SEX DIFFERENCES IN ACTIVITY, ANXIETY, AND SPATIAL MEMORY FOLLOWING ADOLESCENT CANNABINOID EXPOSURE IN LONG EVANS RATS

By

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Abstract

The effects of cannabinoids and the change in food intake of rats have been largely ignored. The decreased food intake of the drug rats should have an effect on the behaviors of the drug rats that is ignored in other research. This study accounted for the feeding changes and measured whether any differences were found across drug use and food intake. Measures were also taken across sexes in Long-Evans rats. The rats were exposed to cannabinoids during adolescence and measured behaviorally after they had reached adulthood.

Overall, the study found that while drugs had an effect on anxiety, it was sex dependent, with male rats expressing significant anxiolytic effects on the elevated plus maze and open field; female drug rats expressed a trend towards anxiolytic effects on the elevated plus maze, but less significantly than the male drug rats.

Performance of rats on the Morris Water Maze spatial memory task showed that decreased food intake correlated with a deficit in acquisition in male rats, but not female. In the reversal-learning task, memory extinction occurred in drug male rats faster than in the male control rats, suggesting that they were capable of learning the new location of the platform faster due to drug use. The male yoked rats also found the platform faster than the control rats, which reinforces the importance of continuing research in long-term food manipulation.

Consistent differences in sex were seen in which female rats were much more active than males. Additionally, in the Morris Water Maze task, females were consistently worse at the task when compared to males, often taking much longer to find the platform and traveling further to do so during acquisition. These findings show the value of studying both male and

female subjects; research assumes that females and males are the same when doing research, but clearly there are differences between them.

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Dedication

I would like to dedicate the work done here to my parents, John Jay and Audrey Ellen Ostrander, without whose constant support and infinite patience in dealing with me from the beginning, none of this would have been possible. You did a fine job raising me, even if I was an impossible smartass at times. Thank you.

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Chapter 1. Introduction

Marijuana (Cannabis sativa) and synthetic cannabinoids are the most commonly abused illicit drugs used by American youth (National Institute of Drug Abuse, 2012). In 2012, the National Institute on Drug Abuse estimated that by twelfth grade, forty-five percent of minors have used marijuana at some point in their lifetime. As well, by the twelfth grade it is expected that eleven percent of minors had partaken in more dangerous synthetic cannabinoids. Spice, a common name for synthetic cannabinoids that can be purchased over the counter at smoke shops, has been shown to have extreme side effects, such as negative reaction in the cardiovascular and central nervous systems, due to the increased potency over marijuana. (Macher, Burke, & Owen, 2012).

In recent years Colorado, Washington state and Washington D.C. repealed the laws criminalizing recreational marijuana use. The legal acquisition of marijuana has become much easier because of the change in legislature. Due to over-the-counter marijuana sales, minors are now much more likely to be able to obtain it. In a study of Colorado adolescents, Thurstone, Tomcho, Salomonsen-Sautel, and Profita (2013) found that adolescents who knew someone with a medical marijuana registration for ownership were more likely to smoke and hold more positive attitudes towards marijuana than adolescents who did not.

With the push for legalizing marijuana, research determining its effects must be obtained and presented to the public to inform them of the risks of its use. With the frequency in which American minors abuse marijuana and synthetic cannabinoids, knowledge regarding what they could be doing to themselves is important and should become household information if marijuana is to become legalized across the country.

In terms of medical marijuana, the ethical issues are numerous. In application, there is no prescription possible for a smoked substance, as the method of inhaling can vary widely across individuals and the THC content of different strains is difficult to regulate. Pill forms of THC exist, but are not sought after as readily by those with medical marijuana licenses due to the delay of and difficulty in controlling the euphoric effects (Wilkinson & D'Souza, 2014).

Ethically, doctors will have to consider a new, intellectual side effect that previously was not common in most prescriptions. Long-term use of marijuana has been shown to cause deficits intellectually in individuals, with younger users having larger deficits. Meier et al. (2012) performed a longitudinal study with 1,037 participants that were monitored from birth to thirty-eight years of age and found that those who used marijuana, especially between the ages of sixteen and nineteen, on average showed lower IQ scores overall than those who did not. The IQ score areas that suffered the most were found in WAIS-IV processing speed and verbal comprehension tests.

Further studies of cannabis have shown that individuals who smoked at an earlier age or frequently as adults show memory deficits. Solowij et al. (2010) used verbal memory tasks to measure memory across time as well as comparing with individuals who regularly use alcohol. When compared to alcohol use, marijuana users performed much worse on the verbal tasks as they showed significant deficits in learning new words, retrieval of known words, and recall of recently learned lists of words. These effects were substantially larger in marijuana users that started during adolescence than those who started later, with even those who smoked and quit during adolescence performing worse than constant adult users.

Design

This study intended to measure three factors across four behavioral tasks to determine the effects of cannabinoids and food manipulation during adolescence on adult rats. The rats' food intakes were manipulated to control for the diminished food intake in adolescent rats exposed to cannabinoids (Biscaia et al., 2003). The rats were injected with CP 55,940 (a synthetic cannabinoid) or vehicle (saline and tween 80) during adolescence (post-natal day (PND) 35-48; Rubino & Parolaro, 2008), administered a four-week washout period (PND 49-76), and tested as adults in four behavioral tasks (PND 77-100).

Behaviorally, the rats were measured on anxiety, spatial memory, and activity. The activity measures were taken across all four behavioral tasks (elevated plus maze, open field, object location recognition, and Morris water maze). The anxiety measures were designed for the elevated plus maze (a task that quantifies anxiety by the interactions of the rats with the open arms of the maze) and the open field (similarly, anxiety is measured by the rats' presence in a large open space, which is typically aversive to rats). Memory measures were taken from the object location recognition task (rats' capability of distinguishing that an object was moved between two trials) and the Morris water maze (a test that tasks the rats with having to find a hidden platform in a large pool of non-translucent water by using distal visual cues to locate the platform).

Cannabinoids

Multiple cannabinoid compounds have been discovered. In their book, Joy, Watson Jr. and Benson Jr (1999) outline the characteristics of different cannabinoids and their effects on animal physiology, including the endogenous cannabinoids, anandamide, which can be found in most mammals, and 2-AG (arachidonyl glycerol)(Table 1).

The cannabinoid agonists found in marijuana, Δ^9-THC and Δ^8-THC, were thought to be the reason that cannabis causes euphoric effects in users. Research shows that with synthetic cannabinoids (WIN 55,212-2), a change in dopamine levels in the nucleus accumbens occurs indirectly. The effects of cannabinoids in the ventral tegmental area were found to be caused by the drug inducing a decrease in the transmission of GABAergic inhibition neurotransmitter, allowing the nucleus accumbens to freely produce more dopamine (Szabo, Siemes, & Wallmichrath, 2002).

When comparing synthetic cannabinoids to Δ^9-THC and anandamide, potency should be considered as well as structure. By using Δ^9-THC as a standard (measure of potency at a baseline of one), anandamide comes in close to half as potent (0.47) and 2-AG, typically much more abundant than anandamide, was only .08 times as potent. In contrast, the synthetic cannabinoid used in this experiment, CP 55,950, is fifty-nine times as potent as Δ^9-THC in CB_1 receptor binding in brain tissue while being structurally similar to Δ^9-THC. Another synthetic to note that is frequently used, HU-210, expresses a variable potency of one hundred to eight hundred times as potent as Δ^9- THC based on the preparation of the drug (Joy, Watson, & Benson, 1999, p. 45-46).

With this considered, the use of CP 55,940 in this study should be observed with an understanding of the choice of this drug. As a potent, clean cannabinoid receptor agonist, CP 55,940 is structurally similar to Δ^9- THC while being potent enough to control for the metabolic difference between humans and rodents, which is something similar to one-hundred times as efficient as ours (Joy, Watson, & Benson, 1999), as well as functions on the same receptors in the brain as Δ^9- THC (Szabo, Siemes, & Wallmichrath, 2002).

Table 1. Cannabinoids. Information found in Felder et al., 1995.

Cannabinoids	**Agonist v. Antagonist**	**Receptor Affinity**
Δ^9- THC	Agonist	CB_1 & CB_2
CP 55,940	Agonist	CB_1 & CB_2
Anadamide	Agonist	CB_2 (minor CB_1)
Cannabinol	Agonist	CB_1 (minor CB_2)
WIN 55,212-2	Agonist	CB_1
HU 210	Agonist	CB_2
SR 141716A	Antagonist	CB_2 (very minor CB_1)

Cannabinoid Receptors

Cannabinoid receptors are found throughout the brain and have differences across species. Herkenheim et al. (1990) explored through autoradiography (a measurement of beta particles and gamma rays) the localization of activity in the brain after exposure to cannabinoids. When compared across species, rodents and human cannabinoid receptors reacted to CP 55,940 in very different ways. Common across the species, the hippocampal cortex was affected by cannabinoids and Δ^9-THC in short-term memory consolidation (Davies, Pertwee, & Riedel, 2002). Receptors in the cerebellum diminished motor control when exposed to WIN 55,212-2, specifically having an effect on the excitatory motor functions in the cerebellum (Yüce et al., 2010). This difference between animal and human distribution was also seen in Herkenham (1990), in which dogs experienced ataxia when exposed to CP 55,940, while humans only express mild motor control depression.

When observing diminished motor function and its relation to CB_1R receptors, Sierra et al., (2014) makes assumptions that through the activation of CB_1R receptors in the basal ganglia, neurotransmission in the striatum was inhibited, causing diminished motor control. These findings were important for observing an experimental model of Parkinson's disease, leading this project to observe the motor function for additional data. If changes to these

receptors can cause changes in the motor activity of rats, it was reasonable to assume that the same changes in motor function could be measured by activity in the behavioral tasks of this project.

Sex Differences and Anxiety

The behavior differences between male and female rats are important factors to consider when running tests. Behaviorally, sex differences can be seen very distinctly in both human and animal comparisons of anxiety. As defined by the Encyclopedia of Psychology (Kazdin, 2000), anxiety is characterized by emotions of worry, stress and tension, which can be expressed through emotional and physical symptoms. In humans, the US National Institute of Mental Health's anxiety disorder prevalence shows a significant difference with females being much more likely to experience anxiety disorders at some point in their life, typically with a more severe expression of symptoms (Pigott, 2003).

There is argument of whether this data is skewed due to the higher likelihood of women to seek help for their issues than men (Mariu, Merry, Robinson, & Watson, 2012). However, there is a large body of research on the biological differences in the brain that induce more powerful HPA (hypothalamic-pituitary-adrenal) axis responses to stress in women than in men, as reviewed by Donner and Lowry (2013).

HPA axis comparisons between rodents and humans create a puzzling issue in cross-species research. In rodents, the HPA axis in females is more active during times of stress, explaining rodent females' increased likelihood to experience higher levels of anxiety and greater endocrine responses when compared to males (Handa, Burgess, Kerr, & O'Keefe, 1994). In humans on the other hand, female HPA axis responses across multiple tests show less reaction in the hypothalamic-pituitary-adrenal axis (HPA)'s human corticotrophin

releasing hormones (CRH) than their male counterparts (Kajantie & Phillips, 2006). Despite the lack of difference in the stress response hormones however, human females still report having an increased feeling of stress during testing (Kelly, Tyrka, Anderson, Price, & Carpenter, 2007). There is an assumption that the difference in the actions of CRF in females may be the reason for increased feelings of anxiety, regardless of the activity of the HPA axis (Bangasser et al., 2010).

In animal models of the sex difference of anxiety responses, there is some debate as to whether the sex chromosomes are truly important as the Y-chromosome and second X-chromosomes do little to change the brain without the difference in gonadal hormones present in the body. A sex difference in females' reactions to corticotrophin-releasing factor (CRF) was found to be due to a different method of trafficking, in which male brains have a receptor internalization with β-arrestin2 which causes a desensitization in males, but is absent in females. Without the desensitization of CRF to the brain, CRF could be the cause of females having a more powerful anxiety response and causing more activity in the locus coeruleus (LC) norepinephrine system, leading to higher incidences of post-traumatic stress disorder (Bangasser et al., 2010).

In a study of maternal deprivation (removal from mother) and CP 55,940 exposure, regardless of the maternal deprivation, females showed significantly lower leptin levels and significantly higher ACTH and corticosterone levels than males, while performing nearly twice as many inner zone entries on an open field task (Llorente-Berzel et al., 2011). This expresses that the female rats, despite increased levels of anxiety cursors, were more active in the tasks, performing more entries into the center zone of the open field than the males.

While comparisons between the males and females are very important, covariates such as activity need to be considered.

The effects of anxiety on the brain can also be seen in pain sensitivity differences between females and males. Women experience higher levels of pain when exposed to the same level of pressure and force against their skin than males (Fillingim, King, Riberiro-Dasilva, Rahim-Williams, & Riley, 2009) despite the complete lack of difference in activity in the spinal cord sending pain signals. Goffaux et al. (2011) showed that if anxiety was controlled, females did not react any differently than males when exposed to the same levels of pain, indicating that anxiety, with a difference in trafficking of CRF, changes the perception of the same stimulus at a perceptual neuronal level, not a physical level.

In the current study, anxiety during adolescence was a major concern. Intraperitoneal injections for adolescent rats (PND 35-48) is a traumatic experience, which is why the rats who were not given drug were given a control vehicle to avoid a bias of anxiety by only the drug rats obtaining injections. Holmes et al. (2005) found that rats exposed to early life trauma suffered from an increase in corticotrophin-releasing factor (CRF) permanently, becoming hypersensitive to both CRF signals and CRF receptor blockades. The effects of the drug may be a traumatic experience; however, it was a trauma that they alone would experience.

Spatial Memory

For this study, the long-term effects of cannabinoids on memory were assessed by measuring memory for the spatial location of three-dimensional objects in the open field and monitoring spatial learning and reversal learning on the Morris water maze. Results of previous cannabinoid research have shown deficits in memory, with antagonists not

expressing opposing effects, when administered daily. The deficits found are likely due to the blockade of presynaptic neurotransmitter release within the hippocampus, inhibiting hippocampal functioning (Robinson et al., 2008). These results could additionally be caused by a decrease in food intake seen in rats exposed to CP 55,940 (Biscaia et al., 2003).

Abush & Akirav (2009)'s study of cannabinoid receptors in the hippocampus have shown that activation of the dorsal hippocampus with cannabinoids, injected with a cannula directed straight into the dorsal hippocampus, has an effect on memory and fear inhibition. When injected with WIN55,212-2 or AM404 (another cannabinoid receptor agonist), it was found that rodents' spatial memory on the water maze task was diminished significantly, both in the latency in which it took the drug rats to find the target platform between trials, and during the first trial of the following day, implying an effect on long-term memory.

Short-term exposure to cannabinoid, HU-210, expressed similar results in memory deficits. Hill, Froc, Fox, Gorzalka, and Christie (2004) exposed male Long-Evans rats to the drug for fifteen days during the dark period. The researchers ran them through the Morris water maze during the day prior to their daily injections. Their performance was extremely diminished by a longer inter-trial delay (300 seconds instead of 30 seconds). This deficit indicated that the rats' working memories were not hindered, but that the drug may have affected their long-term memories.

In another study of long-term cannabinoid effects on memory, researchers gave rats WIN 55,212-2 as adolescents or adults daily for twenty days and measured performance on the standard Morris water maze task after a twenty-day washout period. The rats that were given the cannabinoid as adolescents and then measured as adults, showed a larger increase of thigmotaxis, tactile reaction to the environment to navigate, during the learning process

than control rats. When administered as adults however, rats did not show significant deficits in this task after the washout period (Abboussi, Tazi, Paizanis, & Ganouni, 2014).

CP 55,940 has been shown to express this deficit in rats at a long-term level as well. O'Shea, McGregor, and Mallet's study (2006) on perinatal, adolescent, or early adulthood exposure indicated that doses of .15, .20 or .30 mg/kg all caused deficits on an object recognition task. The rats were measured on the time spent investigating the novel object, finding that drug rats investigated the novel object significantly less than the control rats if the drug was administered for at least twenty-one days between PNDs 4 and 67.

In addition, Mateos et al. (2011) found sex differences in the object location task when rats were exposed to CP 55,940 in adolescence. Females that were exposed to CP 55,940 spent less time investigating the moved object than the male CP rats, but not the control female rats. Interesting to note, when measured on a novel object task (removal of one object and inclusion of a totally new and different object), males showed a deficit when compared to both females and the control rats (Mateos et al., 2011).

Goodman and Packard (2014) measured the effects of WIN 55,212-2 on the impairment of learning and immediate memory recall in rats measured on a cued water maze task, a very similar task to the Morris water maze, but one that is designed to measure immediate capability of rats to perform while under the influence. The rats did not show a deficit based upon the group they were in, making it an important point to be made that the rats' memories were not affected at a working memory level.

Cannabinoids have a history of causing memory problems. In rodent studies, many different cannabinoids have been tested and have found that exposure, both short and long term, causes memory deficits when administered early in life. These findings were important

to the current study to determine whether the memory effects were caused by the action of the drug or by the reduced food intake experienced by drug animals.

Adolescence and Cannabinoids

Due to the increased use of cannabis by adolescents, the effects of long-term marijuana use have become a growing subject of research to determine the chronic effects on the brain during development. Adolescence is a key time in the neural development of humans due to the neural plasticity and rapid changes in the brain, which leaves adolescents more vulnerable to insult. Environmental input, such as the use of cannabis, can alter the brain in both humans and animals through any period of development but less so after adulthood (Rice & Barone, 2000).

When exposed to cannabis at an earlier age, humans tend to have adjustment issues. Difficulty in adjusting can lead to behaviors such as taking of other illicit drugs, partaking in violent crimes, suicide ideation and suicidal attempts (Fergusson et al., 2001). Researchers have also observed an increased likelihood of depression and anxiety disorders later in life in those who used cannabis during adolescence (Patton, Coffey, & Carlin, 2002).

Schneider, Drews and Koch (2005) studied the use of WIN 55-212,2 on juvenile rats (PND 10 – 40). During adulthood, measures of open field anxiety (decreased center zone time) and object recognition (amount of time spent investigating the objects) were taken from rats exposed during childhood. The cannabinoids had long-term anxiogenic effects, as adults spent significantly less time in the center zone than the control adult rats, however, no significant results were found in the object recognition task. This effect helps to support that exposure to cannabinoids during adolescence may have some effect during adulthood on the behavior of rats.

Converse to the Schneider research, Biscaia et al. (2003) measured adolescent exposure to cannabinoids (specifically CP 55,940) on multiple behavioral tasks, including the elevated plus maze and the open field task. On the elevated plus maze, it was found that adolescent rats exposed to cannabinoids spent a significantly greater amount of time in open arms than closed arms as adults, indicating a lower level of anxiety than the control rats. On the open field task, the drug rats moved around more in the center zone of the field than the control rats across sexes. This difference is indicative of a decrease in anxiety that may have been caused by the CP 55,940 exposure.

Renard, Krebs, & Jay (2012) performed a strain comparison of rats against exposure to CP 55,940 during adolescence or adulthood. Measured on the object location recognition task, the rats shared a constant effect regardless of their strain. The rats exposed during adolescence showed a definitive deficit in noticing the new location of the object, which was further hindered by an increase in the inner-trial intervals. Rats who were exposed to the cannabinoids during adulthood did not show this effect, instead relating very closely to the control rats during testing.

An important differentiation needs to be made regarding the effects of cannabinoids having long-term effects in younger users. Research does not support long-term effects after the user has reached adulthood. Due to the highly sensitive time period that is childhood and adolescence, it is highly important that we fully grasp the detrimental effects possible during this time. The current study was designed with this in mind, with previous research from the Radford lab exploring both perinatal and adolescent exposure. In previous research (Hartless, Casazza, Adams, & Jackson, in preparation), results lead us to believe that the diminished food-intake of cannabinoid-exposed rats during lactation resulted in significant deficits in

spatial learning later in life when measured on the T-maze task as adults, similar to the group exposed to CP 55,940.

Nutrition

In addition to the acute effects of cannabinoids on learning and memory, there is often a decrease in food consumption reported in rats while under the influence (Biscaia et al., 2003). Due to the decreased caloric intake of rats exposed to CP 55,940 a yoked-food control group was implemented in the current study such that one group of rats received the same amount of food as consumed by the drug group on the previous day.

As described by Fukuda, Françolin-Silva and Almeida (2002), postnatal malnutrition would be any diet in which the pup consumed a diet deficient in protein during development (post-natal days 0-49), specifically less than sixteen percent of their daily food intake. Malnutrition has been found to have effects on rat body weight and development both behaviorally and neurologically. In all instances of protein deprivation reviewed, including only twenty-five percent of protein deprivation and simply being born to large litters after birth cause a very significant decrease in body weight in rats (Alamy & Bengelloun, 2012).

Behaviorally, rats with a diminished protein intake during postnatal development showed deficits when compared to control rats in distal spatial cue navigation on the Morris water maze task (Alamy & Bengelloun, 2012). The behavioral differences were thought to be caused either by a deficit in the development of the hippocampus or increased emotionality seen as stronger reactions to aversive stimuli caused by early malnutrition in rats (Fukuda et al., 2002). The Morris Water Maze was included in this study to determine whether these effects would be different in any way between the drug and the yoked groups, as both were expected to have deficits.

In open field tests, rats that had a decreased intake of food as pups showed a return to normal activity when returned to a proper nutritional diet as adults. In the Bengelloun (1990) study, it was found that rats that had a lower intake of food during adolescence expressed much higher levels of activity than those who had normal food intake. The increased activity in the malnourished rats subsided after forty-nine days on a regular diet (Bengelloun, 1990).

Similarly, Almeida, Tonkiss, and Galler (1996) found that female rats that are malnourished during adolescence when returned to normal feeding expressed increased activity. When measured on the elevated-plus maze, the malnourished rats spent more time in the open arms and performed more open arm entries than the control rats. The researchers theorized that this was due to increased impulsivity and not a decrease in anxiety behaviors, as their general activity levels were higher, performing more arm entries overall when compared to the control rats.

Our model was designed to only allow litters into the study with between eight and twelve pups to avoid large differences in the intake of protein and possible development differences from being from larger or smaller litters. The nutrition of rats during adolescence is a major factor when looking at adolescent long-term cannabinoid research, as the effects of poor nutrition could be the cause of the deficits and not the drug itself. With research supporting poor memory, but increased activity in malnourished rats, it is important to determine if the same memory and activity changes are occurring in drug rats, or if the two effects are induced by different factors.

Hypotheses

With the influx of legalization of cannabis and cannabinoids, our greatest concern should be in determining the long-term effects of cannabis use. According to current

research, there are potentially several undesirable effects resulting from exposure during puberty, such as anxiety, as well as learning and memory changes. Determining whether some of the deficits in previous rodent studies were due to nutritional changes as a result of cannabinoid use is critical.

