Drew University

College of Liberal Arts

Sex Differences for Developing Anxiety or Depression Related Behaviors After Early Life Exposure to Chronic Variable Stress (CVS) in Long-Evans Rats

A Thesis in Neuroscience

By

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Abstract

Anxiety and depression are two of the leading mental health issues world-wide, affecting people of all ages. Stress and biological sex are believed to play a role in the development of both disorders. Specifically, stress is associated with elevated levels of cortisol, a biomarker of anxiety and depression. Additionally, women are significantly more likely to be diagnosed with anxiety and depression than men. Chronic variable stress (CVS) is a commonly used model in rodents to explore how repeated exposure to various microstressors can lead to long-term behavioral changes. The aim of this present study was to test the hypothesis that female Long-Evans are at a higher risk than males for developing anxiety and depression related behaviors after early life exposure to CVS.

Stressors used were: immobilization, predator odor, cage tilt, strobe lights, ultrasonic deterrent, and cold room. As an additional stressor, rats were housed overnight in isolation cages twice a week. Behavioral tests used were: open field test (OFT), elevated zero maze (EZM), sucrose preference test (SPT), forced swim test (FST), and cocaine-induced locomotor activity (CLA). Body weights were recorded weekly. At the conclusion of the experiment, animals were sacrificed, and organs were weighed. For weekly body weights, as a percentage of baseline weight, males weighed more than females for weeks 2-10. Additionally, control animals weighed more than CVS animals for weeks 2-4. During the OFT, males spent more time in the outer zone than females; however, females spent more time in the middle and center zones. Females also traveled a greater distance than males in all three zones. During the FST, females had a greater latency to immobility on day one than males. During the CLA, females had greater locomotor activity than males.

Key words: Sex differences, chronic variable stress, anxiety, depression

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CHAPTER I: INTRODUCTION

Anxiety and Depression

Affecting 301 million people in 2019, anxiety is the most prevalent mental health issue world-wide (World Health Organization, 2023a). In the United States alone, the statistic is 40 million adults, or about 19% of the population (Anxiety & Depression Association of America, 2022). Within anxiety, there are many specific disorders: generalized anxiety disorder, panic disorder, social anxiety disorder, post-traumatic stress disorder, and phobia-related disorders (National Institute of Mental Health, 2023a). Symptoms can vary in severity and include fatigue, irritability, headaches, difficulty sleeping, and a racing heartbeat. Major depressive disorder, often referred to as depression, is another common mental illness. Out of the approximately 280 million people around the globe who live with depression, 20 million of them are U.S. adults (World Health Organization, 2023b; National Institute of Mental Health, 2023b). While some symptoms of depression may overlap with those of anxiety, others include feelings of helplessness, loss of interest or pleasure in hobbies and activities, changes in appetite, and thoughts of death or suicide.

Anxiety and depression are two distinct mental illnesses; however, it is important to note their high comorbidity (simultaneous presence) rate (Kalin, 2020). Several risk factors that put an individual at risk for developing anxiety and depression have already been identified, two of which are genetics and environment (Anxiety & Depression Association of America, 2022). Additionally, there are many existing treatment methods that have proven effective: psychotherapy (e.g., cognitive behavioral therapy), medication (e.g., antidepressants), support groups, and stress-management techniques (e.g., exercise and meditation) (National Institute of Mental Health, 2023a). Unfortunately, only a fraction of people who are suffering from anxiety

and depression-related symptoms actually receive treatment (Anxiety & Depression Association of America, 2022). This is likely due to the current stigma surrounding mental health, where some individuals may feel embarrassed or not know how to ask for help. Socioeconomic status (SES) also plays an important role: low SES is not only a risk factor for mental illness, but less affluent communities lack opportunities to become educated and afford treatment options (Reiss et al., 2019).

Serotonin (5-hydroxytryptamine, 5-HT), norepinephrine (NE), and dopamine (DA) have all been implicated in the pathogenesis of anxiety and depression (Martin et al., 2009; Maletic et al., 2007). 5-HT has several behavioral effects: mood, perception, memory, anger, aggression, fear, stress response, appetite, addiction, and sexuality (Berger et al., 2009). After being synthesized from tyrosine in the raphe nuclei of the brainstem and enterochromaffin cells of the intestinal mucosa, it is stored in intracellular vesicles (Bamalan et al., 2024). Once 5-HT is released and bound to GPCRs, it is recycled back into the cell via serotonin transporter (SERT) and stored in vesicles or metabolized by monoamine oxidase (MAO) in the cytoplasm.NE is involved in the regulation of arousal, attention, cognitive function, and stress reactions (Hussain et al., 2024). After being synthesized from tyrosine (or phenylalanine) in the chromaffin cells of the adrenal medulla, it is stored in chromaffin granules. After NE is released into the synaptic cleft, it can undergo reuptake or degradation. DA plays an important role in motor control, motivation, reward, cognitive function, maternal, and reproductive behaviors (Klein et al., 2019). It follows the same enzymatic synthesis as NE, because it is actually a precursor in NE synthesis (Sonne et al., 2024). After being released from synaptic vesicles, it can bind to GPCRs D1 and D2. Finally, it can be transported back into the presynaptic neuron via DA transporters (DAT) to be repackaged, taken up by glial cells, or metabolized by the cellular membrane.

By expanding understanding of anxiety and depression through research, the impact mental illness has on individuals and communities can be reduced. Strategies include: creating more targeted prevention and intervention programs, alleviate individual suffering with effective and personalized treatment, lessen the public health and economic burden, and reduce the stigma by increasing understanding and support. On a federal level, Frank et al. (2023) recommend the government to "build the evidence base of preventative mental health care services in nontraditional settings" among other actions addressing the mental health crisis in the U.S. This would be an expansion of multiple initiatives proposed by the Biden administration at the 2022 State of the Union address and budget request for 2023.

Mental Health in Children and Adolescents

Promoting mental health in younger populations is just as important. Childhood and adolescence are both critical periods for development, characterized by significant physical, cognitive, emotional, and psychosocial growth. These changes are largely driven by the reorganization of neural circuitry (Gilmore et al., 2018). In the case of puberty, a surge of gonadal steroid hormones is also a contributing factor (Konrad et al., 2013). Additionally, Cohen et al. (2018) argue that childhood and adolescence are "critical periods for the emergence of anxiety and depression." This is partly due to the brain's plasticity and heightened sensitivity to environmental experiences. With growth curve modeling, their research found that childhood anxiety predicted both adolescent anxiety and adolescent depression, and adolescent depression was predictive of adolescent depression.

While the the COVID-19 pandemic triggered a mental health crisis amongst teens (Gotlib et al., 2022), an upward trend in anxiety and depression was already occurring: from 2016 to

2019, there was a significant increase in anxiety (27%) and depression (24%) in children and adolescents (Lebrun-Harris et al., 2022). This is likely the result of a combination of factors including 1) increasing usage of social media on a daily basis: teens have a tendency to compare themselves to their peers and what they see on the internet, which can be detrimental to self-esteem; 2) online bullying: social media and technology can foster increased bullying and exclusion as it can happen anytime, anywhere; 3) intense academic pressure: parents (and society) can place an exaggerated emphasis on academic achievement, leaving the child to prioritize grades over wellbeing; and 4) environmental stressors: increasing political polarization (specifically in the U.S.), global events, and climate change can all create worry and anxiety about the future.

Additionally, it is worth considering how increased awareness and discussion surrounding mental health may contribute to a *perceived* increase in anxiety and depression in teens. In other words, there is a dramatic change in the diagnosis, rather than in the occurrence. Social media is a large driving force. For example, some individuals who are diagnosed with some sort of mental disorder go online to share what their symptoms look like, and how they impact their daily life. This type of content can help viewers get better at recognizing signs of someone (including themselves) struggling with mental illness, and can seek professional help. Unfortunately, there is also a downside: mental health professionals are now witnessing a growing number of children and teens self-diagnose a mental disorder after watching an influencer talk about it on a social media platform, such as TikTok (McVay, 2023). Without having the appropriate training required and professional expertise, an individual cannot accurately diagnose themselves. This could result in inappropriate or ineffective treatment

strategies, such as self-medication. Self-diagnosis can also be harmful because symptoms can worsen over time if they are not adequately addressed.

Stress as a Factor

Stress involves the activation of two major pathways: the sympathetic nervous system (SNS) and the hypothalamic-pituitary-adrenal (HPA) axis (Chu et al., 2024). Fast activation of the SNS (also known as the "fight or flight" system) leads to increased secretion of norepinephrine (NE) and epinephrine (E) from the adrenal medulla (of the adrenal glands, located right above the kidneys), as well as increased secretion of NE from the sympathetic nerves. NE and E then bind to alpha-adrenergic and beta-adrenergic G-protein coupled receptors (GPCRs). This leads to activation of the cyclic adenosine monophosphate (cAMP) signaling pathway. These cellular responses result in an elevated heart rate, relaxed airways, dilated pupils, and more (McCorry, 2007).

When the HPA axis is activated (slow response), corticotropin releasing hormone (CRH) is released from the hypothalamus to stimulate the release of adrenocorticotropic hormone (ACTH) from the anterior pituitary (Molina, 2023). Both structures are located towards the base of the brain. ACTH then stimulates the adrenal cortex (of the adrenal glands) to release the hormone cortisol. Regulated by a negative feedback loop, cortisol is directly controlled by ACTH, and indirectly controlled by CRH. It binds to type II (glucocorticoid) receptors to activate glucocorticoid response elements (GREs) on DNA. In addition to mediating the stress response, cortisol also regulates metabolism, the inflammatory response, and immune function (Thau et al., 2024). Existing research has found a strong correlation between elevated cortisol

and anxiety and depression, making it a significant biomarker for both disorders (Dziurkowska and Wesolowski, 2021).

Overactivation of the HPA axis can have an effect on several organs. First, it can lead to an increase in adrenal glands size (Ulrich-Lai et al., 2006). Secondly, it can reduce the size of the thymus, which is involved in the immune system (Živković et al., 2005). Lastly, research has implicated that the hippocampus is involved in regulation of the HPA axis, and can decrease in volume as a result of stress (Cole et al., 2022; Kim et al., 2015).

Sex as a Factor

Biological factors are also believed to play a role in the development of both anxiety and depression. Specifically, biological sex. Existing research supports that women are twice as likely to develop anxiety and depression than men (Kundakovic and Rocks, 2022). While specific underlying mechanisms remain unclear, a possible explanation for these differences is the hormonal fluctuations (particularly estrogen) that females experience. Serotonin is a neurotransmitter that regulates several activities in the body, including mood; decreased levels have been linked to anxiety and depression, among other mental disorders (Bamalan et al., 2024). Evidence suggests that estradiol, a form of estrogen, increases serotonergic neurotransmission via modulation of gene expression (Hernández-Hernández et al., 2019). Thereby, decreases in estrogen might play a role in anxiety and depression. While men lack systemic estrogen, research demonstrates that testosterone in the male brain is converted into estrogen by the enzyme aromatase (Albert, 2015). This estrogen could act as a protective factor.

Model of Chronic Variable Stress (CVS)

Implemented in over 1,300 published studies, the chronic mild stress (CMS) model of depression is a robust experimental tool that has made valuable contributions to our understanding of how chronic stress impacts neurobiology (Willner, 2017). In this model, rodents are repeatedly exposed to various microstressors, leading to anxiety-like and depression-like behaviors. In some papers, the term chronic variable stress (CVS) is used instead of CMS; however, the underlying features of the procedure remain the same. Existing research supports that applying a CVS model to rats results in long-term behavioral changes.

Cotella et al. (2019) studied the long-term effects of CVS in adolescent Wistar male rats compared to adults. Their stress paradigm involved two daily stressors (no stressor was repeated on the same day) across two weeks. The stressors were: hypoxia, rotation, cold exposure, lithium chloride injection, foot shock, space restriction, and novel environment. Additionally, an overnight stressor (social isolation or overcrowding) was applied twice a week. After five weeks of recovery, behavior was evaluated with the elevated plus maze (EPM) and forced swim test (FST). These researchers found that CVS exposure during adolescence resulted in decreased exploration of the EPM and increased immobility during the FST compared to the control group Cotella et al., 2019). These changes were not observed in animals exposed to CVS as adults.

Similarly, Bourke and Neigh (2011) studied whether adolescent stress resulted in sustained, depressive-like behavior in male and female Wistar rats. The stress model lasted two weeks and consisted of isolation with exposure to alternating social or restraint stress on a daily basis. Behavioral testing took place during both adolescence and adulthood. These researchers found that female rats exposed to chronic stress during adolescence displayed a decrease in sucrose consumption, hyperactivity in the EPM, and decreased activity in the FST compared to

control groups during adolescence and adulthood (Bourke and Neigh, 2011). No significant behavioral changes were observed in the males.

Hypothesis

Given the current research on stress and sex as factors in the development of mental illness, it was hypothesized that female Long-Evans would exhibit more anxiety and depression related behaviors after early life exposure to chronic variable stress.

CHAPTER II: BEHAVIORAL TESTS

Open Field Test (OFT)

Fear of the unknown is a common symptom of anxiety. This behavior can be modeled in animals using the open field test (OFT), also known as the open field maze (Seibenhener and Wooten, 2015). Rodents are naturally motivated to explore a novel environment, however, this inclination decreases with anxiety (Lecourtier et al., 2023). Thigmotaxis, which refers to an animal's tendency to stay close to the walls, can be related to fear of the unknown and taking risks. Although the walls of the field are still part of a novel environment, rodents feel safer near them because they provide a sense of shelter and protection. When they are in the center of the field, they feel much more exposed and vulnerable to predators. During the OFT, the animal is placed in a large (typically square-shaped) wall-enclosed area and given the opportunity to explore freely. For analysis, video tracking software is used to define parameters within the maze (e.g., outer zone and inner zone) and record the subject's movements. Some examples of data the software can be set up to collect include: distance traveled, average speed traveled, number of zone transitions, and time spent in a specific zone. It can be interpreted that an animal that spends more time in the center of the field is likely less anxious compared to one that spends relatively less time in the center. Additionally, anxiety-reducing agents used to treat humans have been found to suppress thigmotaxis in rats (Treit and Fundytus, 1988).

