats' startle response after sudden sounds at 90, 100, and 110 dB. Before testing days, rats had three days habituation to the chamber for five minutes. On the testing days, the chamber was cleaned with 10 % bleach water one-hour before the testing. A 1500 g and a 750 g weights were used for the calibration of the startle machine. A background white noise of 65 dB was played while each animal was in the chamber. The 30 randomized trials of white noise bursts (90, 100, and 110 dB) were played for 40 ms each, at 30 sec. intervals. The intermittent tone sounds that were played on the stress/sham stress days were played before the trials for the association with the traumatic memory. The output was recorded using Responder X software (Columbus Instruments, Columbus, Ohio) that was connected to the laptop

computer used for scoring. The mean amplitudes of response to each dB sounds were calculated and used for the analysis.

Statistical Analysis

The data from the three behavioral measurements were used to determine each aspect of anxiety behaviors in the PTSD animal model. The effect of social isolation and the drug (Pimavanserin) were determined through the results from the behavioral measurements. For the evaluation of the stressors, One-Way ANOVA followed by post-hoc comparisons (*Fisher*'s test) were used for the analysis of the data measured by open field and EPM. The data of ACSR were analyzed by using a mixed ANOVA model to assess the intensity of the startle response to each decibel sound. In relation to the drug effects, a *t*-test was used for the comparison of the results between saline and Pimavanserin injections in the stressed group.

CHAPTER IV:

RESULTS

The Effects of Pimavanserin Dose in Stressed Rats

The amplitudes of startle response force in grams during ACSR tests were analyzed through the mixed model of ANOVA (Pimavanserin dose x decibel level), with decibel level as a repeated measures variable. The results (Fig. 1, left panels) revealed that there were significant effects of decibel level, $F(2, 53) = 286.84$, $p < .001$ and pima dose, $F(3, 53) = 3.48$, $p = .038$. However, there was no significant interaction effect, $F(4, 53) = 1.45$, $p = .222$. A Fisher's LSD *post-hoc* analysis indicated that rats in the 1.0 mg/kg pima group had significantly lower overall startle response (averaged across all decibel levels) compared to the overall startle response in saline-injected rats, $p = .016$. Figure 2 illustrates the average amplitudes of animals' startle response in the three stressed groups.