In this study, decreased food intake found in the drug rats (Biscaia et al., 2003) was compared by the use of a food manipulation control group yoked to the drug group throughout the injection portion of the study.

The spatial memory capabilities of both drug (O'Shea et al., 2006) and yoked (Alamy & Bengelloun, 1990) were expected to be worse than the control rats when measured on the object location recognition and Morris water maze tasks.

It was expected that there would be differences in the anxiety measures where the yoked and control rats would express higher levels of anxiety than the drug rats in the elevated plus maze and open field tasks. The results of anxiety measures were uncertain, as previous research has shown conflicting results. In Curry (2013), the post-natal drug rats and the yoked rats showed no differences in anxiety when measured in an open field task, boli (frequency of defecation), hide box entries, and hide box exit latency. However, in a pilot study (Ostrander & Davidow, 2014), an increase in boli was found to be significant during the open field testing with control rats showing higher levels of anxiety.

It was lastly anticipated that activity would show a difference between drug groups and sex. Female rats have been shown to be more active than male rats (van Hest, van Haaren, & van de Poll, 1987) on open field and elevated plus maze tasks. As well, activity has found to be increased by decreased food intake (Gutierrez, 2013) and decreased by cannabinoids (Wiley, Evans, Grainger, & Nicholson, 2011).

Chapter 2. Methods

Subjects

Thirty-six naïve female and thirty-nine naïve male Long-Evans rats descended from rats obtained from Charles River Research Laboratories were used in this experiment at the behavioral and cognitive neuroscience animal lab at Radford University. The rats were bred within the Radford lab in plastic cages (44x22x20.5cm) in which the males were present in the cage with the females until gestational day 21 or post-natal day (PND) 2. Litters were culled to a maximum of twelve pups to promote equal nutrition across pups. Additionally, litters were required to consist of at least 8 pups at weaning to be included in the study to assure comparable infantile nutrition. Each of the twelve cohorts used consisted of three males and three females from the same litter whenever possible. One cohort was used consisting of only three males and no females to account for previously excluded rats due to health issues. If two pairs of breeding parents were similar genetically, the combined offspring were used to make a single cohort of six animals. Most cohorts were genetically dissimilar from each other as possible; two cohorts did share a distant ancestor (three generations in the past).

Upon reaching PND 22, each rat litter was weaned and separated by sex. On PND 34, rats were separated based upon which condition they were semi-randomly assigned to: control, food-deprived control (yoked) or CP 55,940 exposure (drug). Each of these experimental groups consisted of twelve females and twelve males, allowing the power (β) requirement of .80 to be exceeded to allow for any error that could have occurred.

After semi-random assignment (based on body weight) on PND 34, the rats were given distinct tail markings and single-housed in metal hanging cages (25cmx20cmx18cm)

fitted with metal mesh on the bottom of the cages to capture all food pieces from falling out of the cage. From PND 34 to PND 50, food was weighed and given to the rats based on their group assignment. Drug and control rats were given free-access to food daily and the yoked-control rats were given food equal to the amount eaten by the drug rats the previous day (±0.5 grams).

Upon reaching PND 35, rats began receiving daily i.p. (intraperitoneal) injections of either a tween-80 vehicle (twenty-four males, twenty-four females) or CP 55,940 (twelve males, twelve females). The injections were administered for fourteen consecutive days, during which the rats were weighed daily.

On PND 50, the rats were weighed and returned to group housing with their same-sex cohort members in a plastic cage. During the group-housed washout period, rats were returned to free-feed. From PND 50 to PND 75, rats were weighed every five days and handled as little as possible. Starting on PND 77, behavioral testing began.

Rats were kept in a 12:12 light/dark cycle, temperature and humidity controlled vivarium during which all testing and drug administration occurred during the light cycle. This project was approved by the IACUC and all procedures were in accordance with the NIH *Guide for the Care and Use of Laboratory Animals.*

Drug Condition

Intraperitoneal (i.p.) injections of 0.35mg/kg (body weight) were administered to all of the rats, consisting of either a vehicle solution (tween 80, saline, and evaporated ethanol) or a synthetic cannabinoid agonist, CP 55,940. Injections began on PND 35 and continued through PND 48. The drug consisted of 3.5mg of CP 55,940 ((-)-*cis*-3-[2-Hydroxy-4-(1,1-dimethylheptyl)phenyl]-*trans*-4-(3-hydroxypropyl)cyclohexanol, (Tocris Bioscience,

Ellisville, MO), dissolved in 0.5mL of ethanol and combined with 75.0 μL of Tween 80 (polyoxyethylene sorbitan monoleate). The solution was mixed thoroughly before evaporating the ethanol using a stream of compressed oxygen. The remainder was then mixed with 9.925mL of saline to produce a final solution of 10.0mL (O'Shea, Singh, McGregor, & Mallet, 2004). The vehicle solution was mixed in the same manner as the drug condition without the CP 55,940.

Behavioral Measures

The behavioral tasks used within this experiment were designed to measure activity, anxiety, spatial learning, and memory for object locations. Rats were tested in the following order of behavioral tasks: the elevated plus maze (EPM), the open field and object location recognition tasks (OF/LocR or LR) and the Morris water maze (MWM). The order was designed to initially measure anxiety and allow the rats to become accustomed to behavioral testing on the EPM, which allowed novel anxiety to be avoided further in the OF/OLR tasks, then finally the Morris water maze to avoid any short-term trauma potentially associated with the water maze task. A four to five day resting period occurred between each behavioral task to allow the rats to recuperate and to reduce interference.

The EPM and open field apparatuses were cleaned with a mixture of nine parts water and one part vinegar between trials to cleanse any odor trails left by the rats that could influence the behavior of subsequent rats. The Morris water maze was drained and cleaned every two to four days. Each experiment took place in testing labs in which overhead lighting and video cameras recording to a DVR (digital video recorder) were used to measure their behavior. The open field/object-recognition location task was processed through the use of

AnyMaze software, while the Morris Water Maze used HVS tracking software. Finally, white noise was used to prevent any exterior noise from affecting their performance on tasks.

Elevated-Plus Maze (EPM)

The elevated plus maze is a standardized task used to measure anxiety in rats in one 10-minute trial. Activity was also measured on this task. Each rat ran this task upon reaching PND 77 and was recorded on a DVR and coded by hand at a later point in time. This task was selected first in order to establish the level of anxiety experienced by each condition and to be able to compare to results on later tasks.

Apparatus. The plus maze was a white-painted wooden apparatus. The maze sat 50 cm above the ground with four 11.5 cm wide arms that measure 61.0 cm in length. The center of the plus-shaped design was 11.5 cm square. Two opposing arms (arm 2 and arm 4) were surrounded by walls that were 40 cm high and open at the top to create the open-closed arm dichotomy integral to the process of anxiety measurement. A DVR camera was mounted directly above the center of the maze to record and playback behavior for coding. Between trials, the elevated plus maze was cleaned with a mixture of one-part vinegar/nine-parts water and a sponge.

Procedure. Rats were placed in the center square zone of the elevated plus maze, facing the closed-arm (arm 2), for one ten-minute trial that would be separated into two pieces when coding but ran simultaneously without any interruption by the experimenter. After being placed in the center of the maze, the experimenter immediately left the room to monitor the rat's activities from the adjacent room. During the session, the amount of time the rat spent in the closed or open arms as well as the number of entries into each of the arms

of the plus maze were recorded in order to determine the level at which the rats experienced anxiety.

Modeled after Arévalo, Miguel, and Hernández-Tristán (2001) and previous studies run within the lab, the measures of anxiety were based on a formula of time spent in open arms/total time multiplied by 100 and the number of entries into the open arms/total time multiplied by 100. If the ratios from these formulas are lower than fifty-percent it indicates avoidance of the open arms, and the rat is described as being more anxious. Additionally, the number of boli were monitored in order to further measure anxiety, as an increase in boli is an indicator of increased anxiety.

When coded, the video was replayed and entries into the arms were measured. Entry into the arm was classified as when the rat's four paws had crossed the line separating the arm from the center platform. Exit of the arm, however, was classified by the center of the rat's back crossing the separating line, based on the reasoning that the rat's cognitive senses would no longer realize itself as being contained within an arm. Time spent in open arms worked as a measure of anxiety, while the number of entries into the arms was used as a measure of activity.

Open Field (OF)

The open field was the second task used to measure anxiety and activity. The open field task began on PND 82 or 83 and ran for two days. Activity was measured by the overall distance traveled in the open field while anxiety was measured on the number of inner zone entries, the amount of time spent in the inner zone as opposed to the outer zone, and the number of boli produced.

Apparatus. The open field is a large 103x103 cm wooden box, painted white, with 46 cm tall walls. When coding the rats' behavior the floor is divided into sixteen 25x25 cm cells in a 4x4 pattern. The outer twelve cells along the wall are considered the outer-zone (OZ), while the inner four cells are called the inner-zone (IZ). The open field was cleaned with the vinegar-water cleaning mix. Posters were placed on the walls of the room in order to give directional cues to the rats.

Procedure. Prior to the first trial on each day, subjects were placed in small holding cells and moved on a cart into the hall outside of the running room for a minimum of 5 minutes. At the start of each trial, rats were placed in the southwestern corner and the experimenter left the room immediately.

Following the procedure of Soffié, Buhot, and Poucet (1992) and Ostrander and Davidow (2014), day 1 and day 2 on the open field was used as habituation for the object location task. The AnyMaze software was used to measure activity (the distance traveled in the empty field and line crossings) and anxiety (entries into and time spent in the center zone). Each of the two trials of the open field task lasted 10 minutes.

Object Location Recognition (OLR)

The object location recognition (OF/OLR) task is one of two tasks used to measure the spatial memory of rats. The object location recognition task started immediately after the open field task. The open field task was used to measure the activity levels and anxiety of the rats. The object location recognition task consisted of two separate five-minute trials on PND 83/84 where two objects were placed within the open field and one object was relocated between sessions. The novel object displacement was used to determine whether the rats recognized the displacement based on an increase in exploration of the relocated object.

Apparatus. The open field was used for this task as well. The cells were fixed with squares of Velcro in the middle of each to secure objects. As well, two small jelly jars, filled with colored marbles, were used as objects for the object location recognition task. The objects were also cleaned between each trial with the vinegar-water cleaning mix. Posters were placed on the walls of the room in order to give directional cues to the rats.

Procedure. Prior to the first trial on each day, subjects were placed in small holding cells and moved on a cart into the hall outside of the running room for a minimum of 5 minutes. At the start of each trial, rats were placed in the southwestern corner and the experimenter left the room immediately.

Following the procedure of Soffié, Buhot, and Poucet (1992) and Ostrander and Davidow (2014) for the object location recognition task, two identical objects were placed in two cells of the open field. During trial 1, objects were placed in cell 7 and cell 11. Exploration of the objects was measured in terms of number of contacts and time spent exploring each during the 5-minute trial. At least 10 minutes after trial 1, but no more than 12 minutes later, the second trial was conducted. The object placed in cell 11 remained, but the object in cell 7 was moved to cell 10 for the second trial. After each session, the rats were placed in their holding cell outside of the running room for return to their home cages or a ten-minute cool down period between object location trials. AnyMaze software was used to record center zone and outer zone entries and time spent, while a blind observer was used to measure the frequency in which the rats observed the objects.

Morris Water Maze

The water maze is a task used to measure spatial learning and memory. This test was run beginning on PND 89, 90, or 91. Each rat was tested with four trials per day across five

days to learn to swim directly to the hidden platform when released from four different starting points of the pool. It was expected that by day five of testing all rats would be able to locate the escape platform, which remains in the same location, in under thirty seconds.

Modeled after Abboussi et al. (2014), on the sixth day, when the escape platform was removed from the water maze, the control rats should have spent more time in the northeast quadrant to find the platform. The learning process for the water maze task uses distal cues to provide rats with the location of objects they cannot see, smell or hear (Morris, 1981).

Apparatus. The water maze was a large, round plastic tub measuring 1.63 m in diameter, filled to the depth of 28 cm high, just above the 26 cm high escape platform. The maze was separated into four quadrants: North, East, South and West, with the escape platform always being placed in the center of the Northeast quadrant. The water in the maze was made opaque by using 950ml of non-toxic white tempera paint. The water was maintained at a constant temperature of 22±2 degrees Celsius to ensure that rats did not suffer from hypothermia. These temperatures have been shown not to have a differing effect on the performance of rats in the water maze (Anderson, Moenk, Barbaro, Clarke, & Matuszewich, 2013).

Procedure. Rats were placed at each starting point of the maze once per day, according to a randomly assigned schedule, with their nose facing the outside of the pool. Rats were allowed to search for the escape platform for up to ninety seconds. When they found it, they were left on the platform for fifteen seconds to reinforce the location of the platform. If the rat did not find the platform after ninety seconds, it was guided to the platform and left on the platform for fifteen seconds. Upon removal from the maze, the rats were placed in a container lined with a dry towel for at least thirty seconds, then placed back

into the maze at a different starting point. The quadrant order was assigned based upon the order suggested by Vorhees and Williams (2006) and reversed after half of the rats had run the water maze to counter balance the distance to the platform from each of the starting points across days. Each of the four starting points were used each day. After four consecutive trials, one starting in each position, the rats were returned to their home cages.

On the sixth day of the Morris water maze task, we ran a probe trial in which the platform was removed. The rat was placed in the south quadrant facing the wall and the time spent and frequency of entries into the northeastern quadrant were measured. After thirty seconds, the rat was removed from the water maze and placed into a holding cage. During this inter-trial interval (on average greater than sixty seconds), the platform was replaced into the Northwestern quadrant and a reversal learning task was performed. The rats were given four trials to attempt to learn the new location of the platform and their search strategies were observed.

Chapter 3: Results

The effects of the vehicle, the drug, and the yoked-food manipulation were assessed using one-way, two-way, and repeated measures Analyses of Variance (ANOVAs), *t*-tests and LSD planned comparison analyses. Data were analyzed and graphs were produced using SPSS 22 and Microsoft Excel. Tasks were analyzed through an omnibus 2x3 ANOVA to identify sex differences between the rats, as the behavior of male and female rats is often radically different. Within the individual sexes, *a priori* comparisons were run between the control rats and each of the manipulation groups throughout the entire set of analyses.

Food Intake

Food intake of the rats was measured daily during the injection period (PND 35-49). Drug and control rats were given an excess amount of food to eat each day while the yoked rats were only given the amount of food that was consumed by the drug rats the previous day. The food was placed in the cage onto a mesh flooring so that no food could be dropped through the wire floor of the hanging cages. Directly before injections, the rats' food was weighed and new food was measured out and placed within the cage for the following twenty-four hours.

A 3 x 2 x 5 repeated measures ANOVA, with group (drug, yoked, and control) and sex (male and female) as between-group factors, was performed on the amount of food consumed across five blocks of three days each (e.g., PND 35, 36 and 37 were analyzed as one datum point, then 38, 39 and 40, and so on). A main effect for sex was seen, $F(1, 66) = 114.69$, $p < .001$, $\eta^2 = 0.63$ in which the male rats ($M = 26.82$, $SD = 1.24$) on average consumed more food than the females did ($M = 21.40$, $SD = 1.24$). An effect for group was also seen, $F(2,66) = 20.41$, $p < .001$, $\eta^2 = 0.38$, where the control rats ($M = 26.37$, $SD = 1.52$)

consistently consumed more food than the drug (M = 23.29, SD = 1.52) or yoked rats (M = 22.67, SD = 1.52). An interaction was seen of blocks by group, $F(8,264) = 3.61$, $p = .001$, $\eta^2 = 0.10$, where it can be seen that the groups gained weight at different rates across the blocks of days. An interaction of blocks by sex was also seen, $F(4,264) = 17.42$, $p < .001$, $\eta^2 = 0.21$, in which the male rats gained weight faster than female rats across blocks of days.

Four 2 x 5 (group by blocks of days) repeated measures ANOVAs were performed comparing each manipulation group to the control rats. It was found that male control rats (M = 29.68, SD = 2.29) consumed significantly ($F(1,22) = 19.94$, $p < .001$, $\eta^2 = 0.48$) more food than male drug rats (M = 25.82, SD = 2.12); no interaction effect was found in this *a priori* comparison. The control males also consumed significantly more food than the yoked (M = 24.95, SD = 2.29) males, $F(1,22) = 25.75$, $p < .001$, $\eta^2 = 0.54$. Additionally, an interaction was found in the yoked and control comparison of blocks by group, $F(4,88) = 3.14$, $p = 018$, $\eta^2 = 0.12$, in which the control rats ate a faster growing amount across the blocks of days than the yoked rats (see Figure 1).

This effect was also seen in the female control rats (M = 23.05, SD = 2.19) when compared to drug females (M = 20.75, SD = 2.19), $F(1,22) = 6.61$, $p = .017$, $\eta^2 = 0.23$, but no significant interaction effect was seen. The yoked females (M = 20.40, SD = 1.79) ate significantly less than the control females, $F(1,22) = 13.17$, $p = .001$, $\eta^2 = 0.37$. An interaction of blocks and groups was seen in the yoked and control comparison, $F(4,88) = 4.78$, $p = .002$, $\eta^2 = 0.18$, reinforcing the group effect previously mentioned (see Figure 2).

As predicted, the drug rats consumed less food than the control rats. Yoked rats were manipulated such that they ate a smaller amount of food than the control rats because they

were given less food, therefore the result found amongst yoked rats was determined by design.

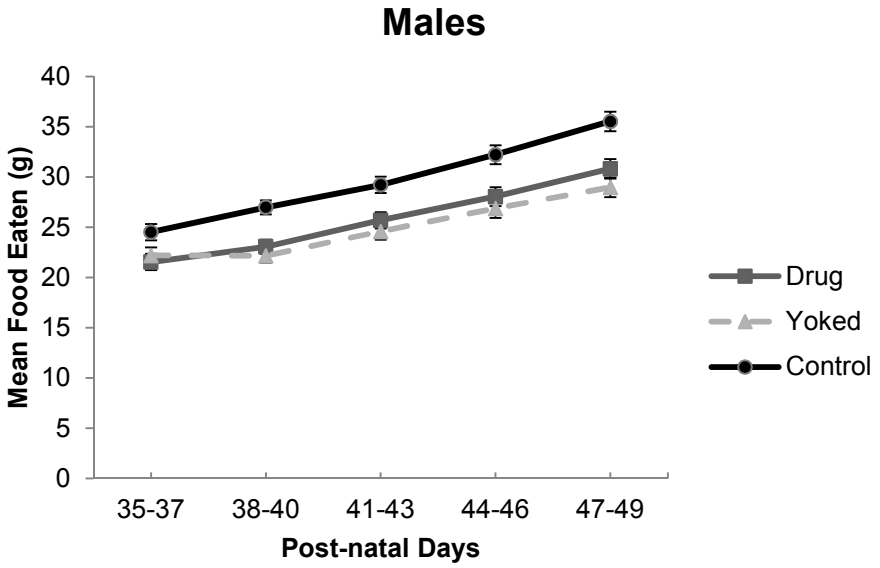

Figure 1. Mean food eaten by males during the injection period (PND 35-49).

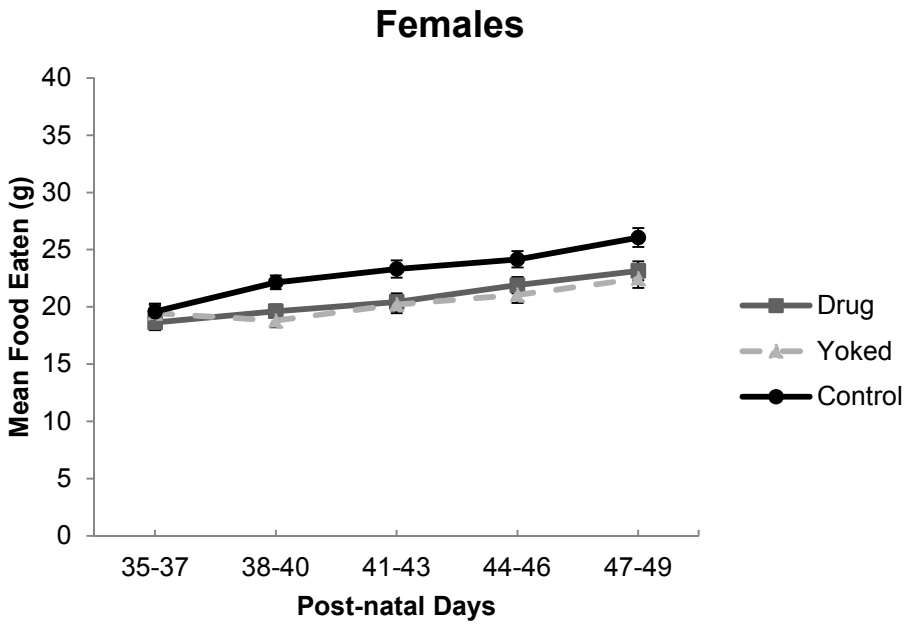

Figure 2. Mean food eaten by females during the injection period (PND 35-49).

Body Weight

The body weights of subjects were collected at planned intervals beginning shortly after birth. Average body weights were compared during the drug/vehicle injection period (PND 35-49) in blocks of 3 days. All rats were weighed at five-day intervals during the washout period from PND 50 until PND 75. They were weighed using an electronic scale zeroed out beforehand.

Prior to injections. On post-natal day 35, the day prior to the injection period, the rats were weighed in order to determine that they were not statistically different at the beginning of the injection period. A 3 x 2 two-way ANOVA with group and sex as factors was used to analyze their weight on PND 35. There was a significant difference between the sexes, $F(1,66) = 46.66$, $p < .001$, $\eta^2 = 0.41$, in which the male rats ($M = 157.61$, $SD = 12.21$) were significantly heavier than the female rats ($M = 136.99$, $SD = 12.86$). There was no significant difference between groups, $F(2,66) = .30$, $p = .740$, $\eta^2 = 0.01$.

Two separate one-way ANOVAs were performed (one for males and one for females) where it was found that there were no significant differences between the male rats across groups, $F = .436$, $p = .650$, $\eta^2 = 0.03$. Female rats also were not significantly different across groups, $F = .120$, $p = .887$, $\eta^2 = 0.01$. Assuring that the rats were similar in weight prior to the injection period allowed us to measure the change in weights from a common baseline (Figure 3).

When analyzed independently using between-samples t-tests, no significance was found between the male drug ($M = 160.08$, $SD = 15.11$) and the male control ($M = 157.38$, $SD = 10.31$), or the male yoked ($M = 155.37$, $SD = 11.28$) to the male control; $t(22) = .51$, $p = .614$, $d = 0.21$ and $t(22) = -.46$, $p = .652$, $d = -0.19$ respectively.