Elevated Zero Maze (EZM)

Fear of the unknown can also be modeled in animals using the elevated plus maze (EPM) (Braun et al., 2011). The apparatus is an elevated platform in the shape of a plus sign, with two opposing quadrants enclosed by walls (these sections are referred to as the closed arms of the

maze). The elevated zero maze (EZM) is a modified version of EPM, with an annular-shaped platform (Braun et al., 2011). This difference in design eliminates any ambiguity posed by the center of the EPM since researchers cannot agree on whether this section should be considered an open arm, closed arm, or its own section. During the test, the animal is placed in the apparatus and given the opportunity to explore freely. Similar to the OFT, this unconditioned, approach-avoidance task "rel[ies] on rodents' innate conflict between approaching and exploring (foraging) vs. avoiding potentially dangerous areas" (Tucker and McCabe, 2017). Rodents feel safer in the closed arm of the maze, and less safe in the open arms.

Sucrose Preference Test (SPT)

Anhedonia, the inability to experience pleasure is a common symptom of depression (Markov, 2022). This behavior can be modeled in animals using the sucrose preference test (SPT). During this test, animals are acclimated to the presence of two bottles; one containing water, and the other containing a 1-2% (weight/volume) sucrose solution (Liu et al., 2018). Once the animals have acclimated, intake from both bottles is recorded (typically at the end of a 24 or 48 hour time period). Sucrose preference is measured by sucrose solution intake over total intake. The SPT assumes that since animals have a natural preference for sweet flavors, sucrose preference can be positively correlated with the amount of pleasure an animal experiences from consumption of the solution (Hoffman, 2016). Therefore, animals with lower sucrose preference are more likely to be depressed.

Forced Swim Test (FST)

Learned helplessness is a common symptom of depression. This behavior can be modeled in animals using the forced swim test (FST) (Yankelevitch-Yahav et al., 2015). The protocol consists of two sessions, 24 hours apart. During the first session, animals are placed in a transparent cylinder filled with room-temperature water (deep enough so that it cannot stand on hind legs) for 15 minutes. They are then removed from the water, dried off, and returned to their cage. This is repeated for the second session, the "test day", except that the animals only spend five minutes in the water. During this session, latency to immobility and total time immobile are recorded. The FST assumes that after the first session, animals learn their efforts to escape are pointless; no amount of thrashing or swimming will help them get out of the water any sooner. When they are exposed to the stressor a second time, animals that "give up" sooner, are likely more depressed. A huge limitation of this approach is that most researchers do not record behavior on the first day because they already assume that there is a change in behavior on the second day. In the event that there are no changes, these changes would go unobserved. If that is the case, it would not be valid to claim that differences in latency and time immobile between groups on the second day are indicators of depression. Additionally, both sessions should be the same length of time (15 minutes).

Cocaine-Induced Locomotor Activity (CLA)

Heightened sensitivity to psychostimulant drugs, such as cocaine and amphetamine, is a common symptom of exposure to early life stress (Maldonado and Kirstein, 2005). This behavior can be modeled in animals using cocaine-induced locomotor activity (CLA). During this test, animals are administered with an acute injection of cocaine (typically 20 or 30 mg/kg/ip) or

saline, then immediately placed in an open field chamber. Behavior is recorded for 30 minutes. A limitation of not recording baseline activity levels is that it does not take into account potential individual differences (e.g., an animal with low baseline activity may still have a significant increase in activity after being injected with cocaine, but still relatively low activity).

CHAPTER III: METHODS

Subjects

12 male and 12 female post-weanling (21 days old) Long-Evans rats were purchased from Envigo (N = 24). They weighed 50-74g and 35-60g upon arrival, respectively. Animals were group-housed (three per cage) in a temperature and humidity controlled vivarium, with a 12-hour light/dark cycle. Each cage (38.1 x 33.0 x 17.8 cm) was supplied with bedding (Teklad Laboratory Grade Aspen Bedding), and enrichment accessories (cardboard hut, wooden block, nylon bones; Bio-Serv). Food (Teklad Laboratory Diet) and water were available *ad libitum*. Identification numbers were assigned to all animals, and written on their tails in permanent marker. Individual weights were recorded on a weekly basis. Cages were also cleaned and changed with fresh bedding each week. All procedures were approved by the Institutional Animal Care & Use Committee (IACUC) at Drew University.

Two days after arrival, baseline weights were measured. Rats were then divided into four groups (n = 6): male control, male CVS, female control, and female CVS. The groups were counterbalanced to avoid any pre-existing differences in weight.

Chronic Variable Stress (CVS) Protocol

The CVS protocol was based on one used by Cotella et al. (2019). Nearly a week after arrival, animals in the CVS groups were exposed to various psychological stressors across a three-week period. All of the stressors were administered in a separate room, and once completed, animals were returned to the same housing room as the control animals. The stressors were given twice a day, every day, and at variable times (AM between 9:00 am-12:00

pm and PM between 1:00-5:30 pm). The six main stressors used were: immobilization (60 min), predator odor (90 min), cage tilt (30 min), strobe lights (60 min), ultrasonic deterrent (60 min), and cold room (60 min). No stressors were repeated on the same day. As an additional stressor, rats were housed overnight in isolation cages twice a week. During this three-week period, animals in the control groups continued to be housed under standard conditions and were only handled for weighing and cage changes. At the end of the protocol, rats within each of the four groups were re-housed into pairs (still counterbalanced for weight). This was done to ensure that as the animals (especially the males) continued to grow, they would still have sufficient room in the cage. It also prevented two bigger, more dominant rats from ganging up on a smaller, more submissive rat.

During immobilization, rats were placed head first into a DecapiCone (Braintree Scientific) that was firmly secured with binder clips. They were then laid in rows on a large, flat metal sheet. During predator odor, two bobcat urine scent release canisters (Foggy Mountain) were taped open to the bottom right corner of each cage. During cage tilt, rats were placed in an empty (with the exception to bedding) cage on top of a moving orbital shaker (150 rounds per minute). For this stressor, all six rats from a group (e.g., female control) were placed in the cage at a time. During strobe lights, cages were placed in a dark room with an LED machine flashing white lights at a high frequency. During ultrasonic deterrent, audio from *Mouse & Rat Ultra Sonic Deterrent. Mice Ultrasonic Repellent. 12 Hours* (Ambience World, 2018) was played from a speaker on a moderate volume. During the cold room, cages were placed in a dark, ventilated room kept at 4 °C. For overnight isolation (~7:00 pm-10:00 am), rats were separated into slightly smaller cages (41.9 x 26.7 x 15.2 cm) without any enrichment accessories. Food and water were still available.

Time Course

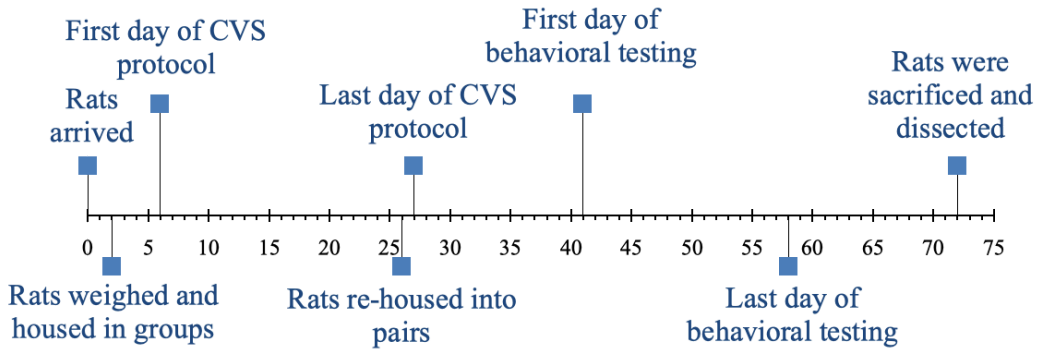


Figure 1. Timeline (in days) featuring key milestones of the experiment. The CVS stressors included: immobilization (60 min), predator odor (90 min), cage tilt (30 min), strobe lights (60 min), ultrasonic deterrent (60 min), and cold room (60 min). A daily schedule of the specific stressors applied can be found in Table 1 of the Appendix.

Table 1. Behavioral test schedule.

Day	Behavioral Test
41-42	OFT
43	EZM
44-49	SPT
50-53	FST
54-58	CLA

Behavioral Testing

Behavioral tests for all 24 rats took place starting two weeks after the last stressor, and ran across a 2-week period. With the exception to the sucrose preference test (SPT), which took

place in the housing room, all tests were administered in a separate room. For each test, animals were given a minimum of 30 minutes to acclimate to the new room. The open field test (OFT) and elevated zero maze (EZM) were used to measure anxiety-related behaviors. The forced swim test (FST) and sucrose preference test (SPT) were used to measure depression-related behaviors. Cocaine-induced locomotor activity (CLA) was also taken as a measurement to compare how rats that experienced early life stressors responded to a stimulant compared to their counterparts.

Procedures for the OFT were based on those used by Seibenhener and Wooten (2015) to measure locomotor and anxiety-like behavior in mice. A ceiling-mounted video camera was connected to AnyMaze behavioral analysis software and used to split the open field chamber (1-meter square box) into three zones (Fig. 2). Each painted box measured 10 x 10 cm. The outer zone represented the two outermost areas of boxes within the open field; the middle zone, which was completely surrounded by the outer zone, was made up by the next two area of boxes; and the center zone consisted of four boxes in the center of the field. At the start of the test, a single rat was placed in the chamber facing one of the corners of the outer zone. It was then given 10 minutes to roam freely. AnyMaze was used to measure total time spent (seconds) and total distance traveled (meters) in each of the three zones. At the end of each trial, the animal was returned to its cage and the chamber was cleaned with 70% ethanol.

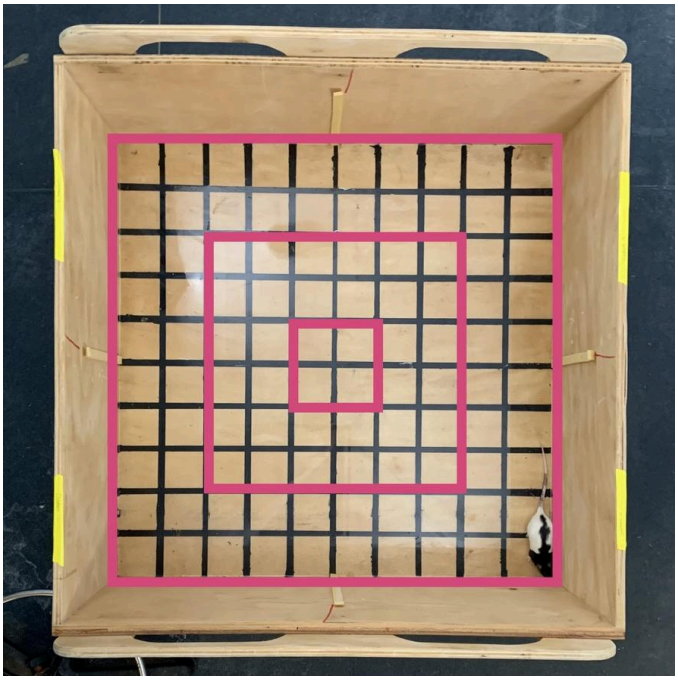


Figure 2. Open field test apparatus. Behavior was monitored for ten minutes. Pink boxes were layered on top of the image to illustrate the three zones. Animals with higher levels of anxiety typically spend more time in the outerzone with less movement. The rat pictured above is currently exhibiting thigmotaxis.

Procedures for the EZM were based on those used by Knight et al. (2011) to examine the effects of anxiolytic (anxiety-reducing) and anxiogenic (anxiety-causing) agents in male rats. At the start of the test, a single rat was placed in the maze so that their lower half was inside of one of the closed arms, and their head and front limbs were in the open arm. They were given five minutes to explore the apparatus (Fig. 3). One timer was used to record latency (seconds) to the open arm, while another was used to record total time (seconds) spent in open arms. A thumb counter was used to record the number of transitions. A transition was defined as both of the back paws crossing the taped line between the open and closed arms (i.e., a rat moving from closed to open and back to closed counted as two transitions). If an animal fell off the platform (which only occurred once), the timer was paused until the animal was put back in the same place it fell from. At the end of each trial, the animal was returned to its cage and the chamber

was cleaned with 70% ethanol.