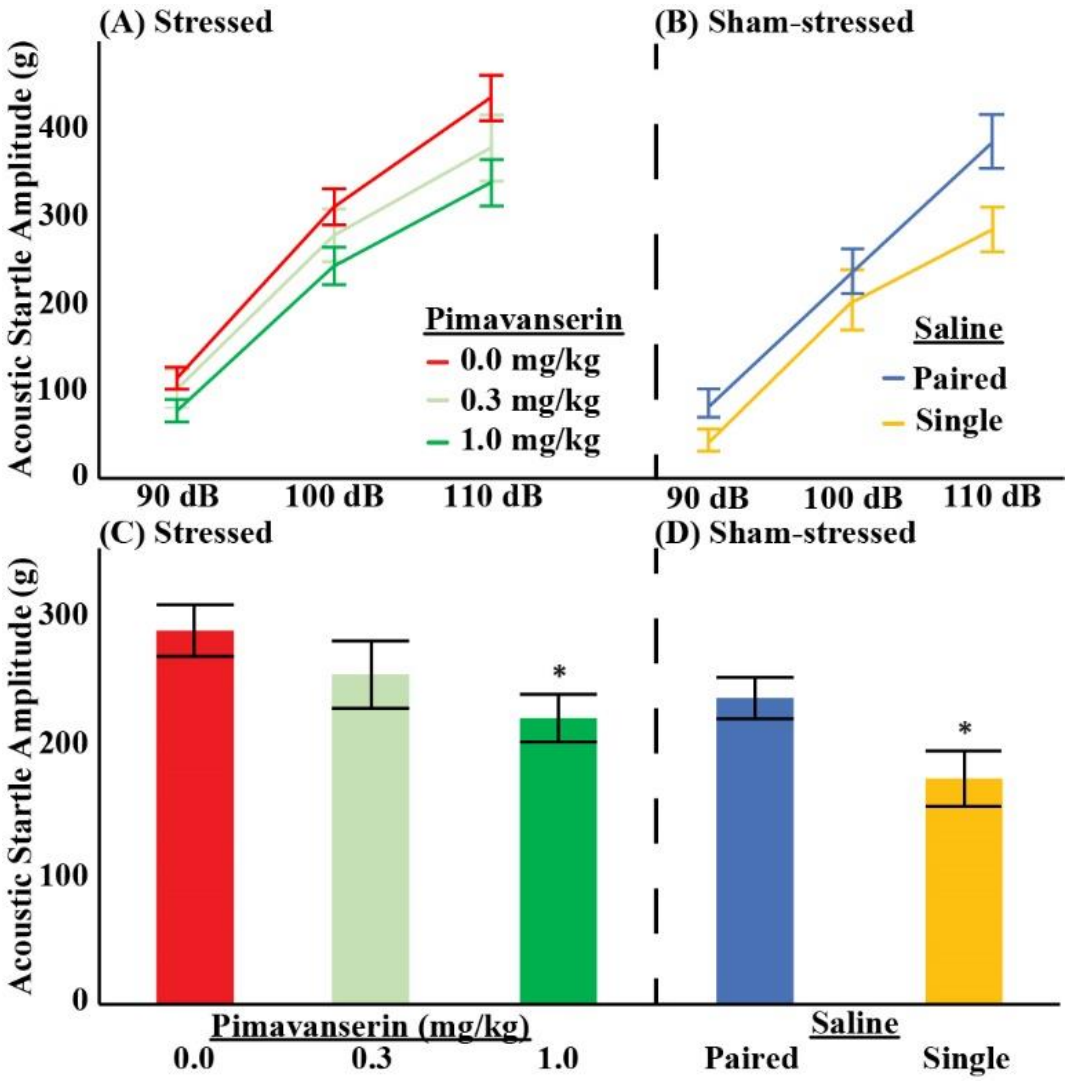

Figure 1. (A) ACSR dose-response under each dB level, (B) ACSR Housing conditions under each dB level, (C) ACSR Avg. over dB dose-response, & (D) ACSR Avg. over dB Housing conditions.

In the elevated plus-maze, the percentage of time that rats spent in the open arm was used to determine the effects of Pimavanserin doses in stressed rats (Figure. 2, left panel). Several rats were removed from the data because they fell off the raised maze. In the comparisons between groups, One-Way ANOVA detected a significant effect of pima dose, F (2, 55) = 3.77, p = .0029. Post-hoc analysis showed that rats injected with 1.0 mg/kg pima spent a significantly higher percentage of time in the open arms compared to rats receiving only a saline injection, p = .016. This suggests a reduction of anxiety.

Time spent in the inner zone and rearing behaviors were collected through the first three minutes of trials in the open field. Freezing behaviors were counted in the last three minutes of the trial. During this time rats were exposed to the same intermittent tone sounded during stress exposure. Figure 3A illustrates the percentage of time rats spent in the inner zone. The reduced time in the exposed inner zone suggests a higher anxiety level. One-way ANOVA of inner zone times was conducted for comparison of the three Pimavanserin doses. The analysis showed that there was a significant difference among groups, F (2, 58) = 5.09, $p < .009$. Fisher's post-hoc test indicated that the animals in the 0.3 mg/kg dose group had a significantly higher percentage of time spent in the inner zone than the animals in saline (p = .001) and 1.0 mg/kg dose group (p = .024).

Episodes of the animals' standing upright were counted as rearing behavior during the open field test (Figure. 3C). In previously stressed rats, a One-Way ANOVA detected a significant effect of Pimavanserin dose, $F(2, 58) = 11.83$, $p < .001$. *Post-hoc* tests showed that the episodes of rearing behaviors of the 1.0 mg/kg group's rats were significantly more frequent than those of rats given saline injections. In terms of the episodes of freezing behavior (Figure. 3E), One-way ANOVA revealed a significant effect of Pimavanserin dose, $F(2, 58) = 5.09$, p = .009. Fisher's test for multiple non-independent pairwise comparisons was conducted for the further analysis. It showed that

the animals in the 1.0 mg/kg pima group had significantly fewer freezing behaviors compared to the 0.3 mg/kg group's (p = .001) and saline injections' animals (p = .002).