Similar results were found in females on independent samples t-testing. The female drug rats (M = 137.83, SD = 13.39) were not significantly different than the female control rats (M = 135.47, SD = 11.92), $t(22) = .46$, $p = .652$, $d = 0.19$. The female yoked rats (M = 137.67, SD = 14.17) were not statistically different from control rats either, $t(22) = .41$, $p = .685$, $d = 0.17$.

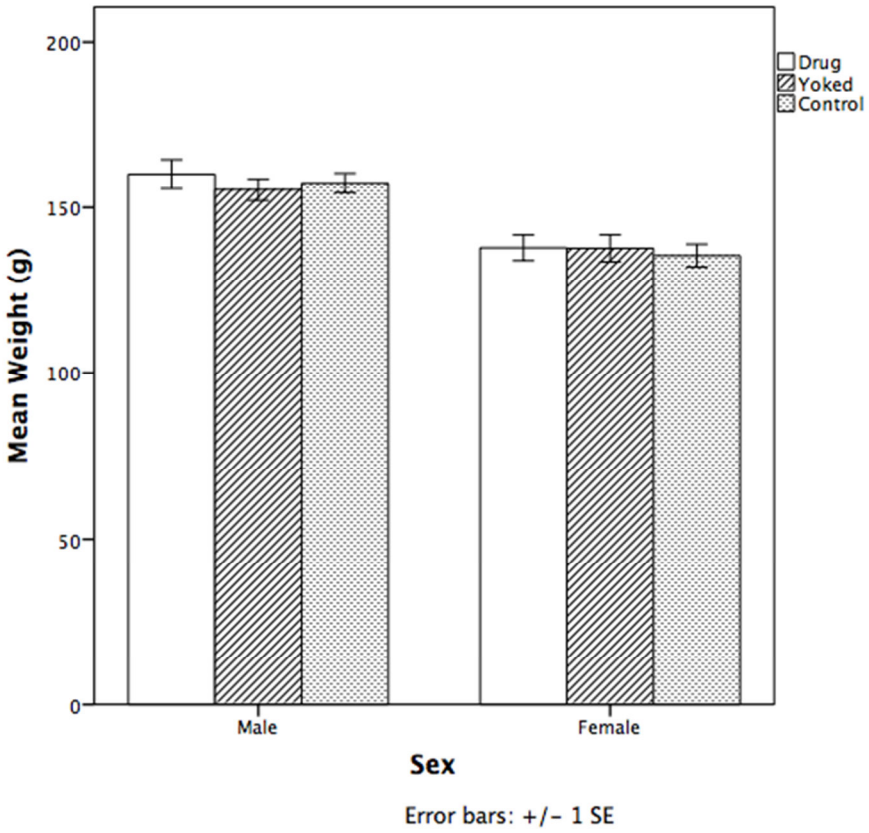

Figure 3. Mean body weights of rats based on drug groups and sex on PND 35.

Body weight during the injection period. The rats were weighed daily as a prerequisite of determining the dosage for their injections. Their weights were analyzed in blocks of three days at a time for analytic purposes. The omnibus 2 (sex) x 3 (group) x 5 (block) repeated-measures ANOVA was performed to observe overall effects and followed

by four separate 2 (group) x 5 (blocks) repeated-measures ANOVAs to individually compare the manipulation groups to the control groups within each sex.

The omnibus repeated measures ANOVA was conducted to assess the effects of treatment on weight gain for three-day blocks. An effect for sex was found, $F(1, 66) = 159.53$, $p < .001$, $\eta^2 = 0.71$, where the male rats ($M = 209.96$, $SD = 8.21$) weighed significantly more than the female rats ($M = 167.61$, $SD = 8.21$) across the injection period. An effect was also found for group, $F(2,66) = 9.68$, $p < .001$, $\eta^2 = 0.23$, where the drug ($M = 186.54$, $SD = 10.06$) and the yoked ($M = 181.09$, $SD = 10.06$) weighed significantly less than the control rats ($M = 198.73$, $SD = 10.06$). Significant interactions were seen between blocks and groups, $F(8,264) = 29.93$, $p < .001$, $\eta^2 = 0.48$, in which the rats all gained weight across blocks, but the control rats did so at a faster rate than the drug or yoked rats. A significant interaction was also seen between blocks and sex, $F(4,264) = 249.01$, $p < .001$, $\eta^2 = 0.79$, with the male rats gaining weight at a faster rate than the female rats across blocks. The three-way interaction of blocks by group by sex was not significant, $F(8,264) = 1.63$, $p = .117$, $\eta^2 = 0.05$.

In the males, separate 2 (group) x 5 (blocks) repeated measures ANOVAs showed effects of group for the *a priori* comparisons. The effect found between the drug ($M = 207.26$, $SD = 15.19$) and control ($M = 222.50$, $SD = 15.19$) rats indicated that the control rats weighed significantly more during the injection period than the drug rats, $F(1,22) = 6.04$, $p = .022$, $\eta^2 = 0.22$. An interaction of group by blocks was also found, $F(4,88) = 29.41$, $p<.001$, $\eta^2 = 0.57$, in which the rate at which the control rats gained weight was significantly faster than the drug rats. A group effect was also seen when comparing the yoked males ($M = 200.12$, $SD = 13.34$) to the control males, $F(1,22) = 16.87$, $p < .001$, $\eta^2 = 0.43$, with the yoked

rats gaining significantly less weight. The interaction of group by blocks was also significant here, $F(4,88) = 30.22$, $p < .001$, $\eta^2 = 0.58$ (Figure 4).

The pattern of results in the female rats was slightly different. The comparison between the drug females ($M = 165.82$, $SD = 14.01$) and the control rats ($M = 174.97$, $SD = 14.01$) was not significant, $F(1,22) = 2.56$, $p = .124$, $\eta^2 = 0.10$, but the interaction of group by blocks was, $F(4,88) = 9.00$, $p < .001$, $\eta^2 = 0.29$, with the control rats gaining weight significantly faster than the drug rats. The comparison between the female yoked ($M = 162.05$, $SD = 12.19$) and control rats was significant, $F(1,22) = 6.74$, $p = .016$, $\eta^2 = 0.23$, as the control female rats weighed significantly more than the yoked females during the injection period. The interaction of group by blocks was also significant for the yoked and control female comparison, $F(4,88) = 20.04$, $p < .001$, $\eta^2 = 0.52$, with the control rats not only weighing significantly more, but gaining weight significantly faster than the yoked rats (Figure 5).

Independent-samples *t*-tests were also conducted on individual days (drug vs. control and yoked vs. control) for all weights collected during the injection period to determine when the manipulation groups became significantly different from control.

The male drug group (M=196.12, SD = 15.71) became significantly different than the male control group (M=210.85, SD = 13.21) on PND 41, $t(22) = -2.49$ $p = .021$, $d = -1.01$, while the male yoked group (M=169.33, SD = 12.98) became significantly different from the male control rats (M=183.58, SD = 12.77) on PND 38, $t(22) = -2.71$, $p = .013$, $d = -1.11$.

Body weights in the manipulated females took longer to differentiate than they had for the males. The female drug group (M=183.53, SD = 18.24) weighed significantly less than the control group (M=197.50, SD = 11.42) for the first time on PND 46 ($t(22) = -2.24$, p

= .035, d = -0.92). Body weights amongst the female yoked rats (M=156.08, SD = 13.49) became significantly different than the female control rats (M=169.38, SD = 11.22) on PND 41, $t(22)$ = -2.62, p = .015, d = -1.07.

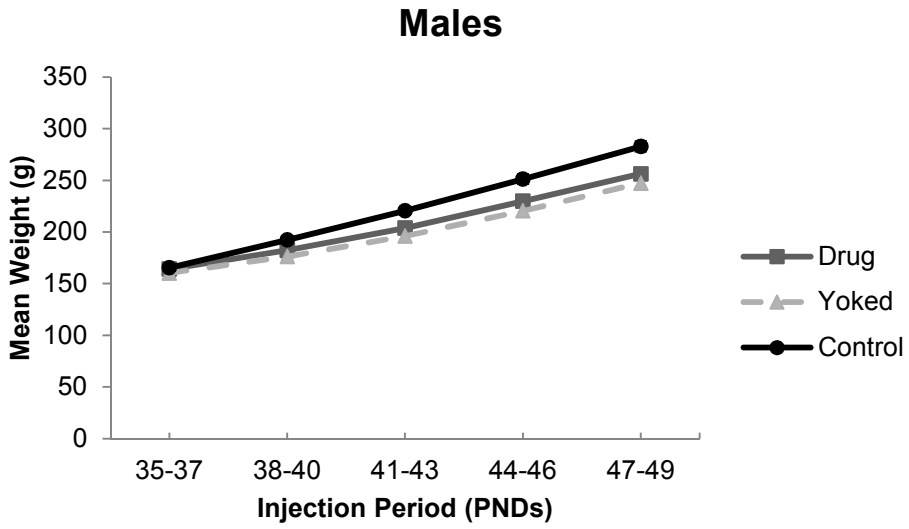

Figure 4. Males mean weight during the injection period (PND 35-49).

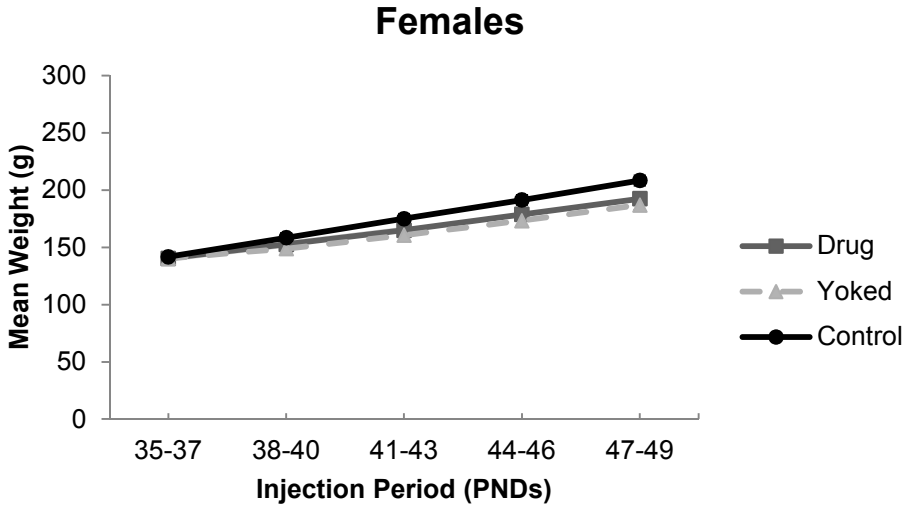

Figure 5. Females mean weight during the injection period (PND 35-49).

Body weight during the post-injection period. After being returned to group housing on PND 50, the rats were weighed every five days (e.g., PND 55, PND 60, etc.) until PND 75. The omnibus 2 (sex) x 3 (group) x 6 (PND) repeated-measures ANOVA was followed by four separate 2 (group) x 6 (day) repeated-measures ANOVAs to individually compare the drug and yoked groups to the control group for each sex.

In the omnibus ANOVA, an effect of sex was found, $F(1,58) = 785.43$, $p < .001$, in which the male rats ($M = 385.80$, $SD = 18.69$) weighed significantly more than the female rats ($M = 254.87$, $SD = 18.70$). An interaction of days by sex was seen, $F(5,290) = 179.25$, $p < .001$, $\eta^2 = 0.93$, in which the male rats gained weight significantly faster than the female rats across the post-injection period. A trend was seen amongst the groups, $F(2,58) = 2.67$, $p = .077$, $\eta^2 = 0.08$, in which the drug ($M = 316.08$, $SD = 18.70$) and the yoked ($M = 316.97$, $SD = 18.70$) groups weighed less than the control ($M = 327.87$, $SD = 18.68$) rats, but not significantly, indicating that over the course of the post-injection, while the manipulation rats were gaining weight, there was still a trending difference over time. A significant interaction of days by groups was also found, $F(10,290) = 3.07$, $p = .001$, $\eta^2 = 0.10$, in which the drug and yoked groups both gained weight significantly faster than the control group in order to catch up to them from the weight differences during the post-injection period. A significant interaction of days by sex was also seen, $F(5,290) = 139.25$, $p < .001$, $\eta^2 = 0.76$.

In the male comparison of drug and control rats, an emerging effect of group was seen, $F(1,20) = 3.78$ $p = .066$, $\eta^2 = 0.16$, in which the control rats ($M = 396.23$, $SD = 18.09$) weighed more than the drug rats ($M = 381.84$, $SD = 18.09$) almost significantly. A trending interaction of days by group was seen here, $F(5,100) = 2.20$, $p = .060$, $\eta^2 = 0.10$, in which the drug rats gained weight at a significantly faster rate than the control rats. An emerging group

effect was seen in the comparison of yoked (M = 379.34, SD = 18.09) rats when compared to the control rats, $F(1,20) = 4.26$, $p = .052$, $\eta^2 = 0.18$, where the yoked rats weighed less than the control rats over the post-injection period. Additionally, an interaction was seen between the yoked and control male rats, $F(10,150) = 3.26$, $p = .009$, $\eta^2 = 0.14$, again with the manipulation group gaining weight across days at a greater rate than the control rats (Figure 6).

Female comparisons of the effects of group showed reduced effects in comparison to the males. The drug females (M = 250.31, SD = 18.00) and control females (M = 259.51, SD = 17.16) were not significantly different in weight during the post-injection period, and no interaction was seen between days and groups. Yoked females (M = 254.59, SD = 19.01) and control females were also not significantly different during the post-injection period, but a trending interaction was seen between days and groups, $F(5.95) = 2.18$, $p = .063$, $\eta^2 = 0.10$, with the yoked rats gaining weight at a faster rate than the control rats to make up for the difference from the injection period (Figure 7).

Apriori t-tests were run comparing each of the manipulation groups to the control groups across each of the days to determine the point at which the two groups were no longer significantly different during the washout period.

The male drug group (M=372.57, SD = 20.13) were no longer significantly different that the male control group (M=385.48, SD = 18.89) starting on PND 60, $t(22) = -1.621$, p = .119, $d = -0.66$. The yoked male group (M=375.63, SD=24.81) also became statistically similar to the control male rats (M=385.48, SD=18.89) on PND 60, $t(22) = -1.094$, $p = .286$, $d = -0.45$.

The female drug group (M=230.55, SD=19.61) were no longer significantly different from the female control rats (M=241.91, SD=15.17) on PND 55, $t(20) = -1.520$, $p = .144$, $d = -0.65$. Like the female drug rats, the female yoked rats (M=234.50, SD=15.07) were no longer significantly different to the female control (M=241.91, SD=15.17) on PND 55, $t(19)= -1.122$, $p = .276$, $d = -0.49$.

Weight changes across the injection period and the post-injection period help us to see the effect that the drug has on the weight of the rats. The rats beginning on PND 35 were determined to be equal across groups; however, by the end of the injection period they were significantly different from each other as the food yoked manipulation and drug groups did not gain weight as quickly as the control rats. While weights did return to normal after the washout period, it took time and significant changes in the feeding patterns of the manipulation rats before it occurred.

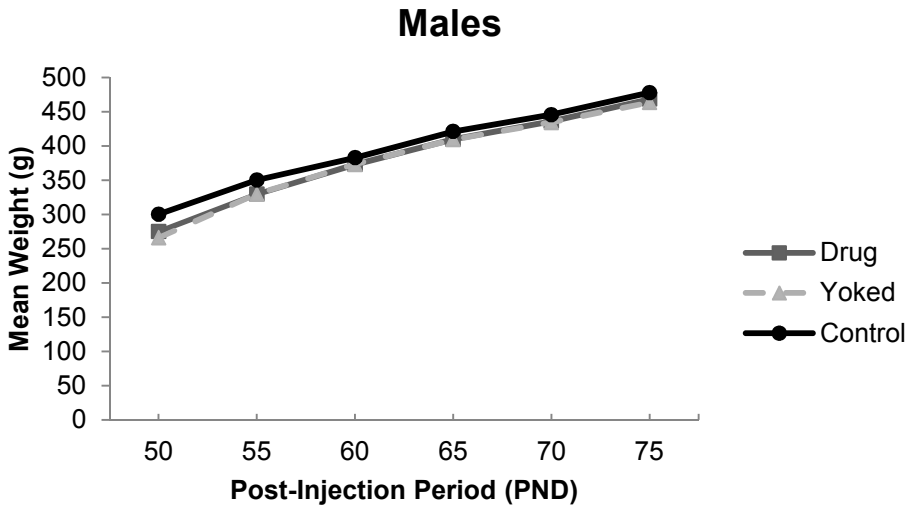

Figure 6. Males post-injection becoming similar after being returned to free-feed.

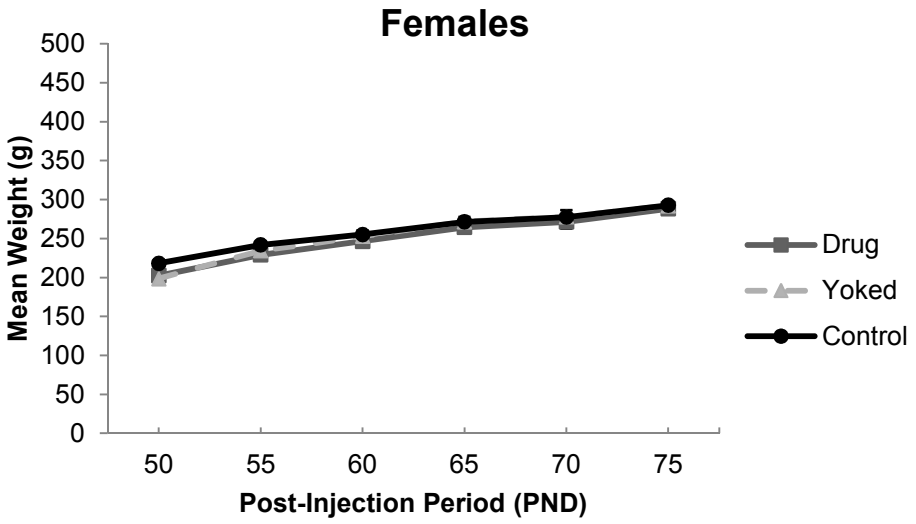

Figure 7. Females post-injection becoming similar after being returned to free-feed.

Elevated Plus Maze – Anxiety

All rats were run on the elevated plus maze (EPM) starting on PND 77. Two types of measures were analyzed: anxiety and activity. Anxiety was measured by observing the percentage of time spent within the open arms when compared to the total amount of time spent in the elevated plus maze, the percent of arm entries into open arms versus the total number of arm entries, and the number of boli produced during the task. Anxiety analyses reported here were taken from the first five minutes of the task, as this is the most common time reported and has been shown to be more sensitive in the analyses than the full ten minutes in which the rats were placed in the EPM, in which many of the effects washed out during the ten minute trials.

Percent of time in open arms. A 2 (sex) x 3 (group) univariate ANOVA was performed on the percent of time spent on the open arms for the omnibus analysis (see Figure 8). There was no effect of sex found in the omnibus analysis, $F(1,66) = 1.21$, $p = .275$, $\eta^2 =$

0.02, as the female and male rats did not differ in the average amount of time spent in the open arms. There was a significant effect of group however, $F(2,66) = 3.33$, $p = .042$, $\eta^2 = 0.09$, in which the drug rats ($M = 32.92$, $SD = 11.07$) spent more time on average in the open arms than the yoked ($M = 24.91$, $SD = 11.37$) and control rats ($M = 25.67$, $SD = 12.65$).

A priori comparisons of male rats did not support this group difference. Independent samples *t*-tests showed that drug ($M = 34.32$, $SD = 13.48$) rats were not significantly different than control ($M = 27.32$, $SD = 13.71$) rats in the percent of time spent in the open arms, $t(22) = 1.26$, $p = .220$, $d = 0.51$. Yoked rats ($M = 26.48$, $SD = 13.86$) were also not significantly different than the control rats, $t(22) = -.15$, $p = .882$, $d = -0.06$.

Comparisons among female rats suggested the same effects. Drug females ($M = 31.51$, $SD = 8.36$) spent a higher percentage of time in the open arms than the control females ($M = 24.01$, $SD = 11.86$), but the effect was not quite significant, $t(22)$ 1.79, $p = .087$, $d = 0.73$. This effect is not present in the comparison of the yoked females ($M = 23.35$, $SD = 8.53$) to the control females however, $t(22) = -.16$, $p = .877$, $d = -0.06$. This implies that the group effect that was seen in the omnibus analysis was primarily due to the drug rats, male and female combined, compared to the control animals. As can be seen in Figure 8, the drug animals spent somewhat more time in the open arms, suggesting reduced anxiety compared to the control animals.

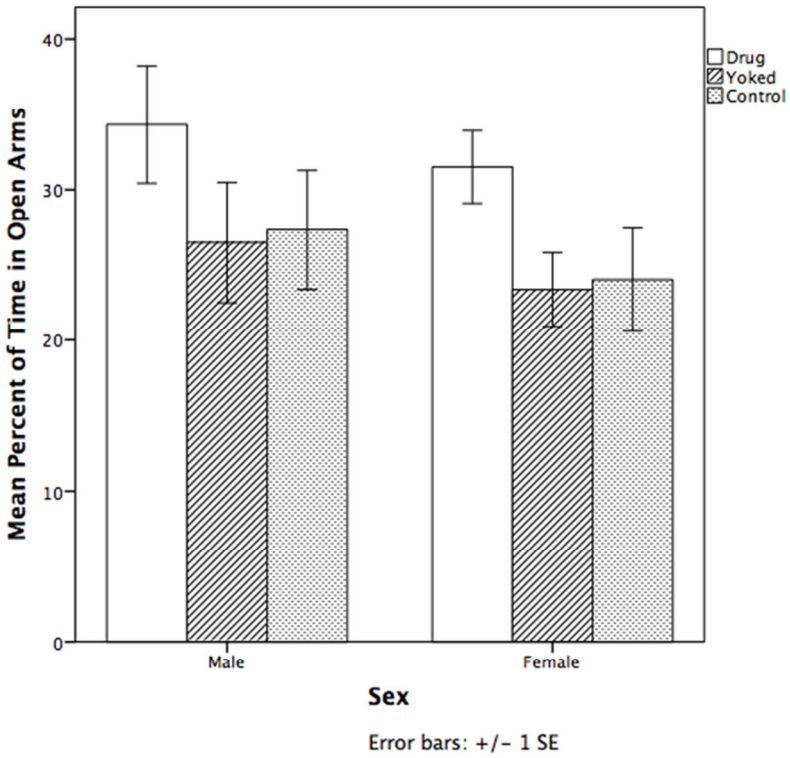

Figure 8. Mean percent of time spent in the open arms of the elevated plus maze task

Number of arm entries into the open arms versus the closed arms. On an omnibus 2 (sex) x 3 (group) x 2 (open versus enclosed arm) repeated measures ANOVA comparing the number of arm entries performed in the first five minutes, it was determined that the rats significantly preferred the enclosed arms (M = 9.90, SD = 2.49) to the open arms (M = 6.32, SD = 2.56), $F(1, 66) = 138.94$, $p<.001$, $\eta^2 = 0.68$. There was a significant effect of sex as well, $F(1,66) = 12.85$, $p = .001$, $\eta^2 = 0.16$, in which the male rats (M = 7.19, SD = 2.17) did not perform as many arm entries as the female rats (M = 9.03, SD = 2.17). An interaction of type of arm by sex was found, $F(1,66) = 14.04$, $p < .001$, $\eta^2 = 0.18$, where the female rats entered the enclosed arms significantly more often than the male rats, likely due to being more active overall than the males. The increase in arm entries, but mostly into the closed arms seen in the females could also be indicative of higher levels of anxiety, as by

comparison, they prefer the enclosed arms to the open arms. There was no significant effect found between drug groups, $F(2,66) = .53$, $p = .590$, $\eta^2 = 0.02$, and there were no significant interactions including drug groups. The lack of group effect on this measure implies that the effect of the drug did not create a significant anxiolytic or anxiogenic effect amongst the subjects in terms of the number of entries into the open and enclosed arms during the first five minutes on the elevated plus maze.