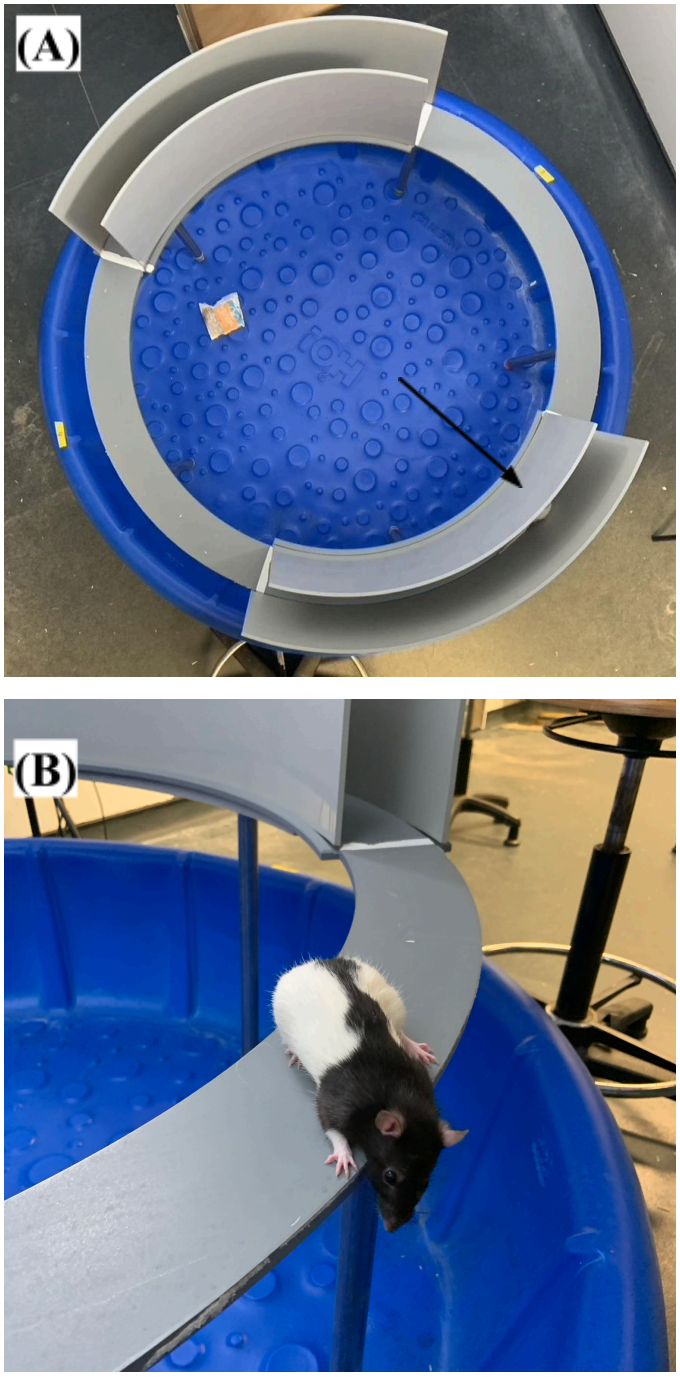


Figure 3. Elevated zero maze apparatus. Behavior was monitored for five minutes. White tape reinforces the borders between open and closed arms. Animals with higher levels of anxiety typically spend more time in the closed arms than open, and make fewer transitions between the two sections. **(A)** Bird's eye view of the maze. The arrow indicates a nearly-hidden rat in the closed arm. **(B)** A rat's relative size to the platform.

Procedures for the SPT were based on those used by Liu et al. (2018) to measure stress-induced anhedonia in mice, with modification. Rats remained in paired-housing. Over a 72-hour period, the animals were habituated to the presence of two drinking bottles (both filled with water). Once acclimated, water in one of the bottles was replaced with a 1% sucrose solution. A single drop of green food coloring was added to the solution to make it easily distinguishable from the water (Fig. 4). After 24 hours, water and sucrose intake for each cage was measured. Sucrose preference was calculated as a percentage of sucrose intake over total fluid intake.

Figure 4. Sucrose preference test setup. Intake was measured after 24 hours. Animals with higher levels of anhedonia, a symptom of depression, typically have a weaker sucrose preference.

Procedures for the FST were based on those used by Yankelevitch-Yahav et al. (2015) to model depressive-like behavior in rats, with modification. A clear testing cylinder was filled with water (25-28 °C) to a level that was too high for the rats to stand on the bottom, but low

enough to prevent them from climbing out (Fig. 5). At the start of the test, the animal was placed into the cylinder head first and a timer was set for 10 minutes. A second timer was used to record the latency to immobility (seconds), while a third timer was used to record the total time spent immobile (seconds). In this study, an animal was considered "immobile" once all four limbs (and tail) went completely still. In many cases, immobility would only last a brief instance. At the conclusion of the swim, the rat was removed from the cylinder, dried off with a fresh towel, and returned to its cage. A small hand net was used to clean feces out of the apparatus before the next trial. The test was repeated 24 hours later.

Figure 5. Forced swim test apparatus. Behavior was monitored for ten minutes. Animals with higher levels of depression typically have a lower latency to immobility, and spend more time immobile.

Procedures for the CLA were based on those used by Maldonado and Kirstein (2005) to compare how mild stress influences cocaine-induced activity in adolescent and adult male rats, with modification. Rats were individually given a 20 mg/kg intraperitoneal injection of cocaine hydrochloride dissolved in saline. After each injection, they were immediately placed in a 40 x

40 cm open field with an infrared motion detection grid for 10 minutes (Fig. 6). Locomotor activity was measured by the number of horizontal beam breaks. At the end of each trial, the animal was returned to its cage and the chamber was cleaned with 70% ethanol.

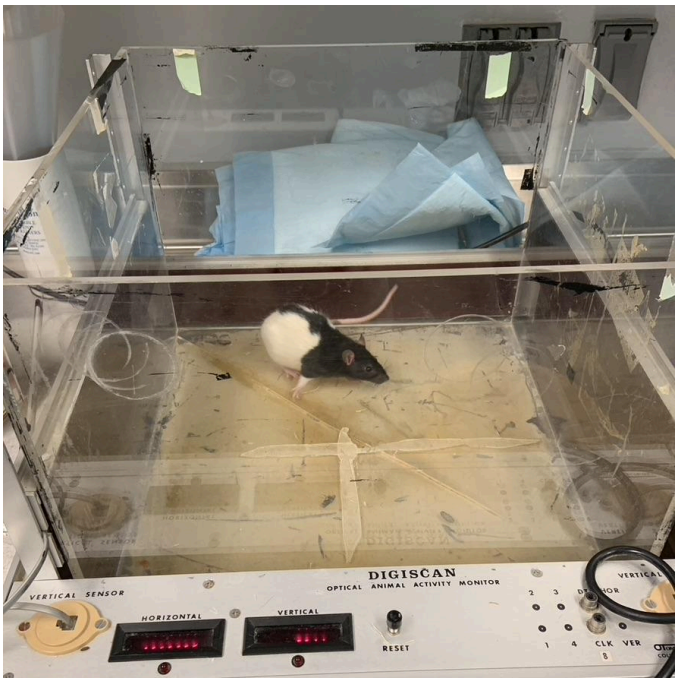


Figure 6. Cocaine-induced locomotor activity apparatus. Behavior was monitored for ten minutes. Animals with past exposure to early life stressors typically have a larger increase in locomotor activity after being given a stimulant.

Dissections

Two weeks after the final behavioral test was administered, the rats were sacrificed via carbon dioxide asphyxiation. Brains were removed, frozen on powdered dry ice, and stored at -80 °C for potential future analysis. The adrenal glands and thymus from each animal were removed and weighed. These weights were then calculated as a percentage of baseline body weights. Animal carcasses were disposed of following Drew University guidelines for hazardous waste disposal. All staff handling contaminated items wore appropriate protective clothing and gloves.

Data Analysis

Statistical Package for the Social Sciences (SPSS) software was used to conduct data analysis. Multiple 2 x 2 two-way analyses of variances (ANOVAs) (manipulating sex and treatment) were run to determine differences. Three-way mixed model ANOVAs were also run for the FST and weekly body weights (with the additional manipulations of day and week, respectively). Differences were found significant at the 0.05 level, and values were reported as mean ± standard error of the mean (SEM). Differences that were not statistically significant, but quite close ($0.05 < p < 0.10$) were recognized as a "trend."

CHAPTER IV: RESULTS

Weekly Body Weights (Fig. 7)

First, it is worth noting that body weights for each week were calculated as a percentage of baseline weight. For this reason, data from week 1 was omitted from statistical analysis (i.e., all groups started at 100 percent for the first week). Additionally, animals in all four groups experienced significant growth each week [$p < 0.001$; $p < 0.001$; $p < 0.001$; $p < 0.001$; $p < 0.001$; $p < 0.001$; $p < 0.001$; $p < 0.001$; $p = 0.003$].

There was a statistically significant main effect of sex on body weight for weeks 2 [$F(1, 20) = 16.00$, $p < 0.001$], 3 [$F(1, 20) = 39.42$, $p < 0.001$], 4 [$F(1, 20) = 50.65$, $p < 0.001$], 5 [$F(1, 20) = 28.49$, $p < 0.001$], 6 [$F(1, 20) = 32.78$, $p < 0.001$], 7 [$F(1, 20) = 37.50$, $p < 0.001$], 8 [$F(1, 20) = 37.41$, $p < 0.001$], 9 [$F(1, 20) = 38.99$, $p < 0.001$], and 10 [$F(1, 20) = 27.99$, $p < 0.001$]. Specifically, males weighed more than females for weeks 2-10, on average (Appendix, Table 2).

There was also a statistically significant main effect of treatment on body weight for weeks 2 [$F(1, 20) = 9.21$, $p = 0.01$], 3 [$F(1, 20) = 19.21$, $p < 0.001$], and 4 [$F(1, 20) = 17.68$, $p < 0.001$]. Specifically, control animals weighed more than CVS animals for weeks 2-4, on average (Appendix, Table 2). Additionally, there was a trend for treatment on body weight for week 5 [$F(1, 20) = 3.59$, $p = 0.07$]. Specifically, control animals trended towards weighing more than CVS animals for week 5, on average (Appendix, Table 2). There was no main effect of treatment on body weight for weeks 6 [$F(1, 20) = 1.88$, $p = 0.19$], 7 [$F(1, 20) = 1.28$, $p = 0.27$], 8 [$F(1, 20) = 0.87$, $p = 0.36$], 9 [$F(1, 20) = 0.27$, $p = 0.61$], and 10 [(1, 20) = 0.003, $p = 0.96$].

There were no interactions between sex and treatment on body weight for weeks 2 [$F(1, 20)$, = 0.53, $p = 0.48$], 3 [$F(1, 20)$, = 0.07, $p = 0.79$], 4 [$F(1, 20)$, = 0.004, $p = 0.95$], 5 [$F(1, 20)$,

= 0.000, p = 0.98], 6 [F(1, 20), = 0.000, p = 0.99], 7 [F(1, 20), = 0.001, p = 0.98], 8 [F(1, 20), = 0.04, p = 0.84], 9 [F(1, 20), = 0.002, p = 0.96], and 10 [F(1, 20), = 0.24, p = 0.63].

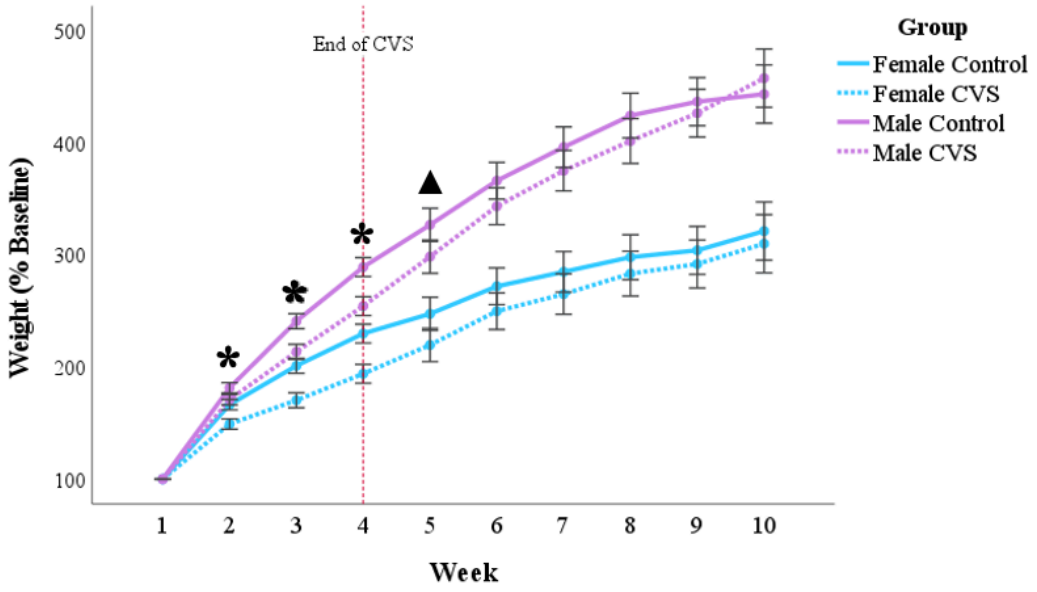


Figure 7. Weekly Body weights. Weights were taken weekly and calculated as a percentage of baseline weight. Males weighed more than females for weeks 2-10. Control animals weighed more than CVS animals for weeks 2-4*. Control animals trended towards weighing more than CVS animals for week 5†. Differences were found significant at the 0.05 level, and values were reported as mean ± SEM.