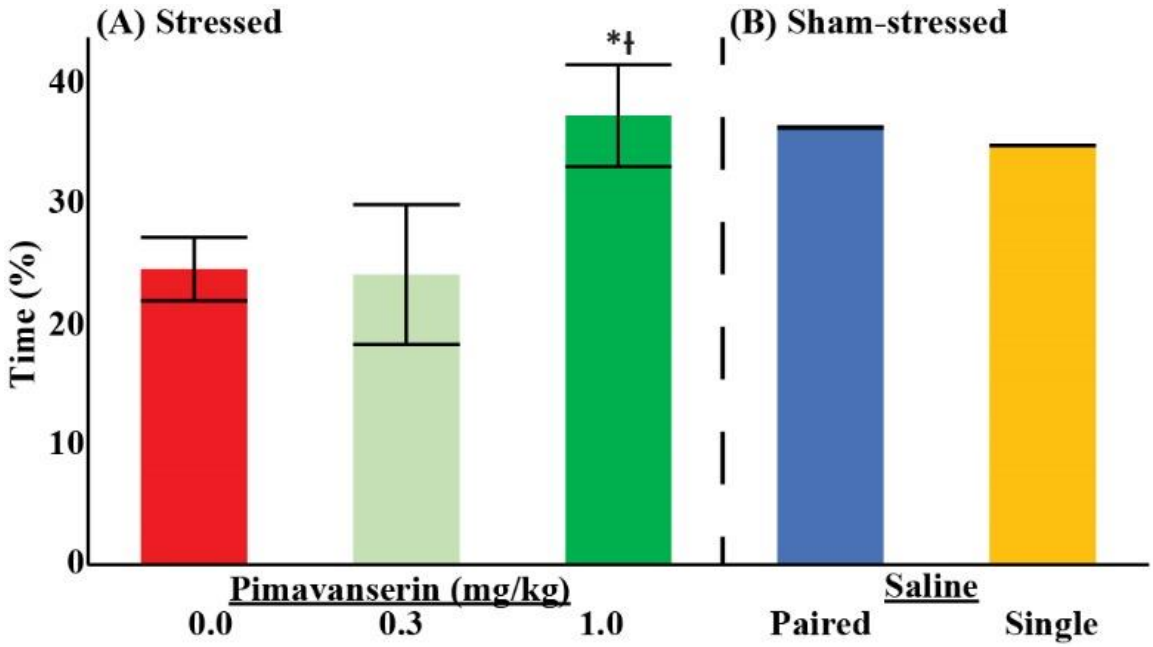

Figure 2. (A) EPM time in open arm % dose-response (B) EPM time in open arm % housing conditions.