In *a priori* comparisons of male and female rats, there were no significant effects of group and no significant interactions of the group on the type of arm. The sex differences and preference for the enclosed arms can be seen in Figure 9 below.

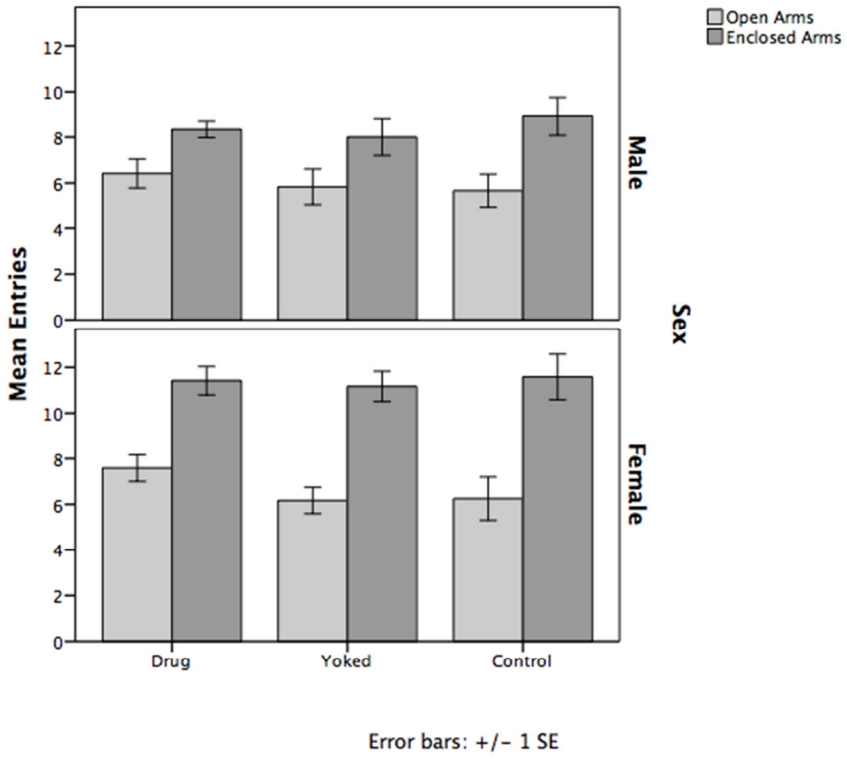

Figure 9. Mean number of entries into the open and enclosed arms on the elevated plus maze

Mean number of boli on the elevated plus maze. The number of boli produced was also used as a measure of anxiety of the rats on the EPM. A 2 (sex) x 3 (group) two-way univariate ANOVA was used to analyze this measure. When measured en masse, it was found that there were no drug group differences, $F(2, 66) = .43$, $p = .652$, $\eta^2 = 0.01$. There were, however, sex differences, $F(1,66) = 7.85$, $p = .007$, $\eta^2 = 0.11$. The male rats produced significantly more boli on the task overall than the female rats. With the male rats producing significantly more boli, it is safe to assume that there was a difference in anxiety across sex, with the male rats being more anxious than the females. There were, however, no significant interactions between the drug group and the sex.

When observing the *a priori* comparisons on this task, independent-samples t-tests were used to analyze the data. There were no significant effects across the comparisons, indicating that there were no group differences in the boli production within sexes (Figure 10).

Overall, the measures of anxiety and activity on the elevated plus maze inform us of two things. The increased number of arm entries in all arms, but not open arms implies higher level of anxiety in the females. Lastly, the time spent in the open arms is greater in drug rats than in the other two groups, indicating that some anxiolytic effect can be seen from the drug exposure.

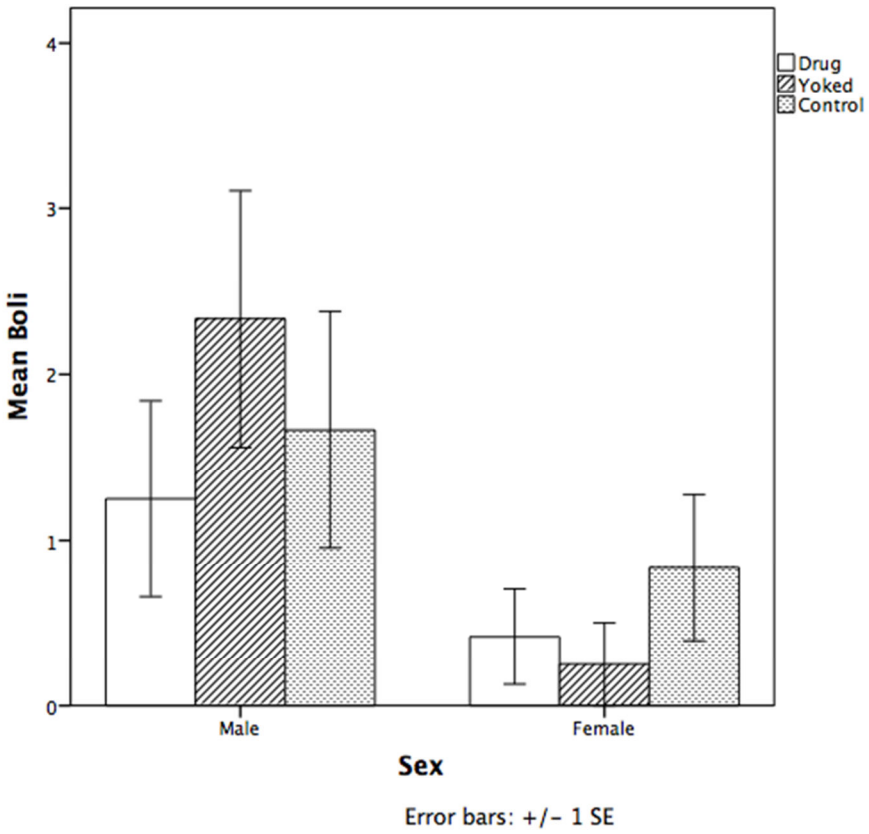

Figure 10. Mean boli across the drug groups and sexes on the elevated plus maze.

Elevated Plus Maze – Activity

Activity on the elevated plus maze was measured by observing the total number of entries into all four of the arms. The analyses reported here were also taken from the first five minutes of the task, as is typical in observations of the elevated plus maze.

Total number of entries into the arms. The total number of entries into the arms is one manner in which to determine how active the rats were in the elevated plus maze task. The omnibus data was analyzed using a 2 (sex) x 3 (group) two-way univariate ANOVA, while the *a priori* measures were run with independent-samples *t*-tests. On the omnibus activity measures of the elevated plus maze, it was determined that there were no differences between drug groups, $F(2,66) = .53$, $p = .590$, $\eta^2 = 0.02$. As expected, there was a sex

difference in activity, $F(1,66) = 12.85$, $p = .001$, $\eta^2 = 0.16$, with the female rats ($M = 18.06$, $SD = 4.34$) entering the arms significantly more frequently than the male rats ($M = 14.39$, $SD = 4.34$)(see Figure 11).

When separated, the *t*-test between the male drug rats (M=14.75, SD=2.67) and male control rats (M=14.58, SD=4.56) showed no significant difference, $t(22) = .11$, $p = .914$, $d = 0.05$. The *t*-test between the male yoked (M=13.83, SD=5.25) and the male control rats also showed no significant difference, $t(22) = -.37$, $p = .712$, $d = -0.15$.

Female comparisons of drug rats (M=19.00, SD=3.54) and control rats (M=17.83, SD=5.86) were not significant, $t(22) = .59$, $p = .561$, $d = 0.24$. The yoked females (M=17.33, SD=3.26) were also not significantly more active than the control rats, $t(22) = -.26$, $p = .799$, $d = -0.11$.

Overall, the female rats were much more active than the male rats. There were no group differences in the EPM on activity, as it seems that the effects of food and drug intake had little effect on the level of explorative activities on this task.

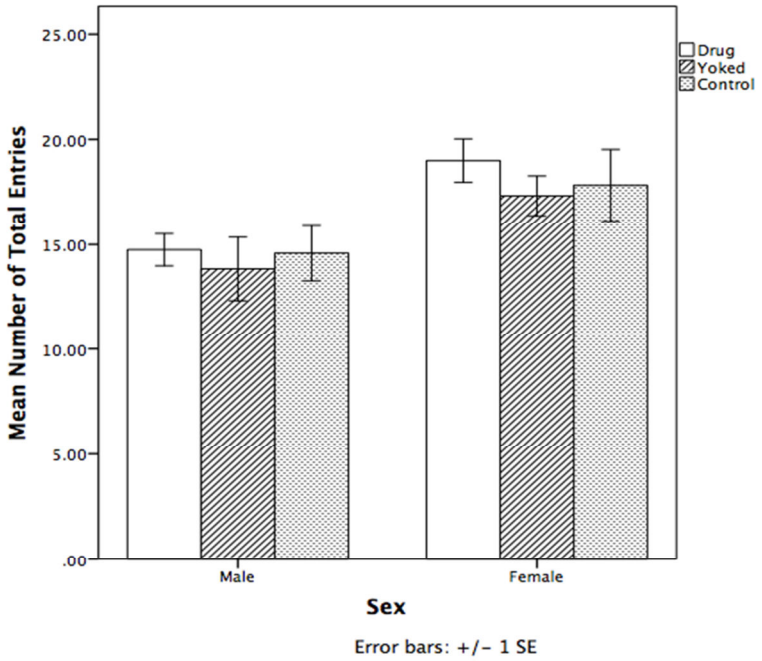

Figure 11. Mean number of total entries on the elevated plus maze

Open Field – Anxiety

All rats began running the open field task on PND 82 or 83. There were two 10-minute trials separated by 24 hours. All data was collected via the AnyMaze tracking program. The open field was used to measure anxiety and activity. To measure anxiety, the mean number of center zone entries, the percentage of time spent in the center zone, the mean time per center zone entry, and boli were used. It should be noted that due to an error during data collection, one control male rat was removed from the analyses, leaving an *n* of 11 for the control males and an *n* of 12 for all other groups.

Mean number of center zone entries. The center zone of the open field was a square quadrant comprised of four 25x25 cm grids in the center of the maze. The outer zone consisted of the 12 grids around the perimeter. Rats entering the center zone more frequently were considered to be less anxious as rats are typically nervous in large open spaces. Entries were measured by AnyMaze, in which the tracking software counted the number of times the

center of the rats' bodies crossed into the center zone from the outer zone. This measure was analyzed using a 2 (sex) x 3 (group) x 2 (trial) repeated-measures ANOVA and the *a priori* comparisons of drug versus control, and yoked versus control, were analyzed with a 2 (group) x 2 (trial) repeated-measures ANOVAs.

For the number of center zone entries' omnibus analysis, there was a significant effect of trial, $F(1,65) = 8.68$, $p = .004$, $\eta^2 = 0.12$, in which the rats entered the center zone more often during the second trial than during the first, indicating that the rats became less anxious of the center zone of the maze between trial one ($M = 12.87$, $SD = 5.73$) and trial two ($M = 15.06$, $SD = 7.74$). Additionally, there was a significant effect of sex on center zone entries, $F(1,65) = 4.56$, $p = .036$, $\eta^2 = 0.07$, with the female rats ($M = 12.43$, $SD = 6.14$) entering the center zone more than male rats ($M = 15.50$, $SD = 5.96$). There was no significant effect of group, $F(2,65) = 1.65$, $p = .200$, $\eta^2 = 0.05$, and no significant interactions (see Figure 12).

No effect of group was found during the *a priori* comparison of male drug ($M = 11.21$, $SD = 4.17$) to male control rats ($M = 13.59$, $SD = 4.18$), $F(1,21) = 1.87$, $p = .186$, $\eta^2 = 0.08$. No effect was seen in the comparison between male yoked ($M = 12.50$, $SD = 6.63$) and control rats either, $F(1,21) = .16$, $p = .697$, $\eta^2 = 0.01$. Additionally, there were no significant interactions found amongst the males, and the male rats did not express habituation across trials.

The females rat comparisons did not express an effect between the drug ($M = 17.83$, $SD = 6.57$) and the control females ($M = 16.83$, $SD = 6.57$), $F(1,22) = .14$, $p = .713$, $\eta^2 = 0.01$. The drug and control females did express a significant level of habituation across trials, $F(1,22) = 4.66$, $p = .042$, $\eta^2 = 0.17$. An emerging group trend was present in the yoked female rats ($M = 11.83$, $SD = 7.09$) when compared to the control females, $F(1,22) = 2.98$, p

= .098, $\eta^2 = 0.12$, in which the yoked rats entered the center zone less than the control rats. The yoked and control females also habituated significantly across trials, $F(1,22) = 5.56$, $p = .028$, $\eta^2 = 0.20$. The interactions were not found to be significant in these analyses.

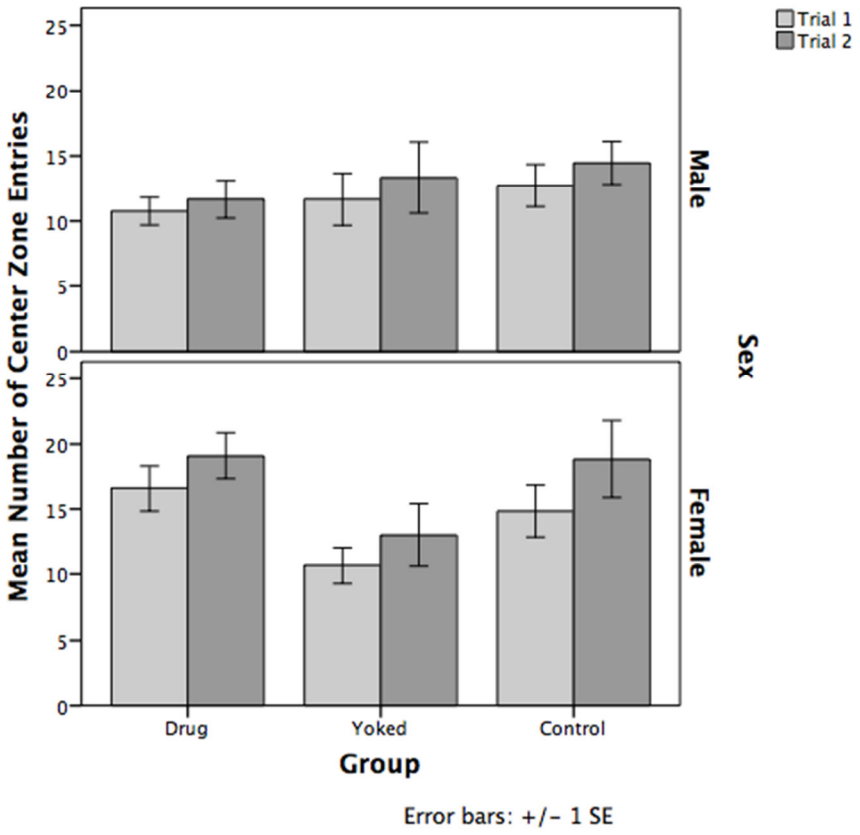

Figure 12. Mean number of center zone entries across trial one and two of the open field.

Percent of time spent in the center zone. The percent of time spent in the center zone was measured by dividing the amount of time spent specifically in the center zone by the total amount of time spent in the open field (10 min). Rats were expected to spend a very small amount of their time in the center zone. The measure was analyzed with a 2 (sex) x 3 (group) x 2 (trial) repeated-measures ANOVA and broken up into four *a priori* comparisons using 2 (group) x 2 (trial) repeated-measures ANOVAs.

On the omnibus analysis of the percent of time spent in the center zone, there was a significant effect of trial, in which the rats spent more time in the center zone during the second trial of the open field than the first, $F(1,65) = 5.72$, $p = .020$, $\eta^2 = 0.08$. There were, however, no significant differences between the effect of group, $F(2, 65) = 1.53$, $p = .224$, $\eta^2 = 0.05$, and the effect of sex, $F(1,65) = 1.51$, $p = .223$, $\eta^2 = 0.02$, on the omnibus analysis. There were also no significant interactions found (Figure 13).

In *a priori* comparisons between male groups, there were no significant effects found. The drug males ($M = 6.71$, $SD = 2.28$) were very similar to their control counterparts ($M = 6.36$, $SD = 2.28$) in percent time spent in the center zone. The yoked males ($M = 6.76$, $SD = 3.58$) were also extremely similar to the control males, with neither comparison being significant, $p = .997$, $\eta^2 = 0.01$ and $p = .791$, $\eta^2 < 0.01$, respectively. Interactions between group and trial in these comparisons were also not significant.

Female comparisons did not express significant findings either. The comparisons of drug females ($M = 8.92$, $SD = 2.30$) to control females ($M = 7.55$, $SD = 2.30$) and of yoked females ($M = 5.93$, $SD = 2.88$) to control females were both not significant, $p = .157$, $\eta^2 = 0.09$ and $p = .183$, $\eta^2 = 0.08$ respectively. A weak trend of group difference between trials in the drug and control comparison was found, $F(1,22) = 2.81$, $p = .108$, $\eta^2 = 0.11$, with a significant difference in the groups between trials, $F(1,22) = 6.18$, $p = .021$, $\eta^2 = 0.22$. When separated, a significant difference was seen during trial 2 between the female drug ($M = 10.25$, $SD = 3.32$), yoked ($M = 6.11$, $SD = 3.68$) and control ($M = 7.81$, $SD = 3.32$) rats, $F(2,33) = 3.84$, $p = .032$, $\eta^2 = 0.10$.

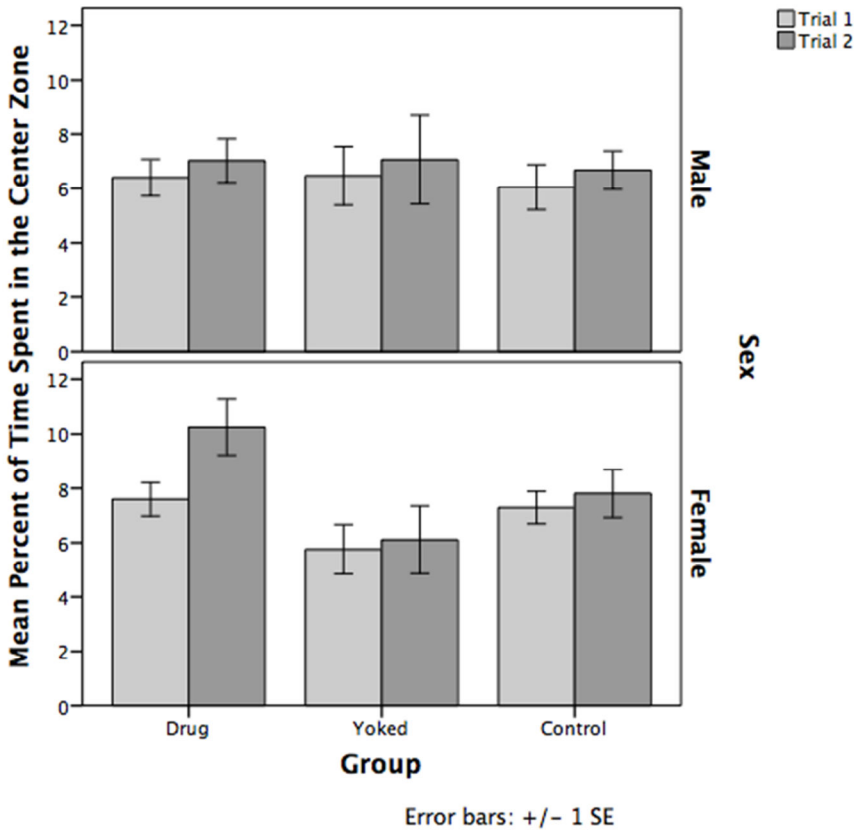

Figure 13. Mean percent of time spent in the center zone during the open field task

Mean time per visit to the center zone. The mean time per visit to the center zone was analyzed in addition because it may be a more sensitive measure than the number of entries or the time spent in the center zone. If the rats spend more time in the center zone per visit, it shows that they are less anxious of the center zone at a level that may reflect their behavior more effectively than just the number of entries into the center zone, which could be an effect of activity. The measure took the total amount of time spent in the center zone and divided it by the number of entries, giving an average amount of time spent per entry for each rat per trial. These averages were analyzed using an omnibus 2 (sex) x 3 (group) x 2 (trial)

repeated-measures ANOVA and the *a priori* analyses were performed using 2 (group) x 2 (trial) repeated-measures ANOVAs.

For the omnibus, there were not a significant main effect of trial on the mean length of time spent per visit to the center zone, $F(1,65) = .10$, $p = .754$, $\eta^2 < 0.01$. No significant sex differences, $F(1,65) = .65$, $p = .422$, $\eta^2 = 0.01$, or overall group differences, $F(2,65) = 2.24$, $p = .114$, $\eta^2 = 0.06$, were found. However, a trending interaction between group and trials was seen, $F(2,65) = 3.01$, $p = .056$, $\eta^2 = 0.08$, with the drug rats' mean time per visit increasing during the second trial while the yoked and control rats' mean time decreased when both male and female data were combined (see Figure 14).

In the group comparisons for the male rats, a significant group effect was found between the drug ($M = 4.00$, $SD = 1.30$) and control rats ($M = 2.88$, $SD = 1.30$), $F(1,21) = 4.31$, $p = .050$, $\eta^2 = 0.17$, indicating that the drug rats spent significantly longer in the center zone per visit. The yoked ($M = 3.34$, $SD = 0.89$) and control rats were not significantly different in their mean time per visit, $F(1,21) = 1.52$, $p = .231$, $\eta^2 = 0.07$. No interactions were found in the male *a priori* comparisons.