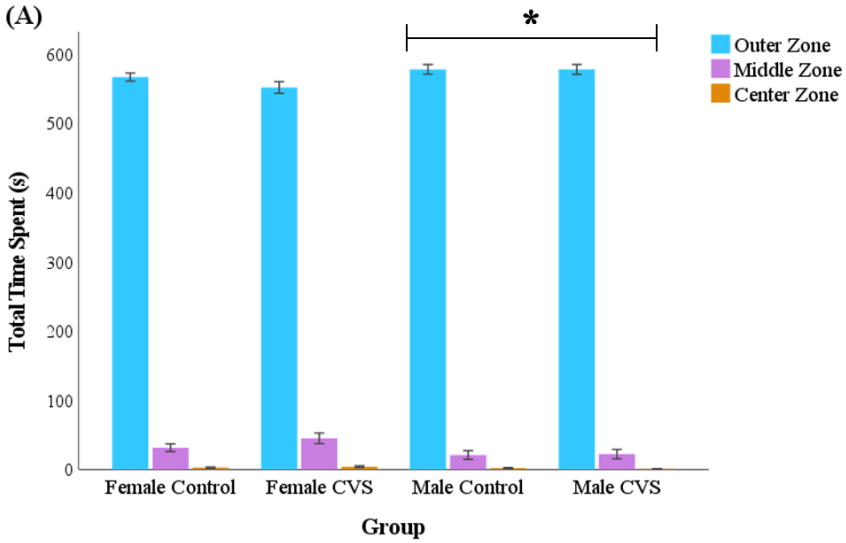
Open Field Test (Fig.8)

There was a statistically significant main effect of sex on total time (s) spent in the outer zone [F(1, 20) = 6.87, p = 0.02], total time (s) spent in the middle zone [F(1, 20) = 6.27, p = 0.02], total time (s) spent in the center zone [F(1, 20) = 5.42, p = 0.03], total distance (m) traveled in the outer zone [F(1, 20) = 11.90, p = 0.003], total distance (m) traveled in the middle zone [F(1, 20) = 9.56, p = 0.01], and total distance (m) traveled in the center zone [F(1, 20) = 16.11, p < 0.001]. Specifically, males spent more time in the outer zone than females; however, females spent more time in the middle and center zones than males, on average (Appendix, Table

3). Females also traveled a greater distance than males in all three zones, on average (Appendix, Table 3).

There was no main effect of treatment on total total time (s) spent in the outer zone [$F(1, 20) = 1.13$, $p = 0.30$], total time (s) spent in the middle zone [$F(1, 20) = 1.25$, $p = 0.28$], total time (s) spent in the center zone [$F(1, 20) = 0.03$, $p = 0.87$], total distance (m) traveled in the outer zone [$F(1, 20) = 1.63$, $p = 0.22$], total distance (m) traveled in the middle zone [$F(1, 20) = 0.37$, $p = 0.55$], and total distance (m) traveled in the center zone [$F(1, 20) = 0.01$, $p = 0.93$].

There were no interactions between sex and treatment on total total time (s) spent in the outer zone [$F(1, 20) = 1.09$, $p = 0.31$], total time (s) spent in the middle zone [$F(1, 20) = 0.86$, $p = 0.36$], total time (s) spent in the center zone [$F(1, 20) = 2.18$, $p = 0.16$], total distance (m) traveled in the outer zone [$F(1, 20) = 0.07$, $p = 0.80$], total distance (m) traveled in the middle zone [$F(1, 20) = 0.06$, $p = 0.81$], and total distance (m) traveled in the center zone [$F(1, 20) = 1.20$, $p = 0.29$].



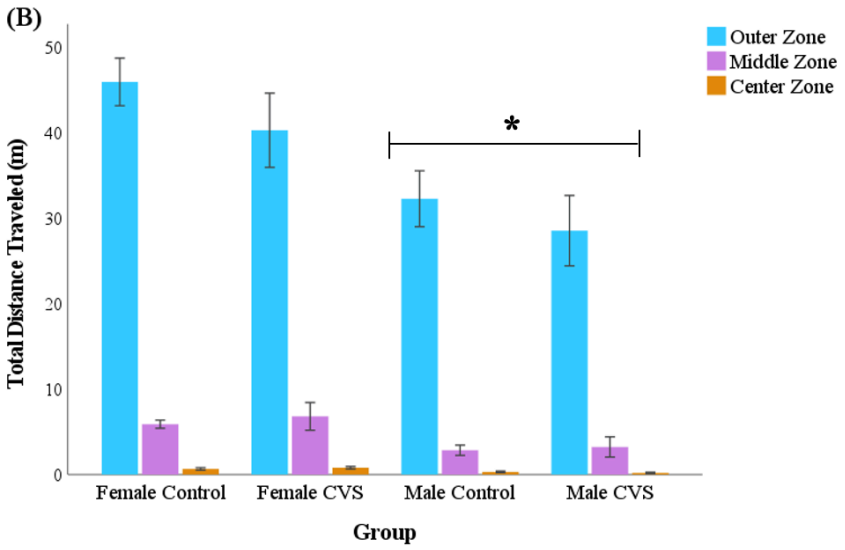


Figure 8. Open field test results. At the start of the test, a single rat was placed in the apparatus facing one of the corners in the outer zone. They were given 10 minutes to freely explore while AnyMaze behavioral software was used to measure behavior in each of the three zones. Differences were found significant at the 0.05 level, and values were reported as mean ± SEM. **(A)** Average total time (s) spent in each zone, by group. While males spent more time in the outer zone, females spent more time in the middle and center zones*. There were no differences in time spent in a zone by treatment. **(B)** Average total distance (m) traveled in each zone, by group. Females traveled a greater distance than males in each of the three zones*. There were no differences in distance traveled in a zone by treatment.

Elevated Zero Maze (Fig. 9)

There was no main effect of sex on latency (s) to open arms [$F(1, 20) = 0.07, p = 0.80$], total time (s) spent in open arms [$F(1, 20) = 0.29, p = 0.59$], or number of transitions [$F(1, 20) = 0.31, p = 0.59$]. There was no main effect of treatment on latency (s) to open arms [$F(1, 20) = 2.86, p = 0.11$], total time (s) spent in open arms [$F(1, 20) = 1.12, p = 0.30$], or number of transitions [$F(1, 20) = 0.34, p = 0.26$]. There were no interactions between sex and treatment on latency (s) to open arms [$F(1, 20) = 0.72, p = 0.41$], total time (s) spent in open arms [$F(1, 20) = 0.27, p = 0.61$], or number of transitions [$F(1, 20) = 0.43, p = 0.52$].

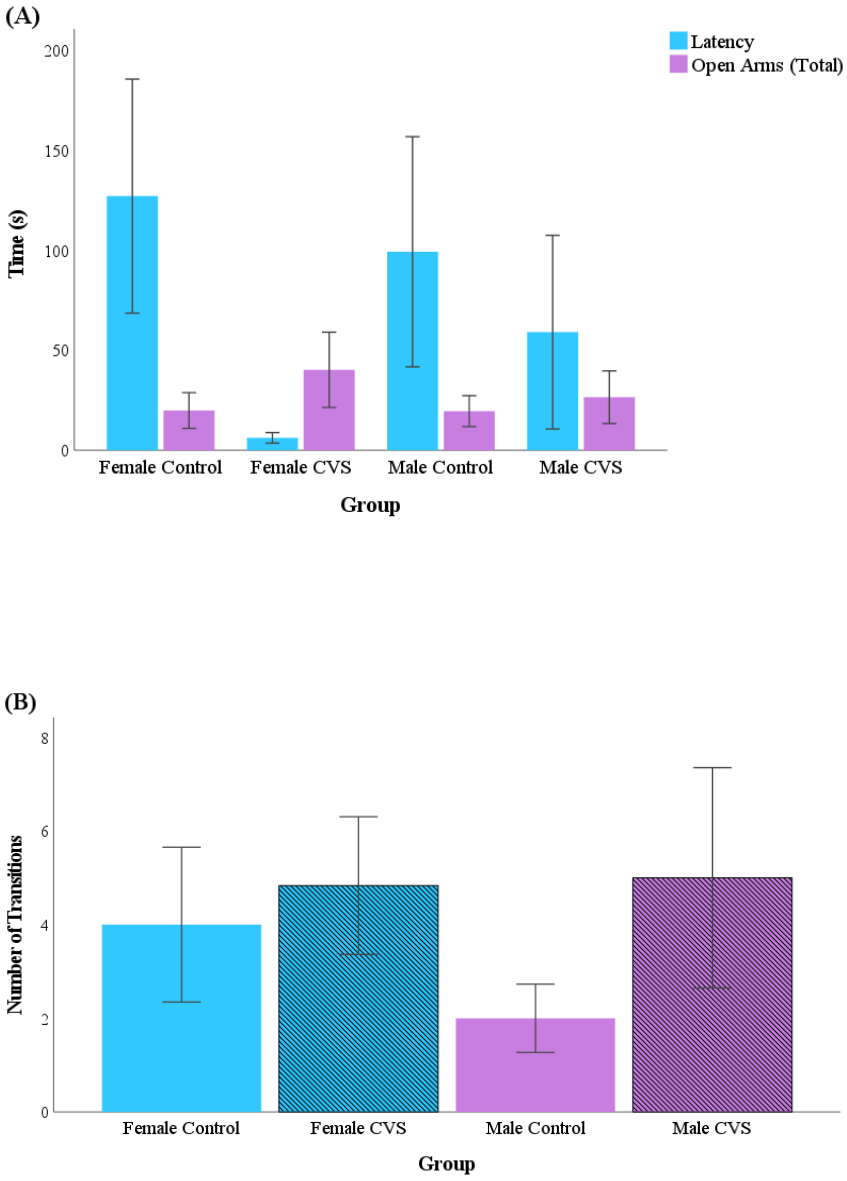


Figure 9. Elevated zero maze results. At the start of the test, a single rat was placed in the maze so that their lower half was inside one of the closed arms, and their upper half was in the open arm. They were given five minutes to explore freely. Values were reported as mean ± SEM. **(A)** Average latency (s) to open arms and total time (s) spent in open arms, by group. There were no differences by sex or treatment. **(B)** Average number of transitions between arms of the maze. There were also no differences by sex or treatment.

Sucrose Preference Test (Fig. 10)

First, it is worth noting that sucrose preference was calculated as a percentage of sucrose intake over total fluid intake. There was no main effect of sex on sucrose preference [$F(1, 8) = 1.50$, $p = 0.26$]. There was no main effect of treatment on sucrose preference [$F(1, 8) = 0.21$, $p = 0.66$]. There was no interaction between sex and treatment on sucrose preference [$F(1, 8) = 0.35$, $p = 0.57$].

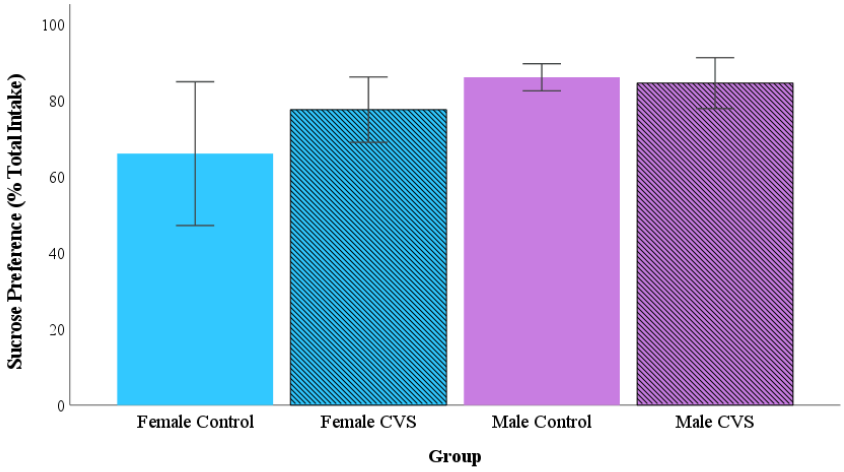


Figure 10. Sucrose preference test results. After a 72-hour acclimation period to the presence of two water bottles, one of the bottles was replaced with a 1% sucrose solution. 24 hours later, water and sucrose intake from each cage-pair was measured. Sucrose preference was calculated as a percentage of sucrose intake over total fluid intake. There were no differences by sex or treatment. Values were reported as mean ± SEM.

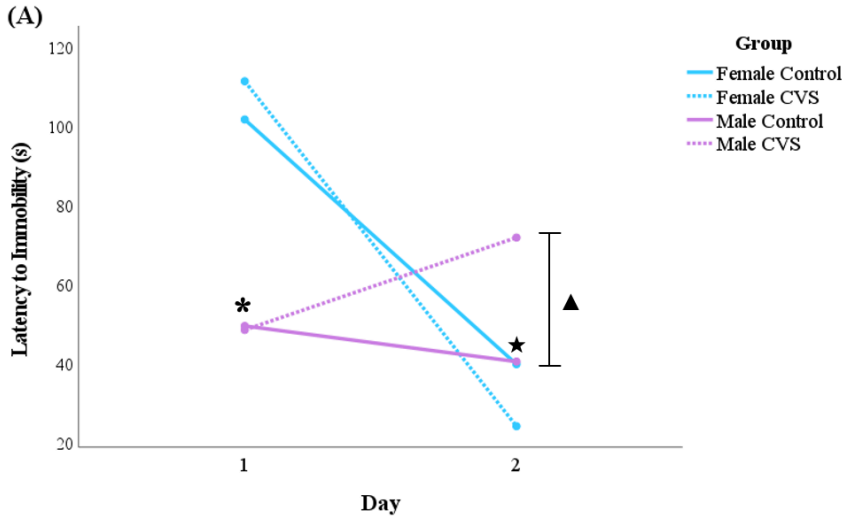
Forced Swim Test (Fig. 11)

There was a statistically significant main effect of sex on Day 1 latency (s) to immobility [$F(1, 20) = 5.87$, $p = 0.03$]. Specifically, females had an increased latency to immobility on Day 1 than males, on average (Appendix, Table 6). There was also a trend of sex on latency (s) to immobility on Day 2 [$F(1, 20) = 3.69$, $p = 0.07$]. Specifically, females trended towards having a decreased latency to immobility than males on Day 2, on average (Appendix, Table 6). There

was no main effect of sex on total time (s) immobile on Day 1 [$F(1, 20) = 0.004$, $p = 0.95$], or total time (s) immobile on Day 2 [$F(1, 20) = 0.17$, $p = 0.68$].