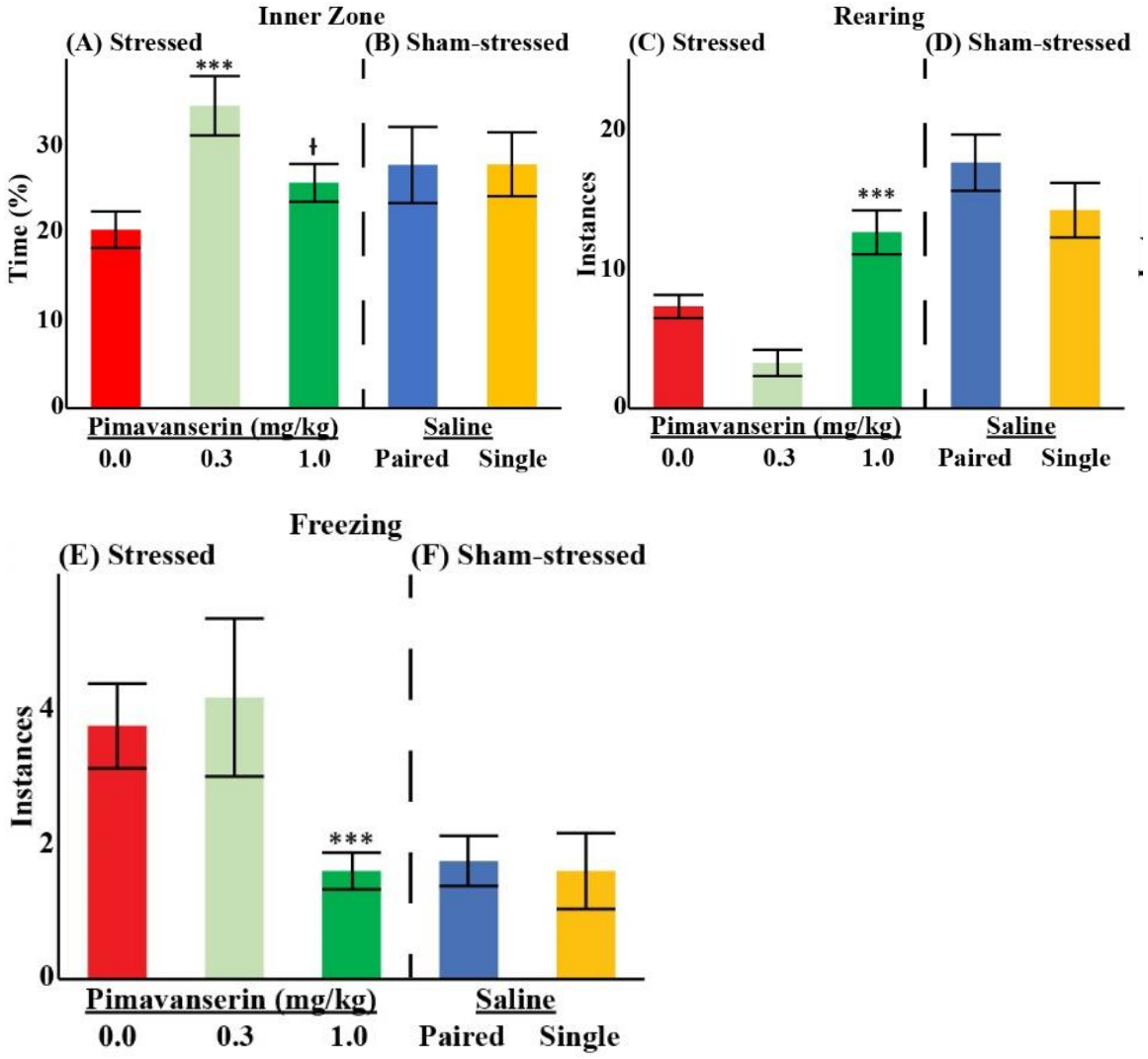

Figure. 3 (A) Pimavanserin dose on time in inner zone of open field in stressed rats. (B) Housing conditions on inner zone time in sham-stressed rats. (C) Pimavanserin dose on rearing episodes in stressed rats. (D) Housing conditions on rearing episodes in sham-stressed rats. (E) Pimavanserin dose on freezing episodes in stressed rats. (F) Housing conditions on freezing episodes in sham-stressed rats.

The Effects of Social Isolation Alone

All the rats in the previous experiment were socially isolated. An experiment was performed to determine whether the three behavioral measures (startle response, elevated plus-maze and open field behavior) might primarily reflect the effects of the social isolation rather than the effects of the stressors. Two sham-stressed groups were compared on these measures: paired-housed and isolated (single-housed) rats. In the acoustic startle measurements (Figure.1, left panels), a two-way ANOVA revealed significant effects of decibel level, $F(2, 40) = 1.37$, $p = .207$, and housing $F(1, 20) = 4.42$, $p = .048$. Additionally, a *t*-test for startle force averaged across decibel levels demonstrated that the isolated animals actually had *weaker* startle responses compared to pair-housed animals, $t(20) = 2.10$, $p = .048$.

As shown in Figure 3 right panels and Table 2, in the elevated plus-maze and the open field, there were no statistically significant differences between paired housed and isolated rats. Thus there is no evidence that the anxiety measures in this study reflect isolation-induced anxiety in the absence of other stressors.

Elevated Plus Maze	**Group(N)**	**Mean ± SEM**	**t value (df)**	***p***
Open Arm % Time	Saline (10)	30.44 ± 7.54%	0.402 (17)	0.69
	Pimavanserin (9)	26.32 ± 6.78%		
Open Field	**Group(N)**	**Mean ± SEM**	**t value (df)**	***p***
Inner Square % Time	Saline (10)	37.87 ± 5.07%	-0.187 (18)	0.85
	Pimavanserin (10)	38.93 ± 2.90%		
Rearing Episodes	Saline (10)	27.4 ± 2.91	0.073 (18)	0.94
	Pimavanserin (10)	27.1 ± 2.92		

Table 2. Effects of Saline and Pimavanserin 1mg/kg in non-stressed rats

CHAPTER V:

DISCUSSION

The purpose of this study was to validate the PTSD animal model that was used in a smaller previous study (Campbell, 2018) and the replication of the effect of the Pimavanserin on the animals conditioned to PTSD. Overall, Pima significantly reduced the rats' anxiety behaviors in three behavioral measurements. However, our results showed that social isolation did not contribute to developing PTSD-like anxiety behaviors with the animal model that was used in the previous study (Campbell, 2018). In relation to the effect of Pimavanserine, four out of five effect sizes were large, indicating robust and consistent effects.