In the female comparisons, the drug ($M = 3.48$, $SD = 1.53$) and control ($M = 3.05$, $SD = 1.53$) rats were not significantly different, $F(1,22) = .47$, $p = .500$, $\eta^2 = 0.02$. The yoked ($M = 2.94$, $SD = 0.97$) and control rats were also not significantly different as they heavily resembled each other, $F(1,22) = .08$, $p = .779$, $\eta^2 < .001$. A significant effect of trial was found between the yoked and control females, $F(1,22) = 5.73$, $p = .026$, $\eta^2 = 0.21$, with the yoked and control animals spending less time during trial 2 per visit in the center zone due to habituation (see Figure 14).

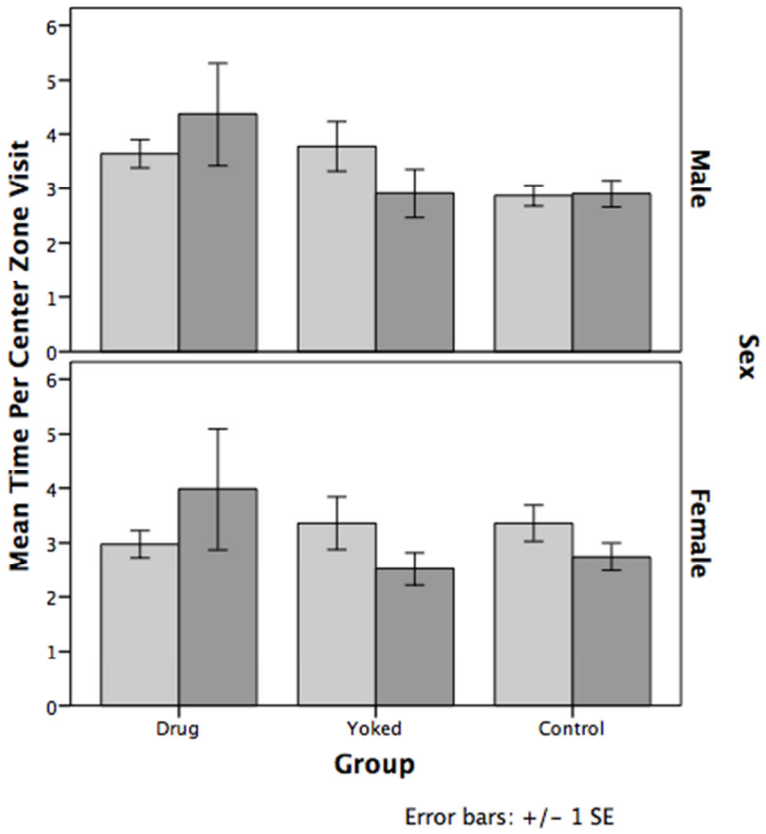

Figure 14. Mean time per center zone visit during the open field task

Boli produced in open field. The final anxiety measure was the production of boli within the open field maze. On the omnibus 2 x 3 x 2 repeated-measures analysis of the boli, there were no differences between the trials in the production of boli, $F(1,65) = .00$, $p = .963$, $\eta^2 < .01$, indicating that regardless of the trial, the rats produced the same amount of boli. Typically it would be expected that after being familiarized with an environment, the rats would experience lower levels of anxiety and produce less boli. The difference between the groups was also not significant, $F(2,65) = 1.88$, $p = .161$, $\eta^2 = 0.05$, at the omnibus level. A sex difference was seen, however, $F(1,65) = 12.18$, $p = .001$, $\eta^2 = 0.16$, in which the male rats ($M = 2.18$, $SD = 1.82$) produced significantly more boli than the female rats ($M = .68$, $SD = 1.81$). This sex difference indicates that in this measure of anxiety, the males experienced

significantly more than the females during this task. No significant interactions were seen in this analysis (the data are graphed in Figure 15).

In *a priori* comparisons of the male and female rats independently, there were no significant differences between pairings of groups (drug-to-control and yoked-to-control), indicating that there was no effect of group on the amount of boli produced in the open field task. There were also no significant interactions found in the *a priori* comparisons. This supports the omnibus finding of a lack of group differences on this measure.

Anxiety in the open field. A sex difference was seen across the measures, with males expressing higher levels of anxiety than the females. Group effects were seen with the drug rats expressing anxiolytic effects when compared to the yoked and control groups, effected by the trial, as while the rats became more habituated to the task by trial 2, the yoked and control rats reduced their average time in the center zone.

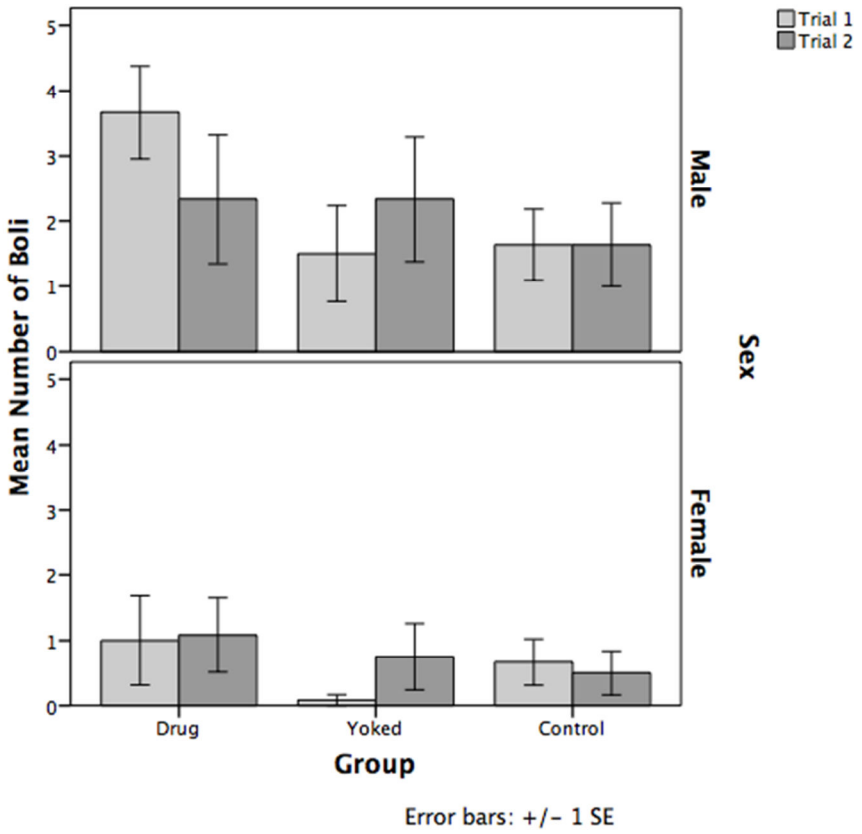

Figure 15. Mean number of boli during open field habituation trials

Open Field – Activity

The distance traveled was used to measure activity level on the open field. This was measured through AnyMaze's tracking software, allowing for an accurate gauge of the amount of distance traveled in meters within the maze. It should be noted that due to an error in running, the single control male rat that was removed from the open field anxiety measures was still absent during this analysis.

Distance traveled. Distance traveled provides a measure of activity in rodents. The AnyMaze software monitored the rats throughout each 10-min trial, and collected the number of meters which each rat traveled on the first and second trials, which were 24 hours apart. These measures were analyzed with a 2 (sex) x 3 (group) x 2 (trial) repeated-measures ANOVA and four *a priori* 2 (group) x 2 (trial) repeated-measures ANOVAs.

In an omnibus analysis of the distance traveled by all of the rats, there was no significant difference between the groups on distance, $F(2,65) = .85$, $p = .430$, $\eta^2 = 0.03$. A significant difference was seen in sex, as expected, $F(1,65) = 19.08$, $p < .001$, $\eta^2 = 0.23$, with the females ($M = 36.46$, $SD = 7.73$) covering more distance than the males ($M = 28.44$, $SD = 7.74$). On average, the female rats' increased levels of activity led to them traveling a further distance within the maze than the male rats. There were no other significant main effects or interactions found in this analysis (see Figure 16).

When comparing drug males to control males, no significant difference was seen between groups, $F(1,21) = 1.07$, $p = .314$, $\eta^2 = 0.05$. Yoked and control comparisons showed even less of a difference between the two groups, $F(1,21) = .00$, $p = .996$, $\eta^2 < .01$, indicating that there was no effect of group on the male rats. Nor was the interaction significant.

Female drug and control rats were also remarkably similar to each other, $F(1,22) = .00$, $p = .973$, $\eta^2 < .01$. The female yoked and control comparison presented a trend, $F(1,22) = 3.189$, $p = .088$, $\eta^2 = 0.13$, with the yoked rats ($M = 32.56$, $SD = 8.12$) moving around the maze less than their control counterparts ($M = 38.47$, $SD = 8.12$). There were no significant interactions found in these analyses. The decrease in distance traveled by the yoked female rats was constant across trials and not the result of a single trial.

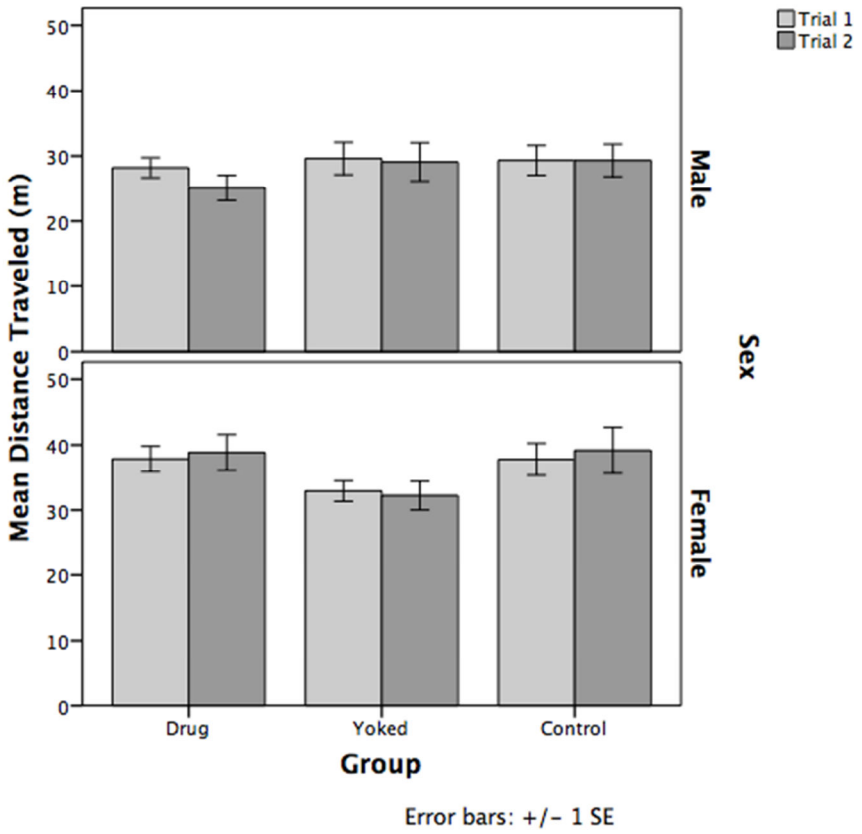

Figure 16. Mean distance traveled during trial one and two on the open field

Object Location Recognition

The object location recognition task measured the rat's ability to notice a change in the location of an object between two separate trials run on the third day in the open field. One of two identical objects was moved on trial two after being introduced to the rats previously for five minutes on trial one. There was a delay of at least ten minutes between the trials. The majority of the object recognition data was collected by hand. Two researchers simultaneously coded this data by observing video recordings of the trials on a digital-video recorder (DVR). The researchers collected this data simultaneously to ensure inter-coder reliability and control for human error during the coding process.

Percent of time spent investigating the moved object. The measure of the percent time spent investigating the moved object on the second trial takes into consideration the total time spent investigating both the moved and the static object and divides it into the time spent investigating just the moved object. As both objects were novel during the first trial, the amount of time spent measuring each of the objects during the first trial alone would not be indicative of anything. These percentages were analyzed using a 2 (sex) x 3 (group) univariate ANOVA and *a priori* independent samples *t*-tests.

In the omnibus measure of the percent of time spent investigating the moved object, there was a significant effect of group, $F(2,66) = 4.13$, $p = .020$, $\eta^2 = 0.11$. The yoked groups ($M = 52.51$, $SD = 10.88$) spent more of their time investigating the moved object than the drug groups ($M = 46.30$, $SD = 10.88$) or the control groups ($M = 43.73$, $SD = 10.88$). There was not a sex difference, $F(1,66) = .07$, $p = .795$, $\eta^2 < 0.01$, and no interaction effect between group and sex, $F(2, 66) = .420$, $p = .659$, $\eta^2 = 0.01$, indicating that the group effect was not sex-dependent.

In the male *a priori* comparisons, the effect of group was absent in the *t*-test between the drug and control groups, $t(22) = .71$, $p = .482$, $d = 0.29$. The effect was also absent in the *t*-test between the yoked and the control groups, $t(22) = 1.28$, $p = .216$, $d = 0.52$. The mean data are graphed in Figure 17.

When looking at the female drug and control comparison, no significant effect was found, $t(22) = .53$, $p = .604$, $d = 0.21$. However, the *a priori t*-test between the yoked and the control group was significant, $t(22) = 2.83$, $p = .010$, $d = 1.16$. This effect indicates that the yoked female rats were more aware of the object location change than the control females (Figure 17).

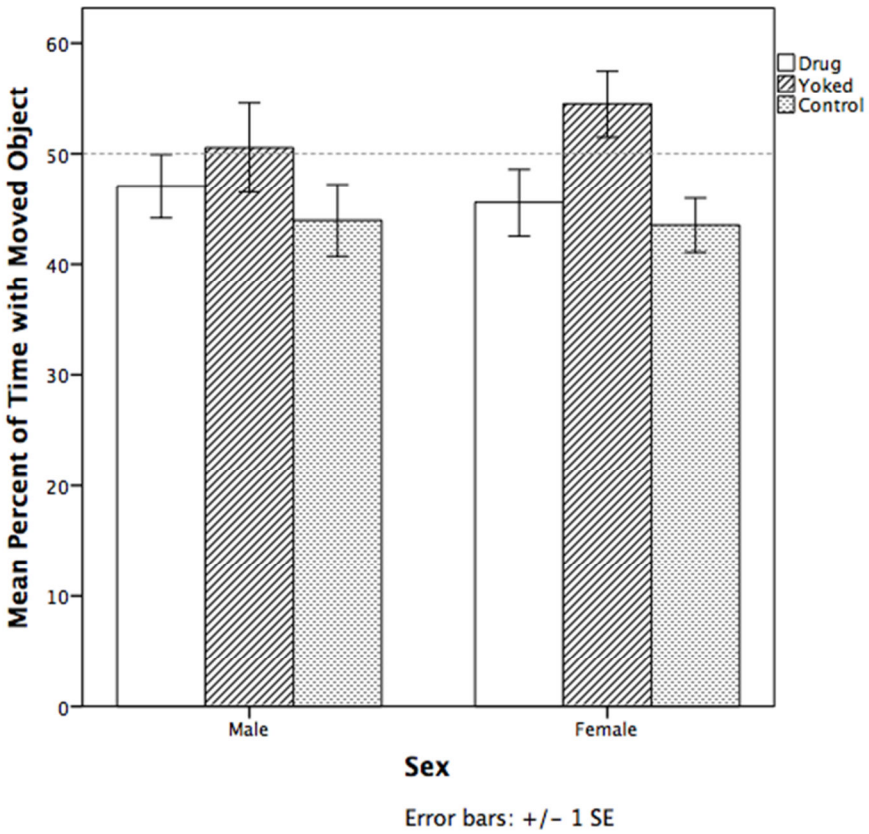

Figure 17. Mean percent of time spent investigating the moved object during trial 2 The dotted line indicates the midpoint of equal investigation across both objects.

Percent of interactions with object. A percentage of the number of investigations that occurred to object A (the object that was moved) relative to investigations to both objects was used to determine the change of attention to the moved object across trials. If the rats noticed the change in the location of the object, the percent of investigations of object A should increase between trials. A percentage was chosen over the number of interactions to account for the difference in activity level across sexes. These data were analyzed using an omnibus 2 (sex) x 3 (group) x 2 (trial) repeated-measures ANOVA and 2 (group) x 2 (trial) *a priori* repeated-measures ANOVAs.

In the omnibus measure of the interactions with the moved object, no effect was seen across sex, $F(1,66) = .98$, $p = .325$, $\eta^2 = 0.01$, or across groups, $F(2,66) = .10$, $p = .909$, $\eta^2 <$

0.01 . As well, a lack of an interaction between group and sex was observed, $F(2,66) = 1.16$, $p = .320$, $\eta^2 = 0.03$. A three-way interaction was found between trial, group, & sex however, $F(2,66) = 3.35$, $p = .041$, $\eta^2 = 0.09$, in which the drug and control males lost attention to object A, while the females increased attention. The yoked males however increased attention from a low point to greater than all of the other groups while the yoked females stayed consistent across trials (see Figure 18). There was a trend of an interaction between the trial and group, $F(2,66) = 2.38$, $p = .100$, $\eta^2 = 0.07$, in which the male yoked rats' change in attentiveness to the moved object was significantly powerful enough to create an effect.

A priori male comparisons of drug and control rats showed no significant effects of group, $F(1,22) = .07$, $p = .800$, $\eta^2 < 0.01$, or in the interaction between group and trial, $F(1,22) = .08$, $p = .777$, $\eta^2 < 0.01$. The yoked and control comparisons, however, showed a significant interaction between the trial and group, $F(1,22) = 7.30$, $p = .013$, $\eta^2 = 0.25$, but not between groups, $F(1,22) = .85$, $p = .367$, $\eta^2 = 0.04$. The male yoked group was the only group that showed a significant increase in attention to the moved object between trials (see Figure 18).

Comparisons of female drug and control rats continued to show no effects of group, $F(1,22) = .07$, $p = .797$, $\eta^2 < 0.01$, or the interaction between group and trial, $F(1,22) = .10$, $p = .754$, $\eta^2 < 0.01$. Yoked and control females did not show a group difference, $F(1,22) = 1.12$, $p = .302$, $\eta^2 = 0.05$, or interaction between group and trial, $F(1,22) = .01$, $p = .918$, $\eta^2 < 0.01$.

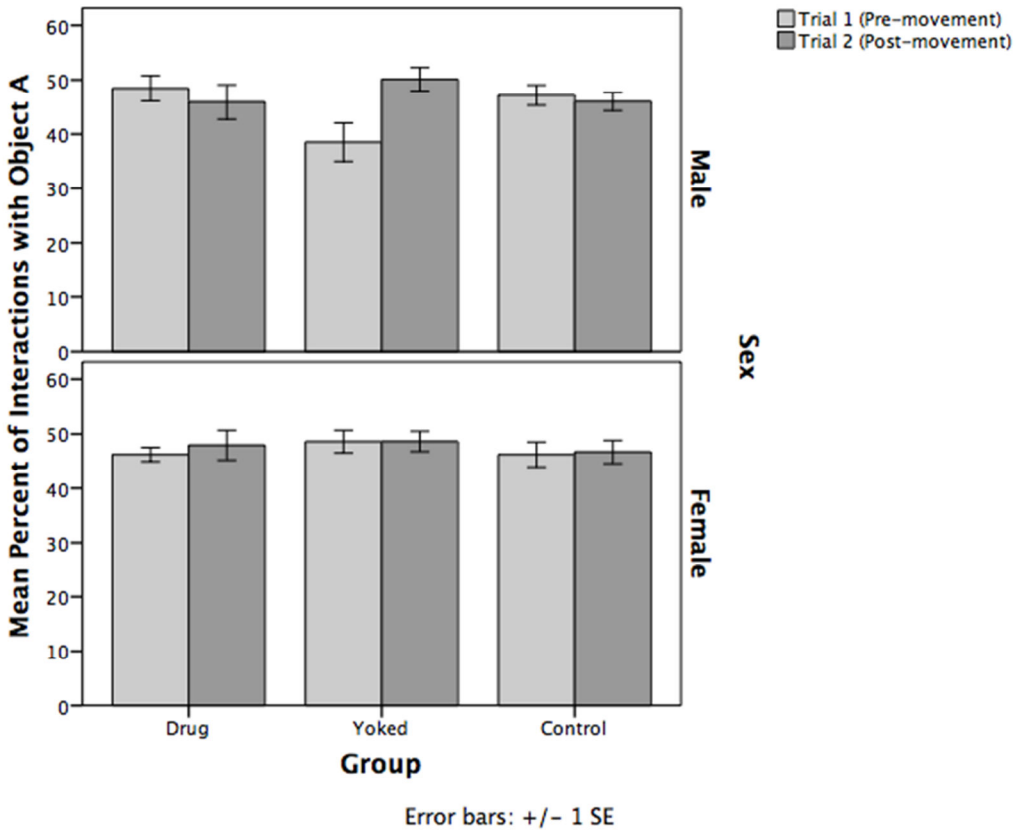

Figure 18. Mean percent of interactions with moved object (A) across trials

Morris Water Maze – Learning

The learning phase of the Morris water maze involved the rats learning over time where the platform was and how fast they would begin finding the platform over the span of five days with each day consisting of four trials. Data were collected in the water maze using the HVS tracking system, which monitored the rats' swimming activities from the beginning of the trial through the fifteen-second time out after they found the platform. The swim speed and distance traveled to find the platforms were used to measure their learning during this phase of the Morris Water Maze. Measures were analyzed with 2 (sex) x 3 (group) x 5 (day) x 4 (trial) repeated-measures ANOVAs, and *a priori* measures were analyzed with 2 (group) x 5 (day) x 4 (trial) repeated-measures ANOVAs. Due to experimenter mistakes and computer errors across the high number of trials required for these analyses, several rats were

not counted within these measures. In the male rats, one yoked rat and a concerning five control rats were eliminated while two rats per group were lost in the female rats.