There was no main effect of treatment on latency (s) to immobility on Day 1 [$F(1, 20) = 0.03$, $p = 0.86$], total time (s) immobile on Day 1 [$F(1, 20) = 1.02$, $p = 0.32$], latency (s) to immobility on Day 1 [$F(1, 20) = 0.39$, $p = 0.54$], or total time (s) immobile on Day 2 [$F(1, 20) = 0.001$, $p = 97$]. There was a trend for an interaction between sex and treatment on latency (s) to immobility on Day 2 [$F(1, 20) = 3.48$, $p = 0.08$]. Specifically, CVS males trended towards having an increased latency to immobility on Day 2 than control males, on average (Appendix, Table 6).

Lastly, it is worth noting that females had a significant decrease in latency (s) to immobility from Day 1 to Day 2 ($p < 0.001$).



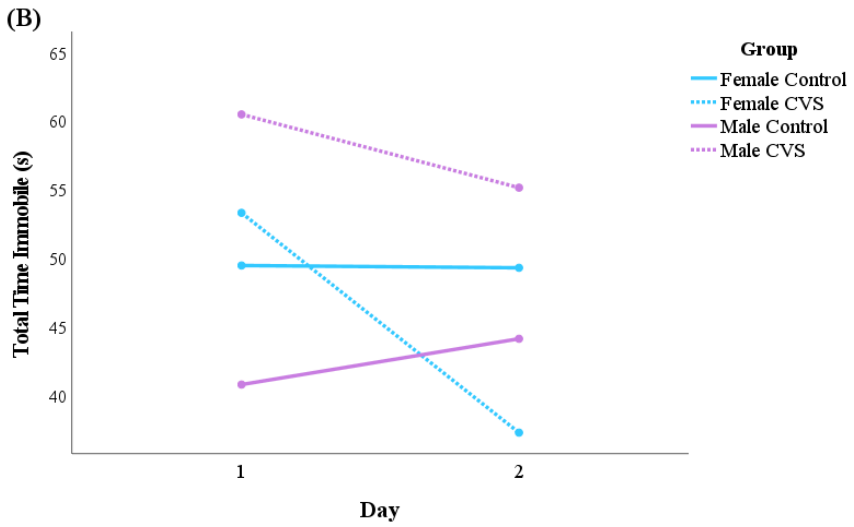


Figure 11. Forced swim test results. A clear testing cylinder was filled with water (25-28 °C) to a level that was high enough to prevent animals from standing on their hind legs, but low enough to keep them from climbing out. At the start of the test, a single rat was placed into the cylinder head first and a timer was set for 10 minutes. A second timer was used to record the latency (s) to immobility, while a third timer was used to record the total time (s) immobile. The test was repeated 24 hours later. Differences were found significant at the 0.05 level, and values were reported as mean ± SEM. **(A)** Changes in the average latency (s) to immobility from Day 1 to Day 2, by group. Females had an increased latency than males on Day 1*. Females trended towards having a decreased latency than males on Day 2▲. CVS males trended towards having an increased latency than control males on Day 2★. **(B)** Changes in total time (s) immobile from Day 1 to Day 2, by group. There were no differences by sex or treatment.

Cocaine-Induced Locomotor Activity (Fig. 12)

First, it is worth noting that cocaine-induced locomotor activity was measured by the number of horizontal beam breaks. There was a statistically significant main effect of sex on cocaine-induced locomotor activity [F(1, 20) = 7.89, p = 0.01]. Specifically, females had greater activity than males, on average (Appendix, Table 7). There was no main effect of treatment on cocaine-induced locomotor activity [$F(1, 20) = 0.24$, $p = 0.63$]. There was no interaction between sex and treatment on cocaine-induced locomotor activity [$F(1, 20) = 2.09$, $p = 0.16$].

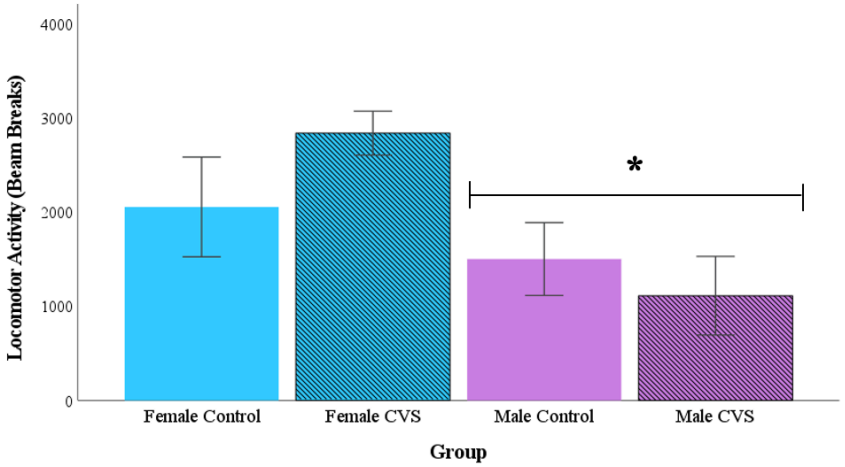


Figure 12. Cocaine-induced locomotor activity results. At the start of the test, a single rat was given a 20 mg/kg intraperitoneal injection of cocaine hydrochloride dissolved in saline. They were then immediately placed in a 40 x 40 cm open field with an infrared motion detection grid for 10 minutes. Locomotor activity was measured by the number of horizontal beam breaks. Females had greater cocaine-induced locomotor activity than males*. There were no differences by treatment. Differences were found significant at the 0.05 level, and values were reported as mean ± SEM.

Organ Weights (Fig. 13)

First, it is worth noting that adrenal weights were calculated as a percentage of baseline body weight. There was no main effect of sex on adrenal glands weight [$F(1, 20) = 2.27$, $p = 0.15$] or thymus weight [$F(1, 20) = 1.14$, $p = 0.30$]. There was no main effect of treatment on adrenal glands weight [$F(1, 20) = 0.13$, $p = 0.73$] or thymus weight [$F(1, 20) = 0.34$, $p = 57$]. There were no interactions between sex and treatment on adrenal glands weight [$F(1, 20) = 0.64$, $p = 0.43$], or thymus weight [$F(1, 20) = 0.28$, $p = 0.60$].

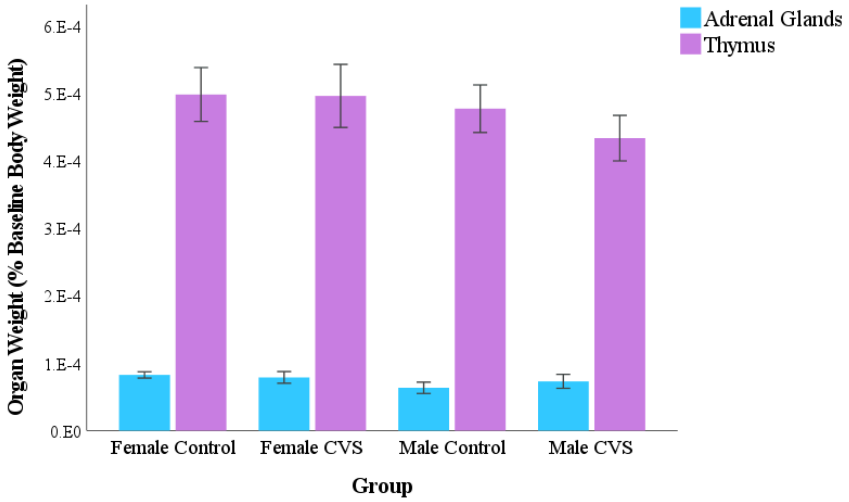


Figure 13. Organ weights. At the end of the experiment, animals were sacrificed; adrenal glands and thymuses were removed and weighed. Organ weights were then calculated as a percentage of baseline body weight. There were no differences by sex or treatment. Values were reported as mean ± SEM.

CHAPTER IV: DISCUSSION

Overview

The purpose of this research was to test the hypothesis that female Long-Evans rats are at a greater risk than males for developing anxiety and depression related behaviors after early life exposure to chronic variable stress (CVS). This was done by manipulating sex and stress (with two levels; control and CVS). Body weights were recorded weekly. Nearly a week after arrival, rats in the CVS groups were exposed to a three-week CVS protocol. Stressors were administered twice a day, every day, at varying times. They included: immobilization, predator odor, cage tilt, strobe lights, ultrasonic deterrent, and cold room. As an additional stressor, rats were housed overnight in isolation cages twice a week. Two weeks after the last stressor was applied, all animals underwent a series of behavioral testing: open field test (OFT), elevated zero maze (EZM), sucrose preference test (SPT), forced swim test (FST), and cocaine-induced locomotor activity (CLA). At the conclusion of the experiment, animals were sacrificed, and organs were weighed.

Findings

For weekly body weights (as a percentage of baseline weight), males weighed more than females for weeks 2-10. This suggests that males have a larger growth rate, possibly the result of increased appetite and food consumption. Control animals weighed more than CVS animals for weeks 2-4, and still trended towards weighing more for week 5. These differences were gone for weeks 6-10. For context, the CVS protocol occurred during weeks 1-3. This finding was very interesting and unexpected, because it suggests that 1) the stressors were effective and had a short-term effect on the growth of CVS animals (perhaps they were eating less); and 2) the CVS

animals had some sort of adaptive resilience that allowed them to catch up in weight shortly after the conclusion of stressors. This resilience may be dependent on age; the rats were still relatively young (adolescent) during the CVS protocol. The adolescent brain (in both humans and rodents) is especially malleable and sensitive to the environment. Originally, it had been predicted that as a result of this sensitivity, the CVS animals would be more likely to develop anxiety and depression related behaviors. However, it appears that this neural flexibility may be acting as a protective factor against long-term effects of chronic stress. In a recent study, Campos-Cardoso et al. (2023) found that early adolescent male Wistar rats were resilient to delayed anxiety-like behavior.

When discussing the following results from the behavioral tests, it is important to keep in mind that behavioral testing started during week 6, after differences in weight between CVS and control animals were no longer detected. So, it is very possible that the stressors resulted in short-term, but not long-term behavioral differences.

During the open field test (OFT), males spent more time in the outer zone than females; however, females spent more time in both the middle and center zones than males. Females also traveled more distance in all three zones compared to males. These findings suggest that females are more active and exploratory in a novel environment than males. They are also supported by previous research that reported female rats spending more time in the center zone of the open field than males (Knight et al., 2021; Domonkos et al., 2017; Pavlova et al., 2020). Knight et al. (2021) and Pavlova et al. (2020) also found that female rats were more active than males. Alternatively, other research does not establish any sex differences in time spent in the center zone (Sakhaie et al., 2020; Scholl et al., 2019; Yang et al., 2019). In the present study, there were no differences between the control and CVS animals in any of the zones, suggesting that the

CVS protocol was not effective in producing long-term adverse effects. This finding was unexpected, as it was predicted that control animals would spend more time in the center zone than CVS animals. Furthermore, CVS males would spend more time in the center zone than CVS females. Previous research is contradictory; Gronli et al. (2005) found that rats exposed to chronic mild stress (CMS) traveled a greater distance in both the outer and center zones of the open field than those in control groups. Farhan et al. (2014) reported that rats in CMS groups had decreased activity in the open field compared to the control groups.

During the elevated zero maze (EZM), there were no differences in latency to open arms, total time spent in open arms, or number of transitions. These findings suggest that the CVS protocol was not effective in producing long-term adverse effects. It was predicted that control animals would spend more time in the open arms than CVS animals. Furthermore, CVS males would spend more time in the open arms than CVS females. Since the EZM is still a relatively new model, there are not a lot of published studies that implement it. However, existing studies looking at sex differences in the elevated plus maze (EPM) have demonstrated that female rats spend more time in the open arms than males (Knight et al., 2021; Scholl et al., 2019). Other studies looking at the effect of CMS have established that male rats exposed to CMS spend less time in the open arms of the EPM than those in the control groups (Wange et al., 2018; Bondi et al., 2018).

For the sucrose preference test (SPT), there were no differences in sucrose preference, suggesting that the CVS protocol was not effective in producing long-term adverse effects. Originally, it was predicted that control animals would have a greater preference than CVS animals. Furthermore, CVS males would have a greater preference than CVS females. This finding was in line with another study that did not observe any sex differences in preference for a

2% sucrose solution (Pupikinia and Sitnikova, 2023). Another study observed that female rats had a higher sucrose preference than males (Grimm et al., 2022). However, this might have been a result of using a 10% sucrose solution which is much higher than the 1 or 2% in typical protocols. Additionally, several CMS studies have reported that the stressors lead to a decrease in sucrose preference (Li et al., 2018; Fan et al., 2018; Franklin et al., 2018).

During the forced swim test (FST,) female rats had a greater latency to immobility on Day 1 than males. On Day 2, they trended towards having a decreased latency to immobility than males. Traditionally, this result would be interpreted as females displaying greater 'learned helplessness', and thus being more depressed. However, discussion of why that might not be the case can be found later in this paper. It was predicted that control animals would have increased latency to immobility and decreased time spent immobile than CVS animals on Day 2. Furthermore, CVS males would have an increased latency to immobility and decreased time spent immobile than CVS females on Day 2. Regardless, this finding is supported by one study that reported adolescent exposure to chronic stress did not have an effect on male rats, but decreased latency in females (Bourke and Neigh, 2011). Alternatively, another study observed that female rats had an increased immobility than males (Lovelock and Deak, 2019). In the present study, CVS males trended towards having an increased latency to immobility than control males, suggesting that the CVS protocol was only effective in producing long-term adverse effects in males. Based on previous research, it was predicted that CVS groups would have an increase in time spent immobile (Fan et al., 2018; Wang et al., 2018; Bansar et al., 2008). Lastly, females in the present study had a significant decrease in latency to immobility from Day 1 on Day 2. This suggests that Day 1 taught them learned helplessness.