The effects of Pimavanserin

The results from four out of five behavioral measurements showed that Pima injections at higher doses significantly reduced anxiety behaviors within stressed animals. The higher 1.0 mg/kg dose Pima injections had significant effects on animal behaviors, although, in four cases, 0.3mg/kg injections did not cause a significant difference from the saline injections' animals. Unexpectedly, in time spent in the center of the open-field, the lower dose (0.3 mg/kg) significantly increased the percentage of time spent in the inner zone. However, 1.0 mg/ kg injections did not differ significantly from saline injections. This result is puzzling. However, Catherine and colleagues (2015) also found that the smaller dose of a 5-HT2A antagonist significantly altered the amount of time spent in the inner zone of the open field compared to the vehicle injections animals, even though the larger dose did not significantly differ from vehicle injections. Four out of five of the effect size of 1.0 mg/kg Pimavanserin vs. vehicle were large. The results indicated that the effects of Pimavanserin were dose-dependent with higher doses having robust effects on animal behaviors.

In the study, the drug was injected before the stressor (social defeat). The results of the study (Catherine et al., 2015) indicated the need for more research on dose-dependent variables. The activation of serotonergic receptors is associated with various stressors (Chaouloff et al., 1999). For example, León and colleagues (2017) used the 5-HT2A antagonist ketanserin to increase the time spent in the open arm of the EPM and to reduce anxiety-like behaviors such as freezing. Furthermore, Adamec and colleagues (2004) injected another antagonist of 5-HT2A receptors, EMD 281014, and measured the ASR and EPM behaviors. Their results also showed that EMD 281014 significantly reduced the effects of predator stress on the behavior of mice. In the study, 5-HT2A antagonist was injected after predator stress exposure, and then behavioral measurements were conducted one week later. Additionally, Xiang and colleagues (2019) found that inactivating 5-HT2A receptors in stressed animals reduced delayed like anxiety-related behaviors. In both of these studies, the 5HT2A antagonists were administered at the time of the stressors. In contrast, Pimavanserin was injected weeks after the stressors in the present study. Therefore, the results of the current study suggest that administering 5HT2A inverse agonists, such as Pimavenserin might be helpful in treating ongoing, chronic PTSD. Since Pimavanserin is already FDA-approved for another disorder, clinical studies on the effects of Pimavanserin on PTSD patients should be considered.

The Effect of Social Isolation

A combined model of acute and chronic stressors was used in the current study. Social isolation was used as the chronic stressor in all animals in Experiment 1. Weiss and colleagues (2000) have shown that social isolation-induced abnormalities of the acoustic startle response, as well as the reduction of locomotor activities, in several species of rodents. This raised the question, might the behavioral measurements in the PTSD model reflect social isolation rather than the acute stressors (predator odor and

immobilization stress)? In the absence of acute stressors, the results from the acoustic startle response did show a significant difference between single-housed and paired-housed rats. However, the socially isolated rats actually had significantly lower anxiety scores on that measure. On the other measures, behavioral measurements did not indicate any significant differences between single-house and paired-housed animals.

Limitation and Future Direction

There were several limitations to this experiment. First, conducting several cohorts of the study during the pandemic under stringent health precautions tended to disorganize the timeline of the experiment. There were also some changes in the experimental design from the earlier cohorts of animals. In the later cohorts, the animals had several blood draws for the measurement of hormonal changes in the other study. These might possibly have affected the animals' anxiety level. In addition, we used female rats exclusively in the experiments because females are more susceptible to develop PTSD symptoms both in the human population and animal models. It remains unknown how the PTSD model would affect male rats and how any such effect would be altered by Pimavanserin. Although single-housing did not intensify anxiogenic effects in the absence of acute stressors, it remains unknown whether social isolation actually intensifies the effects of those stressors. It also remains to be seen whether Pimavanserin given at the time of the stressors might reduce the subsequent development of a PTSD-like state.

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