Swim speed. The rats' speed of swimming (in meters per second) was measured by the HVS tracking system and was used to measure motor function across groups and sex. In the omnibus measure, it was found that there was a significant effect of group, $F(2,54) = 3.87$, $p = .027$, $\eta^2 = 0.59$, in which the drug group ($M = .23$ m/s, $SD = 0.02$) moved slower than the yoked ($M = .25$ m/s, $SD = 0.02$) or control groups ($M = .25$ m/s, $SD = 0.02$). There was also a trend found between sexes, $F(1,54) = 3.26$, $p = .077$, $\eta^2 = 0.38$, in which the female rats ($M = .25$ m/s, $SD = 0.02$) swam faster than the male rats ($M = .24$ m/s, $SD = 0.02$). A significant effect of trial was seen across the rats, $F(3,162) = 14.92$, $p < .001$, $\eta^2 = 0.22$, in which the rats slowed down across trials within days, likely due to fatigue. A significant interaction of days by trial was also seen, $F(12,648) = 4.50$, $p < .001$, $\eta^2 = 0.08$, in which the rats increased their speed across days, due to an increased level of confidence, but still expressed fatigue across trials. A significant interaction of day by sex was also found, $F(4, 216) = 3.56$, $p = .008$, $\eta^2 = 0.06$, where the male rats increased in speed across the days and the female rats decreased in speed across days. There were no other significant effects or interactions in the omnibus swim speed analysis. The data are graphed in Figures 19 and 20.

The independent comparisons between drug and control males continued the group difference, $F(1, 17) = 8.99$, $p = .008$, $\eta^2 = 0.35$, as the drug group ($M = .22$ m/s, $SD = 0.02$) swam slower compared to the control rats ($M = .24$ m/s, $SD = 0.02$). The fatigue effect of trial was consistent in the drug and control male comparison, $F(3,51) = 4.62$, $p = .006$, $\eta^2 = 0.21$, as well as an effect of trials by group, $F(3,51) = 3.03$, $p = .038$, $\eta^2 = 0.15$, in which the drug group showed a much stronger fatigue than the control rats across trials. The yoked (*M*

= .23 m/s, *SD* = 0.02) and control comparison showed no such difference, $F(1,16) = 1.08$, $p = .313$, $\eta^2 = 0.06$. However, there was an effect of trials, $F(3,48) = 8.25$, $p < .001$, $\eta^2 = 0.34$, where the rats moved much faster during the first trial and stayed consistent across the remaining three trials. An effect of days by trials was also seen, $F(12,192) = 2.56$, $p = .004$, $\eta^2 = 0.14$, where fatigue varies across trials within the days. A significant interaction between trials and group was found in the drug and control comparisons of the male rats, $F(3, 51) = 4.62$, $p = .006$, $\eta^2 = 0.15$, that did not appear between the yoked and control rats, $F(3, 48) = .58$, $p = .630$, $\eta^2 = 0.03$. The control group swam much faster on trial 1 (averaged across days) then leveled off at a slower speed for trials 2, 3, and 4; but the drug rats started out slower and slowed down slightly on each trial thereafter (Figure 19).

The female drug (*M* = .23, *SD* = 0.02) and control (*M* = .24, *SD* = 0.02) comparison showed no group effect, $F(1,18) = 1.38$, $p = .255$, $\eta^2 = 0.07$. A significant effect of trial was seen, $F(3,54) = 3.20$, $p = .03$, $\eta^2 = 0.16$, in which the rats always slowed down across trials. A significant interaction of trials by days was found, $F(12,216) = 2.30$, $p = .009$, $\eta^2 = 0.11$, where both groups of rats sped up across days but slowed down across trials within the days. In the female yoked and control rat comparison, a significant effect of days was seen, $F(4,72) = 3.62$, $p = .010$, $\eta^2 = 0.17$, in which the rats all slowed down across days, contrary to the other groups. A significant effect of trials was also seen, $F(3,54) = 4.26$, $p = .009$, $\eta^2 = 0.20$, in which the rats slowed down across trials, likely from fatigue. No group effect or interactions were found between the yoked (*M* = .25, *SD* = 0.03) and control female rats, $F(1,18) = .10$, $p = .758$, $\eta^2 < .01$ (Figure 20).

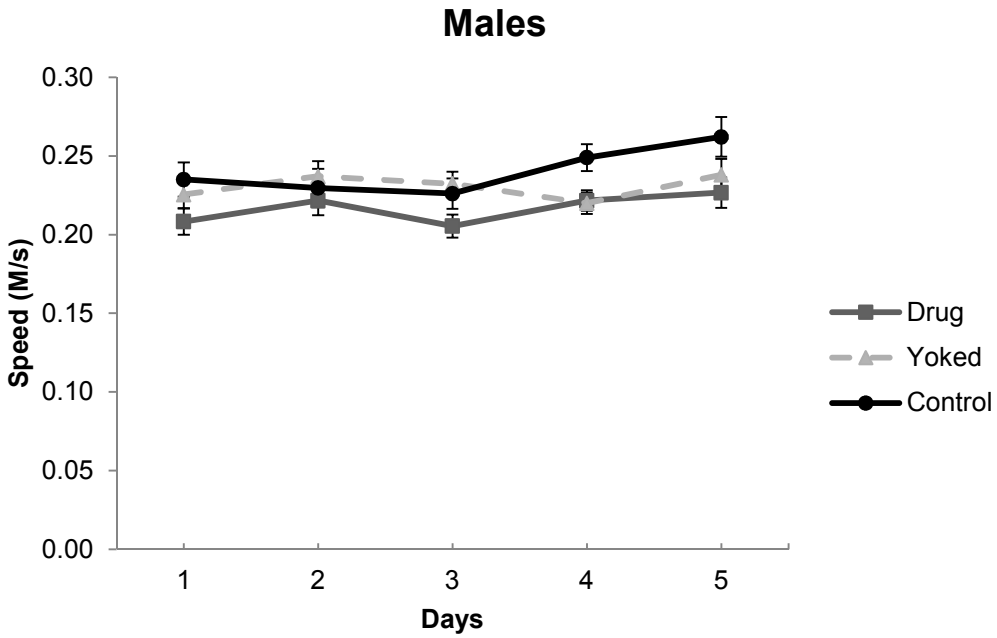

Figure 19. Mean swim speed of male rats across days of learning

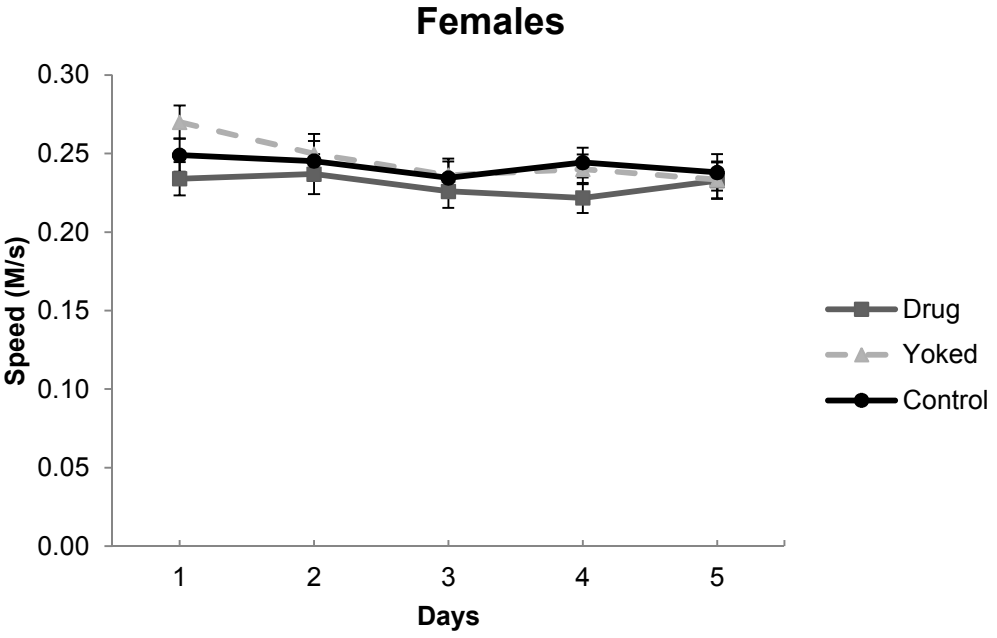

Figure 20. Mean swim speed of female rats across days of learning

Path Length. The length of the path that the rats traveled to find the platform on each trial of the learning task was used as a measure of their competency to remember the platform's location. The path length was measured by the HVS system in meters and was analyzed similarly to the swim speed by using a 2 (sex) x 3 (group) x 5 (days) x 4 (trials) repeated-measures ANOVA. The *a priori* comparisons were also analyzed by using 2 (group) x 5 (days) x 4 (trials) repeated-measures ANOVAs.

In the omnibus measure of path length, a significant effect of sex was found, $F(1,54) = 5.33$, $p = .025$, $\eta^2 = 0.09$, where the females ($M = 6.87$, $SD = 2.16$) traveled further than the males ($M = 5.56$, $SD = 2.22$) to find the platform overall. There was no effect of group, $F(2,54) = .87$, $p = .424$, $\eta^2 = 0.03$, but there was a trending interaction between trials and sex, $F(3, 162) = 2.20$, $p = .090$, $\eta^2 = 0.04$, where the females traveled much further during the first trial than on the following three trials, indicating perhaps a deficit in long-term memory that occurred between days. An effect of days was seen, $F(4,216) = 82.25$, $p < .001$, $\eta^2 = 0.61$, in which the rats decreased their path lengths across days significantly. An effect of trials was found, $F(3,162) = 86.71$, $p < .001$, $\eta^2 = 0.62$, in which the rats all traveled shorter distances across trials, indicating a greater short-term memory. An interaction was seen in days by trials, $F(12,648) = 5.88$, $p < .001$, $\eta^2 = 0.10$, showing that the rats all learned the task well, but a consistent spike in the first trial, indicating issues with long-term memory. No other effects or interactions were significant.

When measured independently, the male drug ($M = 5.62$, $SD = 1.65$) and control rats ($M = 4.76$, $SD = 1.65$) continued to show no significant group differences, $F(1,17) = 1.21$, $p = .286$, $\eta^2 = 0.07$. A significant effect was seen in the days, $F(4,68) = 31.59$, $p < .001$, $\eta^2 = 0.65$, in which the rats traveled less across days to find the platform. An effect was seen in

the trials as well, $F(3,51) = 18.06$, $p < .001$, $\eta^2 = 0.52$, where the rats' path lengths always decreased across trials. A significant interaction was seen between days and trials, $F(12,204) = 2.76$, $p = .002$, $\eta^2 = 0.14$, in which the rats' path lengths decreased steadily during the day, with their first trials frequently being longer than the rest of their trials, likely due to issues with long-term memory of the location of the platform. A trend was found between the yoked ($M = 6.31$, $SD = 1.66$) and control rats, $F(1,16) = 3.76$, $p = .070$, $\eta^2 = 0.19$, where the yoked rats traveled further than the control rats in order to find the platform. An effect was seen between the yoked and the control males for days, $F(4,64) = 32.40$, $p < .001$, $\eta^2 = 0.67$, in which the rats traveled less across days, indicating they had learned the task. An effect was also seen of trials, $F(3,48) = 26.76$, $p < .001$, $\eta^2 = 0.63$, with the rats always needing to cover more distance at the beginning of the running day. The interaction of days by trials was seen here as well, $F(12,192) = 3.56$, $p < .001$, $\eta^2 = 0.18$, with longer path lengths during the first trial of each day, but a constant decrease in the trials succeeding across days (Figure 21).

The female *a priori* comparisons did not show any significant differences between drug ($M = 6.06$, $SD = 2.17$) and control ($M = 7.46$, $SD = 2.17$), $p = .167$, $\eta^2 = 0.10$. A significant effect of days was found, $F(4,72) = 32.34$, $p < .001$, $\eta^2 = 0.64$, with the path lengths decreasing across days. A significant effect of trials was also found, $F(3,54) = 35.44$, $p < .001$, $\eta^2 = 0.66$, with the first trial distance being much longer than the following trials. A significant interaction was seen between days and trials again, $F(12,324) = 2.79$, $p = .001$, $\eta^2 = 0.15$, with the first trial implying poor long-term memory, but decreases in following trials indicating no deficits in working memory. The yoked ($M = 7.08$, $SD = 2.50$) and control group comparison was also not significant, $p = .753$, $\eta^2 = 0.01$. An effect of days was seen, $F(4,72) = 21.88$, $p < .001$, $\eta^2 = 0.55$, with the rats learning the task over days and decreasing

their path lengths needed to find the platform. An effect of trials was also seen, $F(3,54) = 28.42$, $p < .001$, $\eta^2 = 0.61$, also indicative of poor long-term memory as the first trial required further distances to travel. The interaction between days and trials was not significant, $F(12,216) = 1.48$, $p = .132$, $\eta^2 = 0.08$, as while the first trial took longer than the following trials, the following trials were not variable enough to create an interaction (Figure 22).

Overall, the rats all learned the task over the days, with no differences occurring between groups. Sex differences were seen however, as the females appeared to have stronger deficits in their long-term memory, having to travel further during their first trial than the males, but having no issues returning to the task after the first trial.

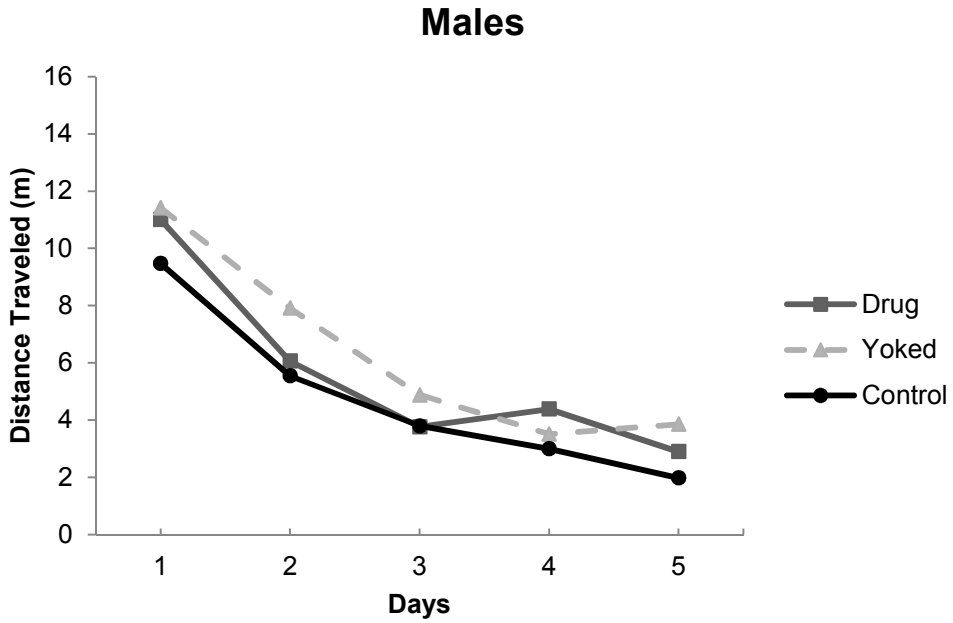

Figure 21. Mean distance traveled (path length) by males during the learning phase of the Morris Water Maze task

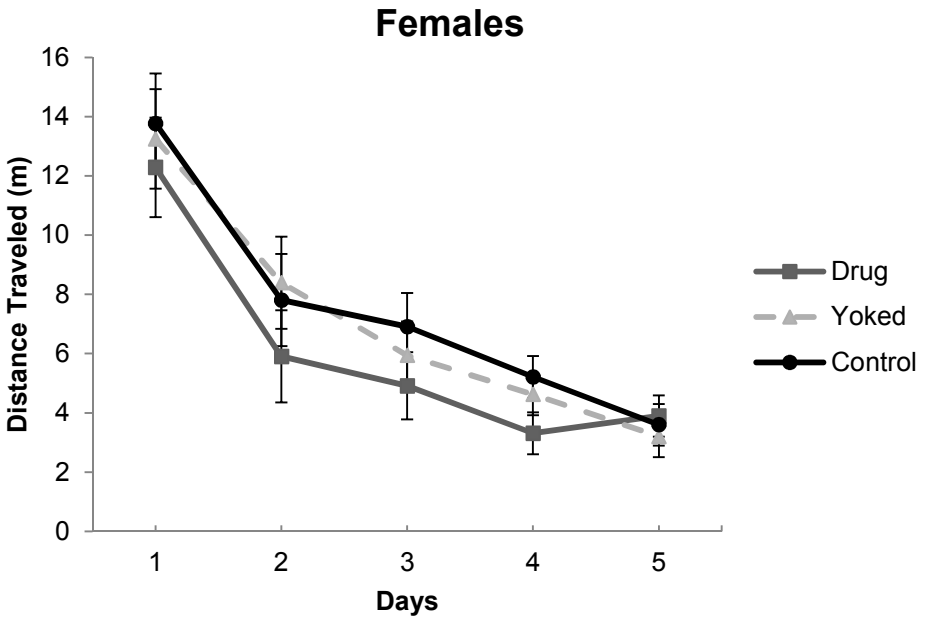

Figure 22. Mean distance traveled (path length) by females during the learning phase of the Morris Water Maze task

Percent of path in thigmotaxis. The final measure for the learning phase was the percent of the rats' path spent in thigmotaxis. Thigmotaxis, a measure of motion with response to the environment through touching of a solid object, was measured by HVS as any instance in which the rats swam close enough to the outer wall to be able to physically contact it.

In the omnibus measure of the percent of the path spent in thigmotaxis during the learning phase, there was no effect of group, $F(2,54) = .37$, $p = .695$, $\eta^2 = 0.01$, or sex, $F(1,54) = .76$, $p = .387$, $\eta^2 = 0.01$. An effect of days was seen, $F(4,216) = 46.94$, $p < .001$, $\eta^2 = 0.47$, in which the percent of the rats' paths spent in thigmotaxis decreased across days. An effect of trials was also seen, $F(3,162) = 136.80$, $p < .001$, $\eta^2 = 0.72$, in which the rats' path began to avoid thigmotaxis across trials within each day. There was a significant interaction between days and trials, $F(12,648) = 25.06$, $p < .001$, $\eta^2 = 0.32$, where rats decreased the

amount of thigmotaxis expressed as they learned the task over time, but consistently increasing their thigmotaxis during the first trial of the day, indicating possible poor long-term memory.

On the *a priori* measures, the male drug and control comparisons showed a lack of a group effect, $F(1,17) = .99$, $p = .335$, $\eta^2 = 0.05$. A consistent significant effect for days was seen, $F(4,68) = 17.48$, $p < .001$, $\eta^2 = 0.51$, as well as a consistent significant effect of trials, $F(3,51) = 36.76$, $p < .001$, $\eta^2 = 0.68$. The continuing interaction between days and trials was also significant, $F(12,204) = 7.00$, $p < .001$, $\eta^2 = 0.29$. The yoked and control comparisons also did not show any effect of group, $F(1,16) = .38$, $p = .547$, $\eta^2 = 0.02$. The same effects of days, $F(4,64) = 14.14$, $p < .001$, $\eta^2 = 0.47$, and trials, $F(3,48) = 46.42$, $p < .001$, $\eta^2 = 0.74$, were found. The same days by trials interaction was also present, $F(12,192) = 16.39$, $p < .001$, $\eta^2 = 0.51$ (Figure 23).

The female drug and control comparisons did not show any effect of group, $F(1,18) = .09$, $p = .765$, $\eta^2 = 0.01$. The same effects of days, $F(4,72) = 23.20$, $p < .001$, $\eta^2 = 0.56$, and trials, $F(3,54) = 46.27$, $p < .001$, $\eta^2 = 0.72$, was seen in this comparison. The days by trials interaction was also significant, $F(12,216) = 9.21$, $p < .001$, $\eta^2 = 0.34$. The yoked and control group comparison was not significant, $F(1,18) = .02$, $p = .903$, $\eta^2 < 0.01$. The constant effects of days, $F(4,72) = 19.02$, $p < .001$, $\eta^2 = 0.51$, and trials, $F(3,54) = 44.95$, $p < .001$, $\eta^2 = 0.71$, were still found. The same significant interaction of days by trials was found, $F(12,216) = 7.25$, $p < .001$, $\eta^2 = 0.29$ (Figure 24).

Overall, the thigmotaxis expressed by the rats did not differ across groups, or sex. The rats all learned the task across the days and trials, with thigmotaxis steadily decreasing across the days, and across the trails. The long-term memory deficit seen previously in the

path length is consistent, as the first trial of each day shows an increase in thigmotaxis when compared to the following trials.

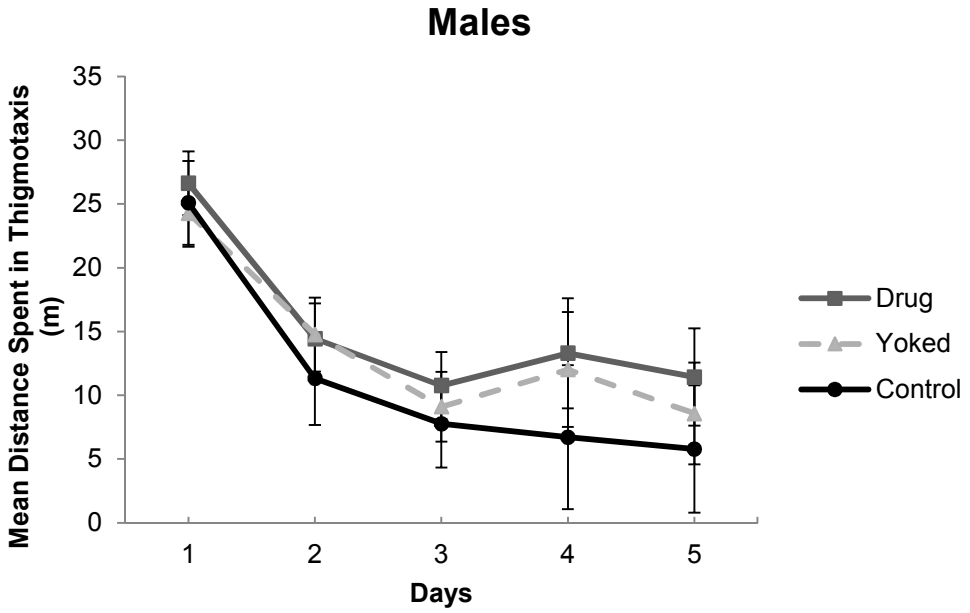

Figure 23. Mean percent of the path spent in thigmotaxis for the male groups across days during acquisition of the MWM task

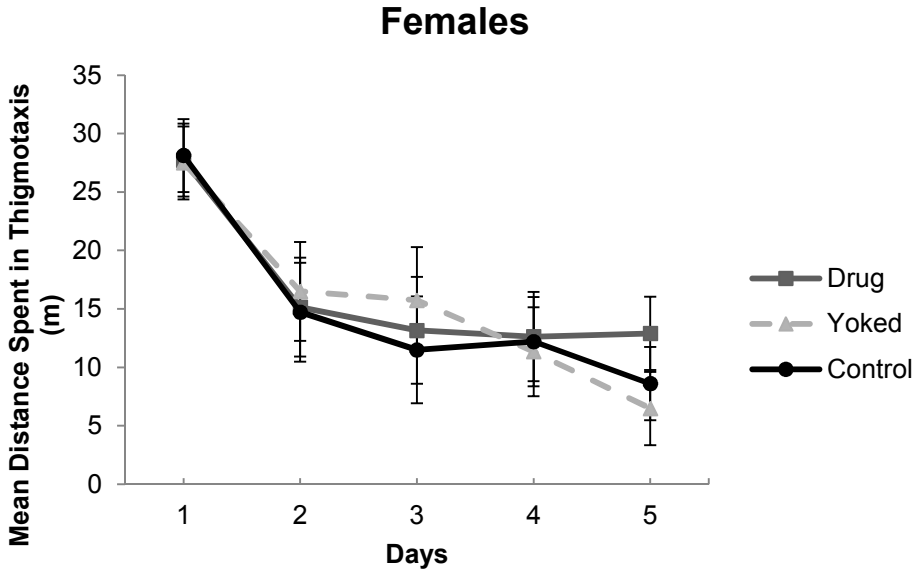

Figure 24. Mean percent of the path spent in thigmotaxis for the female groups across days during acquisition of the MWM task

Morris Water Maze – Probe

The Morris water maze probe trial involved removing the platform altogether on the first trial of Day 6 but limiting the trial to 30 seconds. It was used to measure whether or not the rats would revert to thigmotaxis or continue to swim in the quadrant in which the platform was previously placed. Unfortunately for this thesis, the probe trial found nothing significant across the two measures of percent time in the quadrant or in thigomotaxis.