For cocaine-locomotor activity (CLA), female rats had a greater cocaine-induced locomotor activity than males, suggesting they are more active. This was unsurprising as existing research suggests that females have more baseline activity (Knight et al., 2021; Pavlova et al., 2020). Surprisingly, there were no differences by treatment, as research suggests that stress can increase responsiveness to psychostimulant drugs (Maldonado and Kirstein, 2005). This suggests that the CVS protocol was not effective in producing long-term adverse effects. However, one study established that early life stress did not have an effect on cocaine-induced locomotor activity in male rats (Bolton et al., 2018).

There were no differences in adrenal or thymus weights. This suggests that the CVS protocol was not effective in producing long-term adverse effects. It was predicted that control animals would have increased adrenal gland weights and decreased thymus weights. These findings contradict research that found chronic stress increased adrenal weights (Ulrich-Lai et al., 2006) and decreased thymus weight (Živković et al., 2005).

Strain Differences

Long-Evans rats were chosen for this research for their frequent use in behavioral studies and historically-proven response to stress. In a comparative study, male Long-Evans rats exhibited a greater HPA response to stressors than Sprague-Dawley, Wistar, and Fischer rats (Sanchís-Ollé et al., 2021). In another comparative study, chronic stress significantly reduced corticosterone levels in Long-Evans, but not in Sprague-Dawleys (Bielajew et al., 2002). Based on this research, it is possible that even less behavioral changes would have been detected with another strain.

Effectiveness of CVS Protocol

A statement that was heavily repeated throughout the discussion of findings was that the CVS protocol was not effective in producing long-term adverse effects. It served as a possible explanation for why behavioral differences were not being observed. It was clear that the rats found immobilization very stressful, as a majority of them were exhibiting a condition known as rhinorrhea. This is when nasal inflammation causes mucus to drip out, and can be the result of psychological stress (Yamanaka-Takaichi et al., 2021). Additionally, several of the animals were chewing holes in their DecapiCones, attempting to escape. Initially, predator odor also seemed to also create a stress response, though less severe. After the first few administrations of this stressor, the bobcat urine canisters were completely covered in bedding, and the rats were huddled together in the corner furthest away. However, this type of response began to decline with repetition, suggesting that the animals progressively developed nasal desensitization to the odor. As a result, the effectiveness of predator odor as a stressor may have diminished over time. The animals may have also developed adaptive measures to the other stressors. During cage tilt, strobe lights, ultrasonic deterrent, and cold room, all of the rats could be seen huddling together. It is possible that the specific protocol used in this study did not have an accumulation effect representative of chronic stress, and the stressors were instead perceived by the animals as independent, and acute. Another possibility is that the early stressors caused a vulnerability that would not have emerged until much later in adulthood.

Validity of Behavioral Tests

It is also important to evaluate the validity of the behavioral tests. While the FST is a commonly used procedure in rodent behavioral studies, it is currently being called into question by researchers as to whether it is a reliable measurement of depression. Molendijk and De Kloet (2016) argue that passive behavior during the test is not a sign of depression, but rather is a representation of adaptation. The transition from thrashing in the water to floating is actually a survival mechanism, a way to conserve energy as well as cope with the stressful situation. However, antidepressants have been found to reduce immobility during the FST (Yankelevitch-Yahav et al., 2015). This suggests that immobility may not be reflective of depression, but locomotor behavior. Furthermore, many studies only look at the results from day two. As described in the introduction, the typical protocol follows 15 min on day one versus 5 on day two, with the assumption that behavior will change from the first day. With the purpose of the test being to measure 'learned helplessness', it is crucial to record behavior on both days to determine whether changes actually occurred.

Limitations

One of the greatest limitations of this research was the small sample size ($N = 24$; $n = 6$ per group), a result of three factors: ethics, logistics, and resources. In animal research, the 'three Rs' refer to a set of principles developed by Russell and Burch (1959) to promote the welfare of animals used in laboratory research: *replace* conscious, living animals with insentient material, *reduce* the number of animals required to obtain data, and *refine* the procedures applied to animals by decreasing the severity of inhumanity as much as possible (as cited in Hubrecht and Carter, 2019). It may be clearer to understand why using too large of a sample size is

problematic; however, using too few animals can yield unreliable results which is also wasteful and unethical.

Additionally, the timing of some behavioral tests would have made a larger sample size impossible. For example, the FST takes approximately 15 minutes per animal, per day (ten minutes of testing, plus five minutes for transfers, drying, removing feces from the testing cylinder). If the number of animals were doubled, testing would take around 12 hours on each of the two days. It is important that behavioral tests are administered at the same time of day to eliminate variables associated with animals' circadian rhythms. This would have been impossible with a prolonged time window. There are also limitations in the facilities available to house more animals, as well as in the funds required to purchase and care for the animals.

While a larger sample size was impractical for these reasons, a small sample size poses several issues. First, it can be more difficult to replicate the findings. This is partly due to the impact of each individual within a smaller pool. A small sample also tends to lead to low statistical power, that is, the probability of correctly detecting a true effect. A meta-analysis conducted by Button et al. revealed that while the average sample size for animals used in water maze experiments was 22, an *N* of 134 would be required to achieve 80% power (2013). The authors also emphasized the problem of statistical power in neuroscience literature typically being very low. Finally, it is harder to generalize findings to a broader population. Even if the sample size were greater, the study would be limited by only looking at one strain.

Another limitation was the replicability of the experiment itself. The CVS protocol was especially complex, with six unique stressors administered twice a day in a random order, at various times (in addition to overnight isolation twice a week). While all of the stressors are meant to be psychological and mild, it is likely that each one produces a different level of stress.

Therefore, it is possible that the first stressor given in the day directly influences the amount of stress experienced by an animal during the second stressor of the day. It is also likely that the behavioral tests (especially the FST) caused some level of stress in the control animals, thereby minimizing the differences between control and CVS groups. Hence, small alterations to the protocol could potentially have significant consequences for replicating results.

Future Directions

As discussed previously, a possible explanation for the results is that the CVS protocol was not effective in producing long-term adverse effects. A follow-up experiment could repeat the exact protocol, but only look at one sex, allowing for an increase in size per group. Another, more likely explanation is that the rats experienced short-term effects of chronic stress, but were resilient and able to overcome these changes with time. To investigate this, two identical rounds of behavioral testing would be carried out; one that takes place 24 hours after the last stressor, and another that takes place twelve weeks later.

In regards to the behavioral tests, the SPT could be improved by measuring sucrose consumption over a 48-hour period rather than 24 (which may have been too short). The bottles would be switched after the first 24 hours to counteract any potential preferences to a specific side of the cage. It is also worth considering replacing the FST with another test for depression. The study would further benefit from taking additional biological measurements (e.g., corticosterone levels and brain analysis). It would be expected that the CVS animals would have higher levels of corticosterone, and this would correlate with increased behavioral changes. Additionally, it would be expected to find decreased hippocampal volume in the CVS animals as this structure has been implicated in regulating the HPA axis.

Conclusion

To conclude, the hypothesis that female Long-Evans are at a higher risk than males for developing anxiety and depression related behaviors after early life exposure to CVS was not supported. Rather, this study established that early life exposure to chronic variable stress did not lead to long-term behavioral differences. The significance of these findings is either an indication of an ineffective CVS model, or a resilience to early stress. Results of weekly body weights are more supportive of the latter theory. This study is unique because most of the existing behavioral studies only look at the effects of stress or sex, but not both, or an interaction between the two factors.

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REFERENCES

1. Albert, P. R. (2015). Why is depression more prevalent in women? *Journal of Psychiatry and Neuroscience*, *40*(4), 219–221. https://doi.org/10.1503/jpn.150205
2. Ambience World. (2018, June 9). *Mouse & Rat Ultra Sonic Deterrent. Mice Ultrasonic Repellent. 12 Hours.* https://www.youtube.com/watch?v=ZMpX3vtDICY
3. Anxiety & Depression Association of America. (2022, October 28). *Anxiety Disorders—Facts & Statistics*. Anxiety & Depression Association of America. https://adaa.org/understanding-anxiety/facts-statistics
4. Bamalan, O. A., Moore, M. J., & Al Khalili, Y. (2024). Physiology, Serotonin. In *StatPearls*. StatPearls Publishing. http://www.ncbi.nlm.nih.gov/books/NBK545168/
5. Banasr, M., & Duman, R. S. (2008). Glial Loss in the Prefrontal Cortex Is Sufficient to Induce Depressive-like Behaviors. *Biological Psychiatry*, *64*(10), 863–870. https://doi.org/10.1016/j.biopsych.2008.06.008
6. Bielajew, C., Konkle, A. T. M., & Merali, Z. (2002). The effects of chronic mild stress on male Sprague–Dawley and Long Evans rats. *Behavioural Brain Research*, *136*(2), 583–592. https://doi.org/10.1016/S0166-4328(02)00222-X
7. Bolton, J. L., Ruiz, C. M., Rismanchi, N., Sanchez, G. A., Castillo, E., Huang, J., Cross, C., Baram, T. Z., & Mahler, S. V. (2018). Early-life adversity facilitates acquisition of cocaine self-administration and induces persistent anhedonia. *Neurobiology of Stress*, *8*, 57–67. https://doi.org/10.1016/j.ynstr.2018.01.002
8. Bondi, C. O., Rodriguez, G., Gould, G. G., Frazer, A., & Morilak, D. A. (2008). Chronic Unpredictable Stress Induces a Cognitive Deficit and Anxiety-Like Behavior in Rats that is Prevented by Chronic Antidepressant Drug Treatment. *Neuropsychopharmacology*, *33*(2), 320–331. https://doi.org/10.1038/sj.npp.1301410
9. Bourke, C. H., & Neigh, G. N. (2011). Behavioral effects of chronic adolescent stress are sustained and sexually dimorphic. *Hormones and Behavior*, *60*(1), 112–120. https://doi.org/10.1016/j.yhbeh.2011.03.011
10. Braun, A. A., Skelton, M. R., Vorhees, C. V., & Williams, M. T. (2011). Comparison of the elevated plus and elevated zero mazes in treated and untreated male Sprague–Dawley rats: Effects of anxiolytic and anxiogenic agents. *Pharmacology Biochemistry and Behavior*, *97*(3), 406–415. https://doi.org/10.1016/j.pbb.2010.09.013
11. Button, K. S., Ioannidis, J. P. A., Mokrysz, C., Nosek, B. A., Flint, J., Robinson, E. S. J., & Munafò, M. R. (2013). Power failure: Why small sample size undermines the reliability of neuroscience. *Nature Reviews Neuroscience*, *14*(5), 365–376. https://doi.org/10.1038/nrn3475
12. Campos-Cardoso, R., Novaes, L. S., Godoy, L. D., Dos Santos, N. B., Perfetto, J. G., Lazarini-Lopes, W., Garcia-Cairasco, N., Padovan, C. M., & Munhoz, C. D. (2023). The resilience of adolescent male rats to acute stress-induced delayed anxiety is age-related

and glucocorticoid release-dependent. *Neuropharmacology*, *226*, 109385. https://doi.org/10.1016/j.neuropharm.2022.109385
13. Cavedoni, S., Chirico, A., Pedroli, E., Cipresso, P., & Riva, G. (2020). Digital Biomarkers for the Early Detection of Mild Cognitive Impairment: Artificial Intelligence Meets Virtual Reality. *Frontiers in Human Neuroscience*, *14*, 245. https://doi.org/10.3389/fnhum.2020.00245
14. Cohen, J. R., Andrews, A. R., Davis, M. M., & Rudolph, K. D. (2018). Anxiety and Depression During Childhood and Adolescence: Testing Theoretical Models of Continuity and Discontinuity. *Journal of Abnormal Child Psychology*, *46*(6), 1295–1308. https://doi.org/10.1007/s10802-017-0370-x
15. Cole, A. B., Montgomery, K., Bale, T. L., & Thompson, S. M. (2022). What the hippocampus tells the HPA axis: Hippocampal output attenuates acute stress responses via disynaptic inhibition of CRF+ PVN neurons. *Neurobiology of Stress*, *20*, 100473. https://doi.org/10.1016/j.ynstr.2022.100473
16. Cotella, E. M., Gómez, A. S., Lemen, P., Chen, C., Fernández, G., Hansen, C., Herman, J. P., & Paglini, M. G. (2019). Long-term impact of chronic variable stress in adolescence versus adulthood. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, *88*, 303–310. https://doi.org/10.1016/j.pnpbp.2018.08.003
17. De Kloet, E. R., & Molendijk, M. L. (2016). Coping with the Forced Swim Stressor: Towards Understanding an Adaptive Mechanism. *Neural Plasticity*, *2016*, 1–13. https://doi.org/10.1155/2016/6503162
18. Domonkos, E., Borbélyová, V., Csongová, M., Bosý, M., Kačmárová, M., Ostatníková, D., Hodosy, J., & Celec, P. (2017). Sex differences and sex hormones in anxiety-like behavior of aging rats. *Hormones and Behavior*, *93*, 159–165. https://doi.org/10.1016/j.yhbeh.2017.05.019
19. Dziurkowska, E., & Wesolowski, M. (2021). Cortisol as a Biomarker of Mental Disorder Severity. *Journal of Clinical Medicine*, *10*(21), 5204. https://doi.org/10.3390/jcm10215204
20. Fan, C., Song, Q., Wang, P., Li, Y., Yang, M., & Yu, S. Y. (2018). Neuroprotective Effects of Ginsenoside-Rg1 Against Depression-Like Behaviors via Suppressing Glial Activation, Synaptic Deficits, and Neuronal Apoptosis in Rats. *Frontiers in Immunology*, *9*, 2889. https://doi.org/10.3389/fimmu.2018.02889
21. Farhan, M., Ikram, H., Kanwal, S., & Haleem, D. J. (2014). Unpredictable chronic mild stress induced behavioral deficits: A comparative study in male and female rats. *Pakistan Journal of Pharmaceutical Sciences*, *27*(4), 879–884.
22. Frank, R. G., Glied, S. A., O'Brien, J., & Wachino, V. (2023, July 11). Four Actions the Federal Government Can Take to Address Mental Health in the U.S. *The Commonwealth Fund*. https://www.commonwealthfund.org/blog/2023/four-actions-federal-government-can-take-address-mental-health-us