Percent time in target quadrant. The percent of the thirty-second probe trial spent within the quadrant where the platform had previously been placed was used to measure memory for the previous location.

For the omnibus of the mean percent of time spent in the target quadrant, there was no effect of sex, $F(1,61) = .41$, $p = .523$, $\eta^2 = 0.01$, or of group, $F(2,61) = .54$, $p = .585$, $\eta^2 = 0.02$, nor was there an interaction between sex and group, $F(2,61) = .73$, $p = .488$, $\eta^2 = 0.02$ (Figure 25).

A priori male *t*-tests continued this trend with the drug and control *t*-test showing no difference, $t(20) = -.23$, $p = .818$, $d = -0.10$, as well as the yoked and control t-test, $t(20) = -1.095$, $p = .287$, $d = -0.47$. The female *t*-tests followed suit with drug and control, $t(20) = 1.255$, $p = .224$, $d = 0.54$, and yoked and control, $t(21) = .55$, $p = .587$, $d = 0.23$, also showing no significant differences (Figure 25).

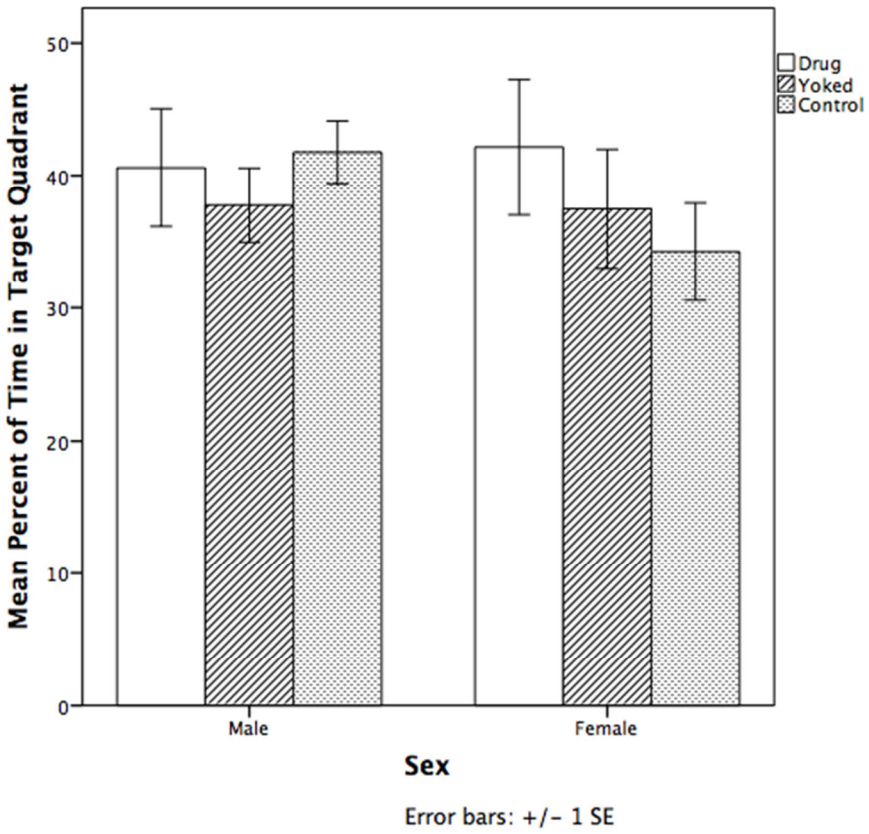

Figure 25. Mean percent of time during the probe trial spent within the target quadrant (NE)

Percent path spent in thigmotaxis on the probe trial. The percent of the path spent in thigmotaxis helps us determine whether the search strategy of the rats was affected by the treatment during adolescence or the gender of the rats upon realizing that the platform was no longer present in the water maze. This data was analyzed with a 2 (sex) x 3 (group) univariate ANOVA, with the *a priori* comparisons being measured with independent-samples *t*-tests.

In measuring the percent of the rats' path spent in thigmotaxis, there were no significant effects of sex, $F(1,61) = .03$, $p = .858$, $\eta^2 < 0.01$, or of group, $F(2,61) = .15$, $p = .864$, $\eta^2 < 0.01$. There was also no significant interaction between group and sex, $F(2,61) = .59$, $p = .559$, $\eta^2 = 0.02$ (Figure 26).

Male *t*-tests were not significant as the drug and control, $t(20) = .07$, $p = .949$, $d = 0.03$, and the yoked and control, $t(20) = -.28$, $p = .786$, $d = -0.12$, showed no differences between the two. Female *t*-tests expressed similar findings with the drug and control, $t(20) = .48$, $p = .617$, $d = 0.20$, and the yoked and control, $t(21) = 1.32$, $p = .200$, $d = 0.56$, coming up with no significant results (Figure 26).

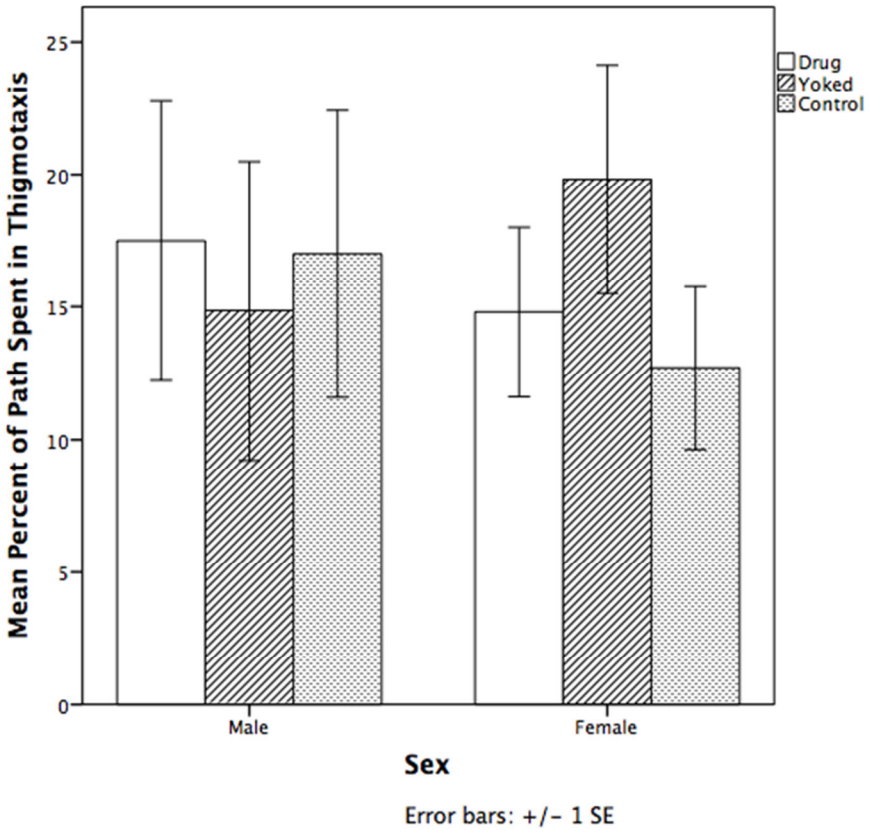

Figure 26. Mean percent of path spent in thigmotaxis during the probe trial

Morris Water Maze – Reversal Learning

The reversal learning portion of the Morris Water Maze was introduced after the probe trial. For the reversal learning, the platform was placed in a new location to measure the memory extinction and learning with changes in the testing apparatus. During the reversal learning, the speed, path length, and the percent of the path spent in thigmotaxis were measured in order to determine if there were group or sex differences in learning the location

of the moved platform after the probe trial. The reversal learning measures aid in determining if the rats are capable of inhibiting the previous spatial memory, and acquiring a new one. Difficulty in memory extinction could be indicative of memory deficits. The reversal learning task was performed on the sixth day of the water maze for four trials after the probe trial was administered. Only one rat was excluded from the analysis: a drug female rat was dropped due to experimenter error.

Swim Speed. Swim speed was again measured in the reversal learning task, as it was in the learning task. Rats' speed was measured in meters per second and the omnibus data was analyzed using a 2 (sex) x 3 (group) x 4 (trial) repeated-measures ANOVA. The *a priori* comparisons were run using 2 (group) x 4 (trial) repeated-measures ANOVAs.

In the omnibus of swim speed, there was no effect of group, $F(2,65) = .70$, $p = .500$, $\eta^2 = 0.02$. There was a trend of sex, as seen previously in measures of speed, $F(1,65) = 3.58$, $p = .067$, $\eta^2 = 0.05$, with the females ($M = .24$ m/s, $SD = 0.02$) moving faster than the males ($M = .22$ m/s, $SD = 0.02$). A trending effect of trials was found, $F(3,195) = 2.46$, $p = .064$, $\eta^2 = 0.04$, in which the rats decreased their swim speeds across trials. There were no significant interactions in the swim speed omnibus analysis (Figure 27).

In separate comparisons, the male drug versus control rats showed no significant effect of group, $F(1,22) = .34$, $p = .567$, $\eta^2 = 0.02$. The same was found when comparing the yoked and control male rats, $F(1,22) = .83$, $p = .373$, $\eta^2 = 0.04$ (Figure 27). The females also showed no significant group effects in the comparisons of the drug and control, $F(1,21) = .60$, $p = .449$, $\eta^2 = 0.04$, or of yoked and control females, $F(1,22) = .23$, $p = .633$, $\eta^2 = .02$ (Figure 28). There were no other significant main effects or interactions amongst the *a priori* comparisons.

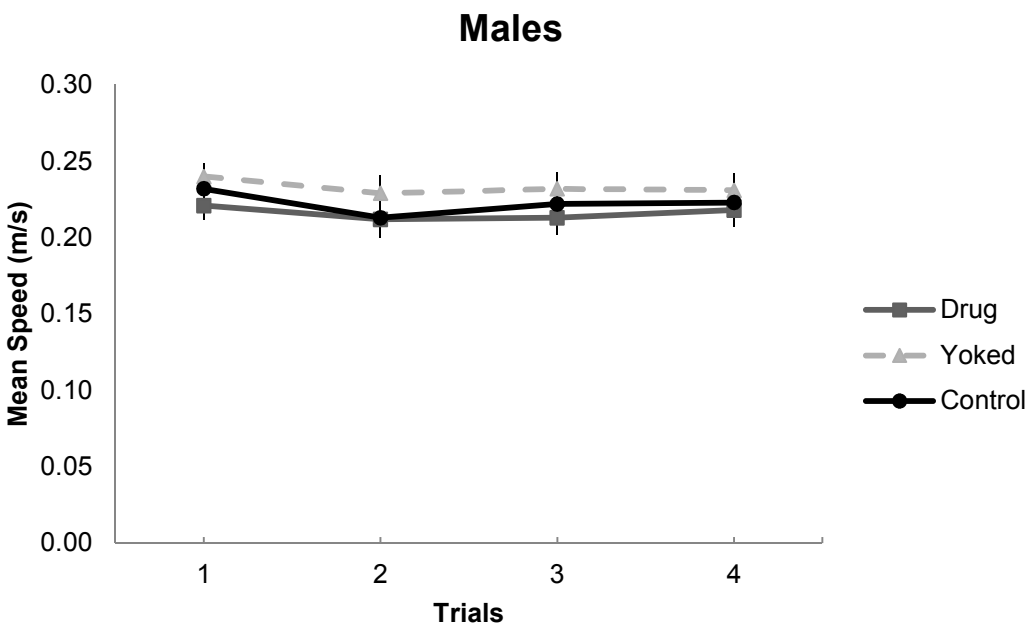

Figure 27. Mean speed of males during reversal learning trials

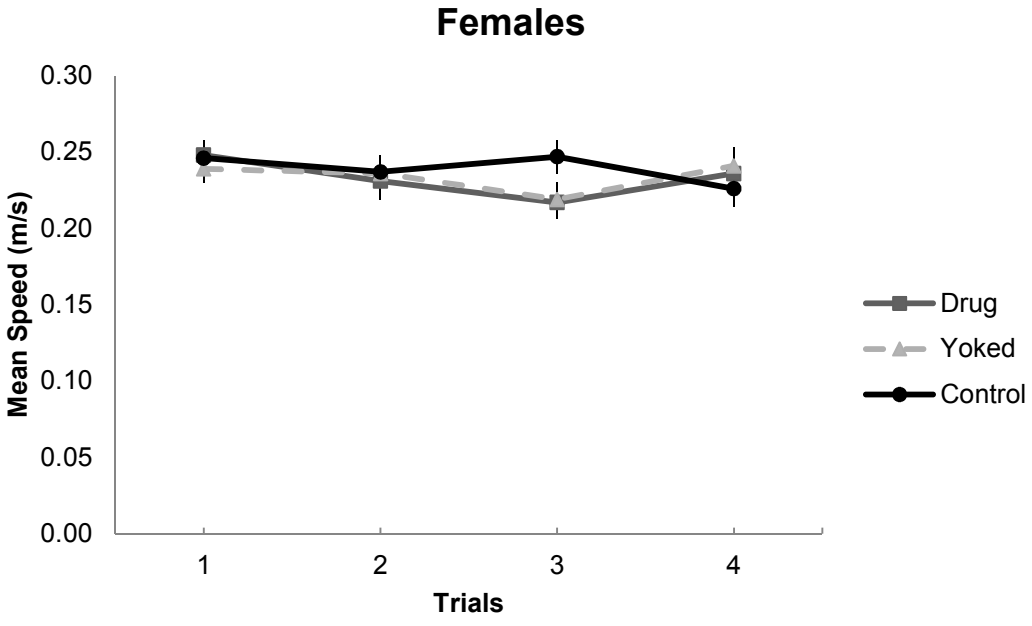

Figure 28. Mean speed of females during reversal learning trials

Path Length. Path length was measured to determine how far the rats had to travel in order to find the newly relocated platform. The data was analyzed with an omnibus 2 (sex) x 3 (group) x 4 (trial) repeated-measures ANOVA, then with four *a priori* 2 (group) x 4 (trial) repeated-measures ANOVAs for comparing each sex's manipulation groups to the controls.

In the omnibus analysis, there was a trend in group, $F(2,65) = 2.41$, $p = .098$, $\eta^2 = 0.07$, with the drug rats ($M = 5.14$, $SD = 3.44$) having overall shorter path lengths than the yoked ($M = 6.12$, $SD = 3.41$) and control rats ($M = 6.92$, $SD = 3.41$). The effect of trials was also significant, $F(3,195) = 83.81$, $p < .001$, $\eta^2 = 0.56$, with the rats learning across trials and reducing their path lengths. There was also an interaction trend between trials and group, $F(6,195) = 1.96$, $p = .073$, $\eta^2 = 0.06$, in which the drug and yoked rats picked up on the change faster on trial 1 than the control rats. There was not a significant difference between sex however, $F(1,65) = .47$, $p = .495$, $\eta^2 = 0.01$ (Figures 29 & 30).

Independent comparisons of drug ($M = 4.62$, $SD = 2.13$) and control ($M = 6.51$, $SD = 2.13$) males show a significant group effect, $F(1,22) = 4.73$, $p = .041$, $\eta^2 = 0.18$, a significant trials effect, $F(3,66) = 33.43$, $p < .001$, $\eta^2 = 0.60$, and a significant interaction between trials and group, $F(3,66) = 3.86$, $p = .013$, $\eta^2 = 0.15$. The three effects appear to be due to the control groups' poor performance during the first trial (Figure 29). The yoked ($M = 6.37$, $SD = 2.66$) and control comparisons do not show the group effect, $F(1,22) = .02$, $p = .899$, $\eta^2 < 0.01$, but the trial effect is still significant, $F(3,66) = 45.07$, $p < .001$, $\eta^2 = 0.81$ and the interaction between group and trials continues to be a trend, $F(3,66) = 2.22$, $p = .094$, $\eta^2 = 0.14$. This furthers that the male control group's longer path lengths during the first trial of the task may be indicative of some form of memory extinction issue not present in the male manipulation groups (Figure 29).

The female drug and control rats did not show any significant effects of group, $F(1,21) = .80$, $p = .382$, $\eta^2 < 0.01$, nor any effect of the interaction between trial and group, $F(3,63) = 1.70$, $p = .176$, $\eta^2 = 0.07$. The effect of trials was significant however, $F(3,63) = 26.59$, $p < .001$, $\eta^2 = 0.56$, with all of the rats learning the task over time, regardless of group. The yoked and control female rat comparison expressed similarly as there was an effect of trials, $F(3,66) = 26.56$, $p < .001$, $\eta^2 = 0.55$, but no effect of group, $F(1,22) = 1.09$, $p = .307$, $\eta^2 = 0.05$, and no interaction between trial and group, $F(3,66) = 1.08$, $p = .365$, $\eta^2 = 0.05$ (Figure 30).

The rats during the reversal learning task expressed an interesting group comparison in which the control animals (males specifically) expressed trouble with learning the new location of the platform, likely due to a failure of memory extinction causing the control rats to return to the previous location more frequently. All of the rats learned the new location of the platform over time, but during the first trial of the task, it was consistent that the drug and yoked animals found the new platform faster than the control animals.

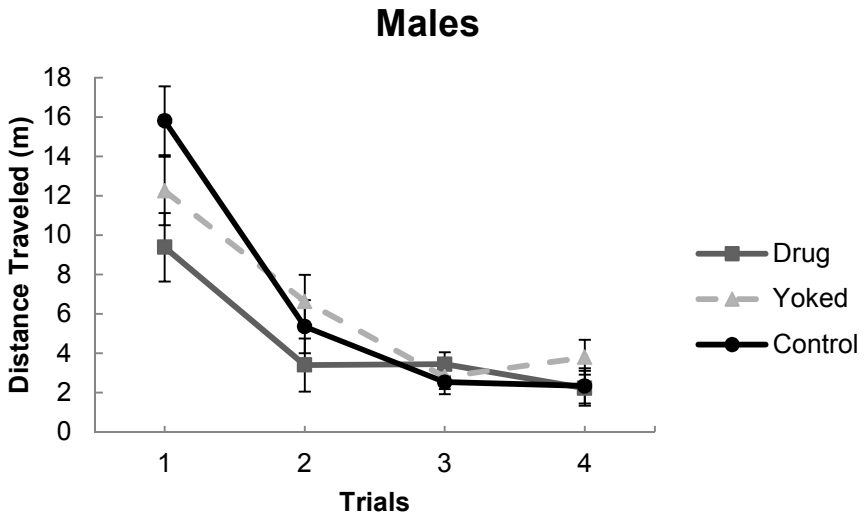

Figure 29. Mean distance traveled (path length) by males during reversal learning

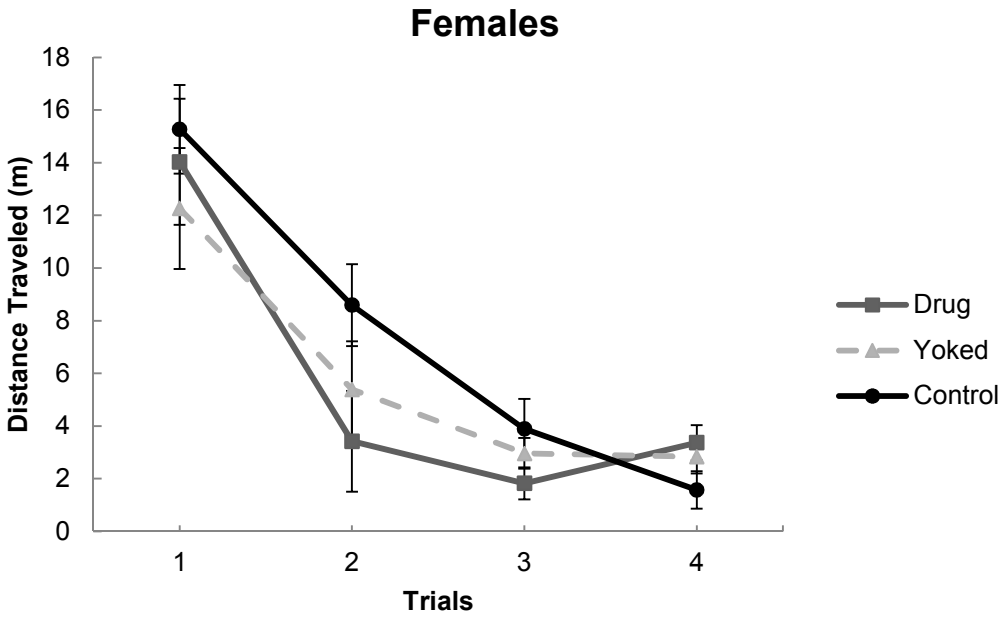

Figure 30. Mean distance traveled (path length) by females during reversal learning

Percent of path spent in thigmotaxis. The percent of the path spent in thigmotaxis (determined by the HVS) during the reversal learning was used to indicate whether the rats had a difficult time adjusting their search strategies to the moved platform by returning to a poor search strategy. This measure was analyzed using a 2 (sex) x 3 (group) x 4 (trial) repeated-measures ANOVA, then comparison of the manipulation groups to control using 2 (group) x 4 (trial) repeated-measures ANOVAs.

In the omnibus, there was no effect of sex, $F(1,65) = .23$, $p = .633$, $\eta^2 < 0.01$, or group, $F(2,65) = .94$, $p = .398$, $\eta^2 = 0.03$. There were no significant interactions on the omnibus of this measure. This is not consistent with the *a priori* comparisons.