23. Franklin, T. C., Wohleb, E. S., Zhang, Y., Fogaça, M., Hare, B., & Duman, R. S. (2018). Persistent Increase in Microglial RAGE Contributes to Chronic Stress–Induced Priming of Depressive-like Behavior. *Biological Psychiatry*, *83*(1), 50–60. https://doi.org/10.1016/j.biopsych.2017.06.034
24. Gilmore, J. H., Knickmeyer, R. C., & Gao, W. (2018). Imaging structural and functional brain development in early childhood. *Nature Reviews Neuroscience*, *19*(3), 123–137. https://doi.org/10.1038/nrn.2018.1
25. Gotlib, I. H., Miller, J. G., Borchers, L. R., Coury, S. M., Costello, L. A., Garcia, J. M., & Ho, T. C. (2023). Effects of the COVID-19 Pandemic on Mental Health and Brain Maturation in Adolescents: Implications for Analyzing Longitudinal Data. *Biological Psychiatry Global Open Science*, *3*(4), 912–918. https://doi.org/10.1016/j.bpsgos.2022.11.002
26. Grimm, J. W., North, K., Hopkins, M., Jiganti, K., McCoy, A., Šulc, J., MacDougall, D., & Sauter, F. (2022). Sex differences in sucrose reinforcement in Long-Evans rats. *Biology of Sex Differences*, *13*(1), 3. https://doi.org/10.1186/s13293-022-00412-8
27. Hernández-Hernández, O. T., Martínez-Mota, L., Herrera-Pérez, J. J., & Jiménez-Rubio, G. (2019). Role of Estradiol in the Expression of Genes Involved in Serotonin Neurotransmission: Implications for Female Depression. *Current Neuropharmacology*, *17*(5), 459–471. https://doi.org/10.2174/1570159X16666180628165107
28. Hoffman, K. L. (2016). What can animal models tell us about depressive disorders? In *Modeling Neuropsychiatric Disorders in Laboratory Animals* (pp. 35–86). Elsevier. https://doi.org/10.1016/B978-0-08-100099-1.00002-9
29. Hubrecht & Carter. (2019). The 3Rs and Humane Experimental Technique: Implementing Change. *Animals*, *9*(10), 754. https://doi.org/10.3390/ani9100754
30. Kalin, N. H. (2020). The Critical Relationship Between Anxiety and Depression. *American Journal of Psychiatry*, *177*(5), 365–367. https://doi.org/10.1176/appi.ajp.2020.20030305
31. Kim, E. J., Pellman, B., & Kim, J. J. (2015). Stress effects on the hippocampus: A critical review. *Learning & Memory (Cold Spring Harbor, N.Y.)*, *22*(9), 411–416. https://doi.org/10.1101/lm.037291.114
32. Knight, P., Chellian, R., Wilson, R., Behnood-Rod, A., Panunzio, S., & Bruijnzeel, A. W. (2021). Sex differences in the elevated plus-maze test and large open field test in adult Wistar rats. *Pharmacology Biochemistry and Behavior*, *204*, 173168. https://doi.org/10.1016/j.pbb.2021.173168
33. Konrad, K., Firk, C., & Uhlhaas, P. J. (2013). Brain Development During Adolescence. *Deutsches Ärzteblatt International*. https://doi.org/10.3238/arztebl.2013.0425
34. Kundakovic, M., & Rocks, D. (2022). Sex hormone fluctuation and increased female risk for depression and anxiety disorders: From clinical evidence to molecular mechanisms. *Frontiers in Neuroendocrinology*, *66*, 101010. https://doi.org/10.1016/j.yfrne.2022.101010

35. Lebrun-Harris, L. A., Ghandour, R. M., Kogan, M. D., & Warren, M. D. (2022). Five-Year Trends in US Children's Health and Well-being, 2016-2020. *JAMA Pediatrics*, *176*(7), e220056. https://doi.org/10.1001/jamapediatrics.2022.0056
36. Lecourtier, L., Durieux, L., & Mathis, V. (2023). Alteration of Lateral Habenula Function Prevents the Proper Exploration of a Novel Environment. *Neuroscience*, *514*, 56–66. https://doi.org/10.1016/j.neuroscience.2023.01.010
37. Li, S.-X., Han, Y., Xu, L.-Z., Yuan, K., Zhang, R.-X., Sun, C.-Y., Xu, D.-F., Yuan, M., Deng, J.-H., Meng, S.-Q., Gao, X.-J., Wen, Q., Liu, L.-J., Zhu, W.-L., Xue, Y.-X., Zhao, M., Shi, J., & Lu, L. (2018). Uncoupling DAPK1 from NMDA receptor GluN2B subunit exerts rapid antidepressant-like effects. *Molecular Psychiatry*, *23*(3), 597–608. https://doi.org/10.1038/mp.2017.85
38. Liu, M.-Y., Yin, C.-Y., Zhu, L.-J., Zhu, X.-H., Xu, C., Luo, C.-X., Chen, H., Zhu, D.-Y., & Zhou, Q.-G. (2018). Sucrose preference test for measurement of stress-induced anhedonia in mice. *Nature Protocols*, *13*(7), 1686–1698. https://doi.org/10.1038/s41596-018-0011-z
39. Lovelock, D. F., & Deak, T. (2019). Acute stress imposed during adolescence yields heightened anxiety in Sprague Dawley rats that persists into adulthood: Sex differences and potential involvement of the Medial Amygdala. *Brain Research*, *1723*, 146392. https://doi.org/10.1016/j.brainres.2019.146392
40. Maldonado, A., & Kirstein, C. (2005). Handling alters cocaine-induced activity in adolescent but not adult male rats. *Physiology & Behavior*, *84*(2), 321–326. https://doi.org/10.1016/j.physbeh.2004.12.007
41. Markov, D. D. (2022). Sucrose Preference Test as a Measure of Anhedonic Behavior in a Chronic Unpredictable Mild Stress Model of Depression: Outstanding Issues. *Brain Sciences*, *12*(10), 1287. https://doi.org/10.3390/brainsci12101287
42. McVay, E. (2023, August 31). Social Media and Self-diagnosis. *Johns Hopkins All Children's Hospital*. https://www.hopkinsmedicine.org/news/articles/2023/08/social-media-and-self-diagnosis
43. Molendijk, M. L., & De Kloet, E. R. (2015). Immobility in the forced swim test is adaptive and does not reflect depression. *Psychoneuroendocrinology*, *62*, 389–391. https://doi.org/10.1016/j.psyneuen.2015.08.028
44. Molina, P. E. (2023). *Endocrine physiology* (Sixth edition). McGraw Hill Medical.
45. National Institute of Mental Health. (2023a, April). *Anxiety Disorders*. National Institute of Mental Health. https://www.nimh.nih.gov/health/topics/anxiety-disorders
46. National Institute of Mental Health. (2023b, July). *Major Depression*. National Institute of Mental Health. https://www.nimh.nih.gov/health/statistics/major-depression
47. Pavlova, I. V., Broshevitskaya, N. D., Onufriev, M. V., & Moiseeva, Yu. V. (2020). Sex-Related Differences in Anxious-Depressive and Defensive Behavior in Wistar Rats. *Neuroscience and Behavioral Physiology*, *50*(9), 1163–1175. https://doi.org/10.1007/s11055-020-01028-4

48. Pupikina, M., & Sitnikova, E. (2023). Sex Differences in Behavior and Learning Abilities in Adult Rats. *Life*, *13*(2), 547. https://doi.org/10.3390/life13020547
49. Reiss, F., Meyrose, A.-K., Otto, C., Lampert, T., Klasen, F., & Ravens-Sieberer, U. (2019). Socioeconomic status, stressful life situations and mental health problems in children and adolescents: Results of the German BELLA cohort-study. *PLOS ONE*, *14*(3), e0213700. https://doi.org/10.1371/journal.pone.0213700
50. Russell, W., & Burch, R. (1959). *The Principles of Humane Experimental Technique*.
51. Sakhaie, N., Sadegzadeh, F., Mohammadnia, A., Dadkhah, M., & Saadati, H. (2020). Sex‐dependent effects of postweaning exposure to an enriched environment on novel objective recognition memory and anxiety‐like behaviors: The role of hippocampal BDNF level. *International Journal of Developmental Neuroscience*, *80*(5), 396–408. https://doi.org/10.1002/jdn.10038
52. Sanchís-Ollé, M., Sánchez-Benito, L., Fuentes, S., Gagliano, H., Belda, X., Molina, P., Carrasco, J., Nadal, R., & Armario, A. (2021). Male long-Evans rats: An outbred model of marked hypothalamic-pituitary-adrenal hyperactivity. *Neurobiology of Stress*, *15*, 100355. https://doi.org/10.1016/j.ynstr.2021.100355
53. Scholl, J. L., Afzal, A., Fox, L. C., Watt, M. J., & Forster, G. L. (2019). Sex differences in anxiety-like behaviors in rats. *Physiology & Behavior*, *211*, 112670. https://doi.org/10.1016/j.physbeh.2019.112670
54. Seibenhener, M. L., & Wooten, M. C. (2015). Use of the Open Field Maze to Measure Locomotor and Anxiety-like Behavior in Mice. *Journal of Visualized Experiments*, *96*, 52434. https://doi.org/10.3791/52434
55. Syed, S. A., & Nemeroff, C. B. (2017). Early Life Stress, Mood, and Anxiety Disorders. *Chronic Stress (Thousand Oaks, Calif.)*, *1*, 2470547017694461. https://doi.org/10.1177/2470547017694461
56. Treit, D., & Fundytus, M. (1988). Thigmotaxis as a test for anxiolytic activity in rats. *Pharmacology Biochemistry and Behavior*, *31*(4), 959–962. https://doi.org/10.1016/0091-3057(88)90413-3
57. Tucker, L. B., & McCabe, J. T. (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. *Frontiers in Behavioral Neuroscience*, *11*. https://doi.org/10.3389/fnbeh.2017.00013
58. Ulrich-Lai, Y. M., Figueiredo, H. F., Ostrander, M. M., Choi, D. C., Engeland, W. C., & Herman, J. P. (2006). Chronic stress induces adrenal hyperplasia and hypertrophy in a subregion-specific manner. *American Journal of Physiology-Endocrinology and Metabolism*, *291*(5), E965–E973. https://doi.org/10.1152/ajpendo.00070.2006
59. Wang, Y.-L., Han, Q.-Q., Gong, W.-Q., Pan, D.-H., Wang, L.-Z., Hu, W., Yang, M., Li, B., Yu, J., & Liu, Q. (2018). Microglial activation mediates chronic mild stress-induced depressive- and anxiety-like behavior in adult rats. *Journal of Neuroinflammation*, *15*(1), 21. https://doi.org/10.1186/s12974-018-1054-3

60. Westenbroek, C. (2003). Gender-specific effects of social housing in rats after chronic mild stress exposure. *Progress in Neuro-Psychopharmacology and Biological Psychiatry*, *27*(1), 21–30. https://doi.org/10.1016/S0278-5846(02)00310-X
61. Willner, P. (2017). The chronic mild stress (CMS) model of depression: History, evaluation and usage. *Neurobiology of Stress*, *6*, 78–93. https://doi.org/10.1016/j.ynstr.2016.08.002
62. World Health Organization. (2023a, March 31). *Depressive disorder (depression)*. World Health Organization. https://www.who.int/news-room/fact-sheets/detail/depression
63. World Health Organization. (2023b, September 27). *Anxiety disorders*. World Health Organization. https://www.who.int/news-room/fact-sheets/detail/anxiety-disorders
64. Yamanaka-Takaichi, M., Mizukami, Y., Sugawara, K., Sunami, K., Teranishi, Y., Kira, Y., Paus, R., & Tsuruta, D. (2021). Stress and Nasal Allergy: Corticotropin-Releasing Hormone Stimulates Mast Cell Degranulation and Proliferation in Human Nasal Mucosa. *International Journal of Molecular Sciences*, *22*(5), 2773. https://doi.org/10.3390/ijms22052773
65. Yang, R., Sun, H., Wu, Y., Lu, G., Wang, Y., Li, Q., Zhou, J., Sun, H., & Sun, L. (2019). Long-Lasting Sex-Specific Effects Based On Emotion- and Cognition-Related Behavioral Assessment of Adult Rats After Post-Traumatic Stress Disorder From Different Lengths of Maternal Separation. *Frontiers in Psychiatry*, *10*, 289. https://doi.org/10.3389/fpsyt.2019.00289
66. Yankelevitch-Yahav, R., Franko, M., Huly, A., & Doron, R. (2015a). The forced swim test as a model of depressive-like behavior. *Journal of Visualized Experiments: JoVE*, *97*, 52587. https://doi.org/10.3791/52587
67. Yankelevitch-Yahav, R., Franko, M., Huly, A., & Doron, R. (2015b). The forced swim test as a model of depressive-like behavior. *Journal of Visualized Experiments: JoVE*, *97*, 52587. https://doi.org/10.3791/52587

APPENDIX

Table 1. CVS Schedule *Also underwent overnight isolation

Day	AM Stressor/Time	PM Stressor/Time
6	Strobe lights/10:30 am	Predator odor/3:45 pm
7	Immobilization/10:45 am	Ultrasonic deterrent/2:00 pm
8*	Cold room/10:30 am	Strobe lights/2:30 pm
9	Cage tilt/11:45 am	Ultrasonic deterrent/2:00 pm
10*	Cold room/10:20-11:20 am	Predator odor/1:05 pm
11	Immobilization/9:30 am	Ultrasonic deterrent 1:35 pm
12	Cold room/10:05 am	Cage tilt/4:15 pm
13	Strobe lights/10:30 am	Predator odor/1:00 pm
14	Immobilization/10:35 am	Cold room/2:00 pm
15*	Ultrasonic deterrent/10:30 am	Strobe lights/1:00 pm
16	Cage tilt/10:30 am	Immobilization 1:50 pm
17	Predator odor/10:30 am	Cold room/1:00 pm
18*	Ultrasonic deterrent/11:15 am	Cage tilt/4:30 pm
19	Strobe lights/11:00 am	Predator odor/3:45 pm
20	Ultrasonic deterrent/10:30 am	Cage tilt/1:00 pm
21	Immobilization/10:30 am	Cold room/1:00 pm
22*	Strobe lights/10:30 am	Ultrasonic deterrent/2:00 pm
23	Cage tilt/11:00 am	Immobilization/2:00 pm
24	Ultrasonic deterrent/11:00 am	Cold room/2:30 pm
25*	Strobe lights/12:00 pm	Cage tilt/4:30 pm
26	Predator odor/10:30 am	Strobe lights/1:15 pm

Table. 2 Raw Descriptive Statistics for Open Field Test

	Sex	Treatment	Mean	Std. Deviation	N
Total Time Spent in Outer Zone (s)	Female	Control	566.200	14.2604	6
		Experimental	551.233	20.3482	6
		Total	558.717	18.4860	12
	Male	Control	577.383	17.0109	6
		Experimental	577.250	17.3717	6
		Total	577.317	16.3923	12
	Total	Control	571.792	16.0648	12
		Experimental	564.242	22.5826	12
		Total	568.017	19.5499	24
Total Time Spent in Middle Zone (s)	Female	Control	31.217	13.5955	6
		Experimental	44.800	18.8858	6
		Total	38.008	17.2180	12
	Male	Control	20.767	15.4354	6
		Experimental	22.017	16.6298	6
		Total	21.392	15.3110	12
	Total	Control	25.992	14.9029	12
		Experimental	33.408	20.7219	12
		Total	29.700	18.0536	24
Total Time Spent in Center Zone (s)	Female	Control	2.567	2.0393	6
		Experimental	3.950	2.9426	6
		Total	3.258	2.5195	12
	Male	Control	1.850	1.8918	6
		Experimental	.750	.7714	6
		Total	1.300	1.4924	12
	Total	Control	2.208	1.9124	12
		Experimental	2.350	2.6456	12
		Total	2.279	2.2587	24
Total Distance Traveled in Outer Zone (m)	Female	Control	45.87267	6.812274	6
		Experimental	40.22700	10.611703	6
		Total	43.04983	8.998471	12
	Male	Control	32.22683	7.995298	6
		Experimental	28.49283	10.087745	6
		Total	30.35983	8.894663	12
	Total	Control	39.04975	10.046644	12
		Experimental	34.35992	11.618661	12
		Total	36.70483	10.889123	24
Total Distance Traveled in	Female	Control	5.87867	1.149209	6
		Experimental	6.79517	3.957338	6
		Total	6.33692	2.819187	12

Outer Zone (m)	Male	Control	2.83383	1.464133	6
		Experimental	3.21833	2.898919	6
		Total	3.02608	2.198773	12
	Total	Control	4.35625	2.025628	12
		Experimental	5.00675	3.798355	12
		Total	4.68150	2.995477	24
Total Distance Traveled in Outer Zone (m)	Female	Control	.65200	.353165	6
		Experimental	.79183	.361731	6
		Total	.72192	.348573	12
	Male	Control	.30583	.214340	6
		Experimental	.18583	.189888	6
		Total	.24583	.202977	12
	Total	Control	.47892	.332050	12
		Experimental	.48883	.419550	12
		Total	.48388	.370056	24

Table. 3 Raw Descriptive Statistics for Elevated Zero Maze.

	Sex	**Treatment**	**Mean**	**Std. Deviation**	**N**
Latency to Open Arms (s)	Female	Control	127.00	143.279	6
		Experimental	6.17	6.432	6
		Total	66.58	115.465	12
	Male	Control	99.17	140.879	6
		Experimental	59.00	118.516	6
		Total	79.08	125.881	12
	Total	Control	113.08	136.249	12
		Experimental	32.58	84.644	12
		Total	72.83	118.302	24
Total Time in Open Arms (s)	Female	Control	19.83	21.858	6
		Experimental	40.17	46.067	6
		Total	30.00	35.980	12
	Male	Control	19.50	18.950	6
		Experimental	26.50	32.235	6
		Total	23.00	25.474	12
	Total	Control	19.67	19.504	12
		Experimental	33.33	38.573	12
		Total	26.50	30.696	24
Number of Transitions	Female	Control	4.00	4.050	6
		Experimental	4.83	3.601	6
		Total	4.42	3.679	12
	Male	Control	2.00	1.789	6
		Experimental	5.00	5.762	6
		Total	3.50	4.359	12

	Total	Control	3.00	3.162	12
		Experimental	4.92	4.582	12
		Total	3.96	3.973	24

Table. 4 Raw Descriptive Statistics for Sucrose Preference Test

	Sex	Treatment	Mean	Std. Deviation	N
Water Intake (mL)	Female	Control	18.33	17.039	3
		Experimental	13.00	7.211	3
		Total	15.67	12.061	6
	Male	Control	10.33	4.509	3
		Experimental	13.33	7.506	3
		Total	11.83	5.776	6
	Total	Control	14.33	11.978	6
		Experimental	13.17	6.585	6
		Total	13.75	9.236	12
1% Sucrose Solution Intake (mL)	Female	Control	37.00	19.468	3
		Experimental	53.33	25.502	3
		Total	45.17	22.176	6
	Male	Control	64.00	5.000	3
		Experimental	80.67	24.007	3
		Total	72.33	17.996	6
	Total	Control	50.50	19.501	6
		Experimental	67.00	26.736	6
		Total	58.75	23.917	12

Table 5. Raw Descriptive Statistics for Forced Swim Test

	Sex	Treatment	Mean	Std. Deviation	N
Day 1 Latency to Immobility (s)	Female	Control	101.67	53.906	6
		Experimental	111.33	83.971	6
		Total	106.50	67.464	12
	Male	Control	49.50	42.585	6
		Experimental	48.50	41.712	6
		Total	49.00	40.193	12
	Total	Control	75.58	53.734	12
		Experimental	79.92	71.222	12
		Total	77.75	61.740	24
Day 1 Total Time Immobile (s)	Female	Control	49.50	19.624	6
		Experimental	53.33	23.947	6
		Total	51.42	20.969	12
	Male	Control	40.83	32.664	6
		Experimental	60.50	34.887	6
		Total	50.67	33.819	12

	Total	Control	45.17	26.087	12
		Experimental	56.92	28.773	12
		Total	51.04	27.522	24
Day 2 Latency to Immobility (s)	Female	Control	39.83	21.245	6
		Experimental	24.17	17.011	6
		Total	32.00	20.091	12
	Male	Control	40.50	39.348	6
		Experimental	71.83	38.923	6
		Total	56.17	40.745	12
	Total	Control	40.17	30.151	12
		Experimental	48.00	37.945	12
		Total	44.08	33.755	24
Day 2 Total Time Immobile (s)	Female	Control	49.33	39.358	6
		Experimental	37.33	21.768	6
		Total	43.33	30.964	12
	Male	Control	44.17	54.905	6
		Experimental	55.17	23.284	6
		Total	49.67	40.616	12
	Total	Control	46.75	45.625	12
		Experimental	46.25	23.422	12
		Total	46.50	35.468	24

Table 6. Raw Descriptive Statistics for Cocaine-Induced Locomotor Activity

	Sex	Treatment	Mean	Std. Deviation	N
Baseline Locomotor Activity (Horizontal Beam Breaks)	Female	Control	1849.50	317.129	6
		Experimental	2420.67	567.043	6
		Total	2135.08	529.943	12
	Male	Control	1672.17	389.006	6
		Experimental	1948.33	462.101	6
		Total	1810.25	432.027	12
	Total	Control	1760.83	350.820	12
		Experimental	2184.50	551.417	12
		Total	1972.67	501.106	24
Cocaine-Induced Locomotor Activity (Horizontal Beam Breaks)	Female	Control	2055.00	1297.500	6
		Experimental	2839.00	571.319	6
		Total	2447.00	1039.822	12
	Male	Control	1502.33	947.081	6
		Experimental	1112.17	1023.129	6
		Total	1307.25	961.791	12
	Total	Control	1778.67	1120.822	12
		Experimental	1975.58	1198.933	12
		Total	1877.12	1139.473	24

Table 7. Raw Descriptive Statistics for Body Weights (g)

	Sex	Treatment	Mean	Std. Deviation	N
Week 1	Female	Control	77.00	5.692	6
		Experimental	76.33	8.165	6
		Total	76.67	6.719	12
	Male	Control	88.83	12.287	6
		Experimental	88.83	4.579	6
		Total	88.83	8.840	12
	Total	Control	82.92	11.024	12
		Experimental	82.58	9.080	12
		Total	82.75	9.879	24
Week 2	Female	Control	127.83	8.565	6
		Experimental	113.33	7.174	6
		Total	120.58	10.681	12
	Male	Control	160.17	15.639	6
		Experimental	151.83	14.634	6
		Total	156.00	15.082	12
	Total	Control	144.00	20.728	12
		Experimental	132.58	22.913	12
		Total	138.29	22.149	24
Week 3	Female	Control	154.50	9.072	6
		Experimental	129.33	7.737	6
		Total	141.92	15.406	12
	Male	Control	212.33	20.265	6
		Experimental	190.00	20.229	6
		Total	201.17	22.554	12
	Total	Control	183.42	33.709	12
		Experimental	159.67	34.885	12
		Total	171.54	35.674	24
Week 4	Female	Control	176.67	9.913	6
		Experimental	147.33	9.352	6
		Total	162.00	17.863	12
	Male	Control	254.83	26.506	6
		Experimental	226.33	25.928	6
		Total	240.58	29.094	12
	Total	Control	215.75	45.060	12
		Experimental	186.83	45.248	12
		Total	201.29	46.566	24

Week 5	Female	Control	189.83	13.527	6
		Experimental	166.17	9.968	6
		Total	178.00	16.766	12
	Male	Control	285.67	31.226	6
		Experimental	265.50	29.548	6
		Total	275.58	30.838	12
	Total	Control	237.75	55.056	12
		Experimental	215.83	55.974	12
		Total	226.79	55.438	24
Week 6	Female	Control	208.67	15.069	6
		Experimental	189.17	12.384	6
		Total	198.92	16.632	12
	Male	Control	320.17	33.409	6
		Experimental	305.67	35.820	6
		Total	312.92	33.881	12
	Total	Control	264.42	63.255	12
		Experimental	247.42	65.988	12
		Total	255.92	63.809	24
Week 7	Female	Control	218.67	15.501	6
		Experimental	200.50	10.464	6
		Total	209.58	15.779	12
	Male	Control	346.17	35.986	6
		Experimental	334.00	41.236	6
		Total	340.08	37.442	12
	Total	Control	282.42	71.633	12
		Experimental	267.25	75.388	12
		Total	274.83	72.334	24
Week 8	Female	Control	228.67	16.096	6
		Experimental	214.17	13.106	6
		Total	221.42	15.911	12
	Male	Control	370.83	40.375	6
		Experimental	357.50	45.860	6
		Total	364.17	41.778	12
	Total	Control	299.75	79.818	12
		Experimental	285.83	81.468	12
		Total	292.79	79.194	24
Week 9	Female	Control	233.33	18.608	6
		Experimental	220.67	12.144	6
		Total	227.00	16.376	12
	Male	Control	381.50	45.081	6
		Experimental	379.67	49.099	6
		Total	380.58	44.949	12

	Total	Control	307.42	84.074	12
		Experimental	300.17	89.764	12
		Total	303.79	85.135	24
Week 10	Female	Control	246.50	19.522	6
		Experimental	234.17	10.610	6
		Total	240.33	16.306	12
	Male	Control	388.00	75.816	6
		Experimental	407.50	50.520	6
		Total	397.75	62.262	12
	Total	Control	317.25	90.811	12
		Experimental	320.83	96.981	12
		Total	319.04	91.900	24

Table 8. Raw Descriptive Statistics For Organ Weights (mg)

	Sex	Treatment	Mean	Std. Deviation	N
Adrenal Glands	Female	Control	.063167	.0055755	6
		Experimental	.058950	.0094910	6
		Total	.061058	.0077411	12
	Male	Control	.055867	.0172037	6
		Experimental	.065850	.0256913	6
		Total	.060858	.0214879	12
	Total	Control	.059517	.0127748	12
		Experimental	.062400	.0188135	12
		Total	.060958	.0157955	24
Thymus	Female	Control	.380050	.0574169	6
		Experimental	.372717	.0529828	6
		Total	.376383	.0528125	12
	Male	Control	.418583	.0557258	6
		Experimental	.385700	.0804123	6
		Total	.402142	.0681585	12
	Total	Control	.399317	.0575758	12
		Experimental	.379208	.0652772	12
		Total	.389262	.0610641	24