Male drug and control comparisons showed similar results with the effects of group not being significant, $F(1,22) = .01$, $p = .931$, $\eta^2 < 0.01$, and the group by trials interaction

also not being significant, $F(3,66) = .031$, $p = .993$, $\eta^2 < 0.01$. The effect of trials was significant however, $F(3,66) = 6.40$, $p = .001$, $\eta^2 = 0.23$. The yoked and control comparison of group was also not significant, $F(1,22) = .38$, $p = .547$, $\eta^2 = 0.02$, and there was no significant interaction between groups and trials, $F(1,22) = .502$, $p = .682$, $\eta^2 = 0.02$, but there was a significant effect of trials, $F(3,66) = 11.11$, $p < .001$, $\eta^2 = 0.34$. This indicates that while the rats learned the task, there was no difference in the amount of thigmotaxis expressed by the rats during the reversal learning task (Figure 31).

The female drug and control comparisons were similar with no significant effect of group, $F(1,21) = 1.68$, $p = .210$, $\eta^2 = 0.02$. A significant effect of trials, $F(3,63) = 11.10$, $p < .001$, $\eta^2 = 0.35$, and a significant interaction between trials and group, $F(3,63) = 3.57$, $p = .019$, $\eta^2 = 0.15$, were found, though. Yoked and control comparisons followed suit with the group effect being not significant, $F(1,22) = 2.48$, $p = .130$, $\eta^2 = 0.10$, but the effect of trial, $F(3,66) = 12.02$, $p < .001$, $\eta^2 = 0.35$, and a significant interaction between trials and group, $F(3,66) = 2.88$, $p = .043$, $\eta^2 = 0.12$. These significant interactions indicated that in both the comparisons of the drug and the yoked groups to the controls, that the control female rats spent more of their paths in thigmotaxis. This increase in thigmotaxis was very prominent during the first two trials by the controls, but disappeared during the following trials, leaving the control rats looking identical to the manipulation groups (Figure 32).

Reversal learning. The reversal learning task was meant to determine how difficult it would be for the rats to learn the location of the platform after it had been moved from where they had grown accustomed to it for the previous five days. It was determined that contrary to what was expected, the control rats actually had more difficulty in learning this task, traveling further to find the platform, and spending a larger percent of their paths in

thigmotaxis than the manipulation groups. It was still seen that the female rats swam faster than the male rats, and that all of the rats learned the task over time, despite fatigue decreasing their swim speeds.

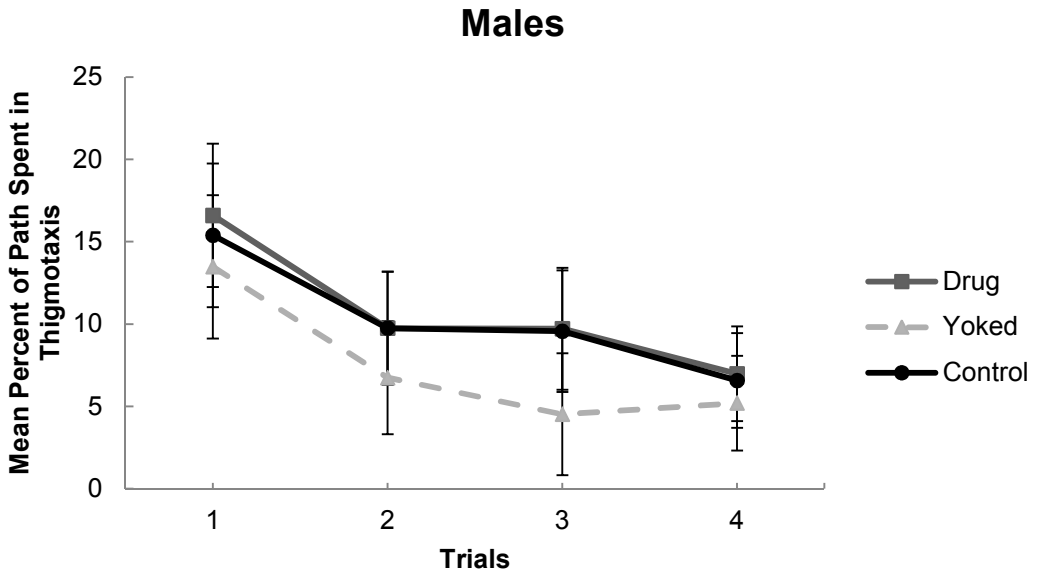

Figure 31. Mean percent of path spent in thigmotaxis for males during reversal learning

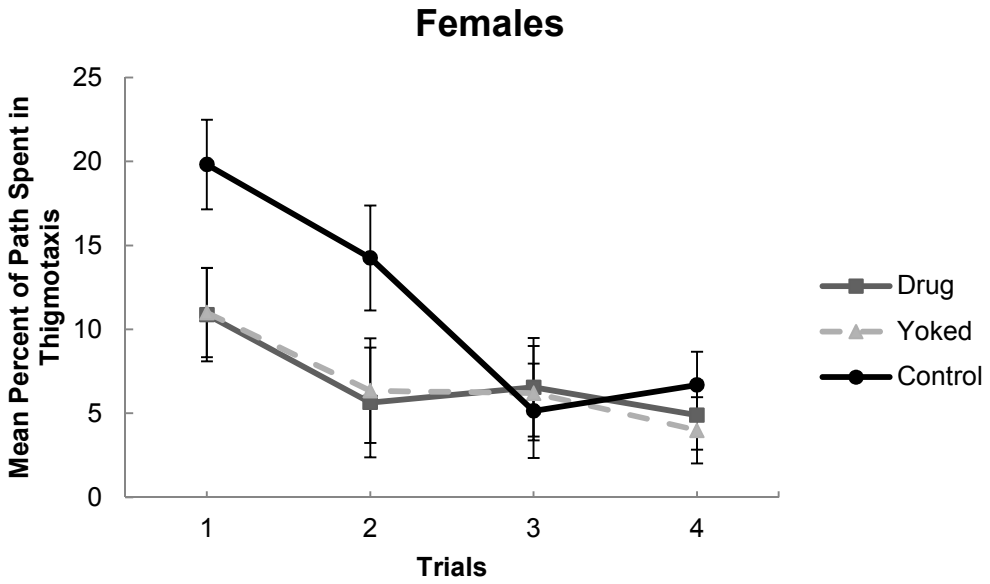

Figure 32. Mean percent of path spent in thigmotaxis for females during reversal learning

Chapter 4. Discussion

It was hypothesized that, based on Biscaia et al., 2003, exposure to CP 55,940 during adolescence (PND 35-48) would cause a decrease in the amount of food that the rats consumed overall during exposure. It was also hypothesized that a manipulation of their food intake would have a direct effect on their weights, in which rats that were being given less food or CP 55,940, would weigh less than rats on free-feed that were not exposed to the drug. These hypotheses were confirmed by the findings of this study. The drug rats willingly consumed significantly less food than the control rats due to exposure to the drug. The drug and yoked groups' diminished food intake led to lesser body weights across the injection period when compared to the control rats.

The significant differences between the drug and control rats' weights during the injection period do support that the use of CP 55,940 within rat studies results in the consumption of less food and reduced weight gain. Possible causes of this effect may relate to the form of food available to the rats within these studies, where the rodents were only given the same form of pellet food throughout the entire study. Perhaps if given a choice of two different forms of food, such as a higher fat and protein form of food and a carbohydrate heavy form of food, the marijuana rats may not experience reduced weight growth during drug exposure.

Cannabinoid receptor location may also have some effect on the feeding habits of the rats. Soria-Goméz et al. (2014) found that the location of the CB1 receptors influenced the occurrence of hyperphagia when cannabinoid receptor antagonists (AM241) were locally administered, specifically to the caudate putamen, the lateral hypothalamus and the paraventricular nucleus. This expression of hyperphagia when antagonizing CB1 receptors

implies that if activated, the cannabinoid receptors should induce something similar to hypophagia, much as we see in the rats used in this study. It was out of the scope of this study to analyze the cannabinoid receptors directly; however, in future studies it may shed light on the occurrence of this hypophagia.

The male rats were no longer statistically different in weight by PND 60 and the female rats were no longer statistically different in weight by PND 55, after the conclusion of the injection period on PND 48. The return of the rats' weights to a common standard before running was both quick and aided in assuring that all behavioral data run were not due to a current difference in weight across groups. The sex difference between the rats' weights, with males weighing an average of one hundred thirty grams more than the females, could have had a large effect on the overall finding across behaviors that the females were generally more active than the males. Previous research, however, does support that females are generally more active than males despite differences in weight (Van Hest, Van Haaren, & Van De Poll, 1987).

The elevated plus maze was run on PND 77, as the first behavioral task for all rats. It was chosen as a baseline anxiety measure and served to accustom the rats to behavioral testing in general. The hypotheses of the elevated plus maze were that the drug-exposed rats would express lower levels of anxiety than the yoked and control rats (Crowe, Nass, Gabella, & Kinsey, 2014). When assessing anxiety via the number of entries into the open arms versus the enclosed arms, there was no effect seen based on the experimental manipulation group. However, when comparing groups across the percent of time spent in the open arms, there was a significant effect such that drug rats spent more time in the open arms than the control rats, indicating that via time, but not entries, the drug rats were less anxious due to the effect

of adolescent cannabinoid exposure. This effect only reached significance in the female rats though, indicating that the anxiolytic effects of the cannabinoids may differ as a function of sex. This lack of effect could indicate that the elevated plus maze task run in this experiment was not causing enough stress to the rats or that their living environments cause a common baseline of stress that does not differ across groups. In Kinden and Zhang (2015), it was shown that when given HU-210, a CB1R agonist, it created anxiolytic effects on a baseline of stress, but when exposed to an acute stressor, such as the elevated plus maze (EPM), there were no such anxiolytic effects.

As hypothesized, there were significant differences in activity levels between the males and females on the EPM. The female rats actively entered the enclosed arms more frequently than the males, but not the open arms. This difference in arm entries preference could be indicative of a higher level of anxiety experienced by the females, as while they are more active, they did not explore the open arms more than the males did. The female rats expressed an increase in the number of entries into the enclosed arms, but did not enter the open arms any more frequently than the males. This increased activity in the females caused an increase in the entries of the arms, but not a significant difference in the amount of time spent in open arms. This is contrary to previous research (Llorente-Berzal et al., 2011), which demonstrated an increase in activity of the female rats, but also an increase in the percent of entries into the open arm in females as well.

When measuring the boli produced during the elevated plus maze, it was found that the male rats produced significantly more boli than the females. Using boli as a measure of anxiety, this presents a conflict in the findings. The male rats produced more boli than the female rats overall regardless of the group. Studies of males in social environments, similar

to the group housing cages we used, have shown that there are no differences in males' open field measured anxiety behaviors when group housed, however females were shown to benefit from the group housing, expressing lesser levels of anxiety (Westenbroek et al., 2003). This boli difference could be a presentation of anxiety in males or simply that male rats were larger and ate more than the female rats on average and, therefore, produced more boli in general. In a sex and strain study, Archer (1974) found that on average, rats did not differ in the number of boli produced across sexes or strains. This suggests that a smaller group housing effect may be in play, as Archer housed all of his rats in cages of five, while we housed our rats in cages of three.

Hypotheses for the open field task stated that expected levels of anxiety were to be lessened in the drug rats when compared to the yoked and control rats. This was not found in the frequency of center zone entries or time spent in the center zone, but was supported in the mean time per visit into the center zone for the male rats exclusively. This effect reinforces that cannabinoid exposure in the current study caused some level of decreased anxiety as is seen in Biscaia et al., 2003. Although Biscaia et al. found that both males and females exposed to CP 55,940 expressed lower levels of anxiety on the elevated plus maze and the open field.

Pamplona, Bitencourt, and Takahashi (2008) report research that short and long-term memory extinction can be manipulated by cannabinoids and the endocannabinoid system. Exposing rats to a conditioning chamber and administering foot shocks allowed the experimenters to use their freezing behavior to measure their fear index upon second exposure. No differences were seen in control rats of their levels of anxiety upon a second exposure to the fear-inducing stimulus, but the rats given cannabinoids (WIN 55,212-2)

decreased their freezing behavior during the second trial. This effect could explain the differences seen in the male drug group during the open field, as the drug rats were not spending more time in the center zone, due to not decreasing their anxiety level of this new environment across days, effectively removing their memory of their previous experience within the open field.

The female yoked rats expressed higher levels of anxiety on the open field when measured on the mean number of entries into the center zone. This finding is contrary to Almeida, Tonkiss, and Galler (1996), in which rats who were malnourished prenatally expressed lower levels of anxiety on the elevated plus maze, possibly as an effect of increased exploratory behavior. An argument could be made that the yoked rats may be more inclined to dramatic anxiety responses in the face of the open field, as they have been exposed to higher levels of anxiety than the drug or control groups starting on PND 35. This difference could have been caused by a strictly decreased food intake, disallowing them the option of eating more while the drug rats were given an excess of food they chose not to eat. Other previous research has found that poor nutrition during the perinatal stage can cause an increase in anxiety behaviors in the open field, although still expressing increased exploratory behavior (Belluscio, Berardino, Ferroni, Ceruti, & Cánepa, 2014). It is also possible that after experiencing a higher level of anxiety, the yoked rats were more hyper-alert of their environments, as seen in Grillon and Davis (1997), where humans who were exposed to an acoustic startle stimuli and electric shock administered through the wrists showed a hyper alertness to similar stimuli when presented to the participants. This effect should be pursued in future research.

Activity level was also measured on the open field in this study. A sex difference in activity was expected in the hypotheses again. This effect was found to be significant in which the female rats were more active than the males, typically traveling on average around ten meters further than the males (roughly forty meters for females, thirty meters for males per trial). This effect supported Van Hest et al., 1987, where Wistar rats were compared in the open field on general activity and their responding capabilities. The study found females to be much more active than males on average, even in states of severe malnutrition. The effects of group did not differ the levels of activity.

Boli data across the elevated plus maze and open field tasks tended to show a similar difference across sex but not any differences between groups. As a measure of anxiety, boli would suggest that the males were more anxious than the females. This is difficult to defend, however, as in overall anxiety measures, the females showed signs of higher levels of anxiety in their preference to avoid the open arms, which leads us to assume that the difference in boli was more a product of the significant difference in size and food intake amongst the males when compared to the females.

In the memory task for the object location, the hypotheses anticipated that exposure to the drugs would cause a deficit in memory for the location of the object, with a sex interaction occurring where female rats would be worse than male rats. Observing this through the use of the percent of time spent investigating the moved object during trial two, the expected effect was not seen, implying no difference was made by exposure to the drug.

An effect was seen in which the yoked female rats increased their exploration of the moved object compared to the static object. This effect could have been a result similar to what was considered in the open field where, due to higher levels of anxiety in the yoked

female rats, they may be hyper-alert to their environments. Grillon and Davis (1997) ran studies on Yale students in which a positive correlation was seen such that as anxiety of shock threat and acoustic startle increased, so did the alertness to the external stimuli. Further research on the effects of yoking and general alertness of rats could provide answers for this finding, as very little research could be found on the topic.

When observing the percent of interactions with the two objects in the object location recognition task, it was seen that the rats had a natural preference for investigating the static object (B) during the first trial. Additionally, when looking at the percent of time spent investigating the two objects during the second trial, only the yoked groups investigated the moved object (A) more than the static object. This could have been caused by a place preference factor involved in the location of the object. The rats were placed within the maze at the southeast corner of the maze in which object B was closer to the place the rats were introduced into the open field, regardless of the trial, than was object A. This proximity may have caused an inflation of the interactions with object B, with the drug and control groups not being less likely to notice the change, but to continue to prefer object B simply due to proximity. In further studies of the open field, it would be better to have the objects in an equal distance or at least a distance away from the entry point that would require the rats to cross the maze before interacting with the objects.

The percent of interactions measure further supports the place preference point, as the yoked male rats' percent of interactions with object A was only thirty-eight percent. It was not until the second trial, after the object had moved, that the male yoked rats increased to fifty percent of their interactions with the moved object. It is very possible that they did not interact with object A as much as object B during the first trial due to place preference

caused by the closer proximity of object B. After the movement of object A, it was clear that the male yoked rats noticed it as they increased their interactions with object A, but only due to the novelty of its movement. This does assist in supporting that the yoked rats excel in noticing environmental change, as indicated by the males showed a similar hyper-alertness to the change, but not as strongly as the female yoked rats.

The fewer investigations seen of the moved object could have been an issue in the measurement of the interactions with the objects. Dix and Aggleton (1998) showed that in their object location recognition task, the most sensitive period of measurement for recognition occurs during the first two to three minutes, where we measured for the entirety of five minutes. In future analyses, it would be wise to separate the data analyses into sections that could be easily separated into minutes and allow for more precise measurement of the interactions.

The Morris water maze task was broken up into three phases: the initial learning, probe, and reversal learning phases. The intention of the learning phase was to teach the rats the location of the platform followed by an examination of their search strategy during the probe trial (Vorhees & Williams, 2003).

Hypotheses during the learning phase anticipated finding that the activity levels (measured by swim speed) and learning (measured by path length) (Beiko et al., 2004) of female rats would be significantly greater than in male rats. Previous research suggested that the swim speed of females should be theoretically slower than the males (Warner, Libman, Wooten, & Drugan, 2013), but the weight differences between the male and female rats were dramatic enough to assume otherwise. The data found a trend for the female rats swimming faster than the male rats, but it was not quite significant. It is possible that the weight

differences between the males and females caused a switch in the effect that would have otherwise shown male rats swimming faster than the female rats. The activity measures from the elevated plus maze and the open field task as well as previous research suggests that females are generally more active than males (Van Hest et al., 1987), which the findings of this project support.

It was hypothesized that the swim speed would be different across groups as well, with the drug rats moving faster than the yoked and control rats. The research defending this (Robinson et al., 2007) used an acute exposure model with HU210 and found that the drug rats on average swam faster than controls across trials. The findings for this study found the opposite effect of speed across groups in which the drug rats were slower than the yoked and control rats. It is possible that effects of cannabinoids affecting swim speed acutely do not have the same effect long-term. The effect found of the drug rats moving slower than the control rats was found solely in the male rats; however, this was not supported by a memory deficit in the drug rats, as no significant effects could be found to suggest memory deficits in the male rats during the Morris water maze task. Further investigation of this effect should consider the long-term effects against the immediate exposure model, as well as determine if there is a sex covariate involved, as this effect was only present in the male rats.

It was hypothesized that there would be a sex difference in path length, as the females took longer on average to discover the location of the platform than males, as also seen in Warner et al. (2003)'s study of sex differences and intermittent swim stress. This effect was supported by the data, in which the females traveled further on average to find the location of the platform. This could be indicative of the female rats having a memory deficit in finding the platform in comparison to the male rats.

Contrary to Alamy and Bengelloun (2012) and Fukuda, Françolin-Silva, and Almeida (2002), a trending effect was found in the yoked male rats in the path length during the learning phase. The previous research found that malnutrition did not express deficits in learning on the Morris Water Maze, however, our findings indicated that the male yoked rats expressed difficulty in learning the location of the platform when compared to controls. This effect should be investigated further, as it is possible that this effect may be important.

Hypotheses for the probe trial assumed that the drug and yoked rats would show deficits when compared to the control rats. The manipulation rats were expected to travel further in thigmotaxis than the controls rats and spend less time in the target quadrant (the quadrant where the platform was previously located) than the control rats. Unfortunately, the probe trial of the water maze task was a wash, as no significant effects were seen in comparing thigmotaxis or path length. It is possible that the probe trials' thirty second length was not long enough to pick up on some of the effects, however, most probe trials are only run for thirty seconds during water maze protocols (e.g., Vorhees & Williams, 2003). The best assumption of this lack of results would indicate that due to the washout period, there were no powerful effects of drug or reduced food intake that could be identified from a thirty second probe trial. Previous research by Abboussi et al. (2014) found that exposure to WIN 55,212-2 during adolescence and testing during adulthood caused deficits in the cannabinoid exposed rats. The high level of variability in the results and null results may indicate some error in running has occurred.

Hypotheses in the reversal learning task expected to see a continuance of the sex difference previously seen in the learning task, with the female rats traveling further and faster than the male rats. The effect of swim speed between males and females only presented

itself as a trend, however it was again in the same direction with the females traveling faster. It is possible that the trend did not become an effect simply due to only having four trials to compare across the measures when compared to the learning phase that had five times as many trials.

In measures of the path length, there was no effect of sex, as the male and female rats' swim path lengths were very similar, but an effect of group, in which the male drug and yoked rats performed better than the control rats during the first trial. This difference was absent in the female rats for path length. Explanations of this for the yoked rats could return to the Grillon and Davis (1997) study, indicating that due to the yoked rats' hyper-alertness to change due to high stress, they were better at locating the new platform and remembering it. In regards to the drug male rats performing better than the controls, this effect is further supporting the previous anomaly found, as Abboussi et al. (2014)'s data still expressed learning difficulties in rats exposed to cannabinoids during adolescence.

Measures of the amount of time spent in thigmotaxis were hypothesized to show that the manipulation groups would travel further in thigmotaxis than the control groups, as they were expected to have an inferior search strategy when looking for the new platform. Overall, this was not the case. In fact, when looking at the female rats independently from the males, it was seen that the opposite was true. In a trial by group interaction, it was seen that the female control rats traveled a lot more of their paths during the first two trials of the reversal learning in thigmotaxis when compared to the manipulation groups. It is possible this is not a difference in search strategy, but instead an anxiogenic effect of now being clueless to where the platform is, but after learning it has just moved, dramatically reduce their path spent in thigmotaxis. This group effect was absent in the male rats.

It was also hypothesized that the drug rats specifically would have more difficulty finding the platform than the yoked or control rats, as it was expected that the effect of the drug itself may be responsible for spatial learning deficits. An interesting effect occurred, however. There was a trend implying that the drug rats picked up on the reversal learning task faster than the control rats, traveling less of a distance to find the platform than the control rats. The only literature that could be found expressing the same effect was at a poster at the 11th Biennal meeting of the European Behavioral Pharmacology Society. Blain and Brett (2005) acutely exposed mice to two differing doses of THC and measured their abilities in an acquisition phase and a reversal learning phase. They found that rats given the lower of the two doses experienced trouble during the learning phase but outperformed the control group during the beginning of the reversal learning, possibly indicating a sense of memory extinction of the location of the platform that benefited them.

The trouble with this hypothesis, however, is that it was not simply that the control rats did worse than the drug rats, but they also did worse than the yoked rats. The yoked rats additionally had shorter path lengths than the control rats, indicating that they learned the task faster. A reasonable explanation for this would be that the anxiogenic effect of the change in the platform location on the probe trial and the first reversal trial made the yoked rats again hyper alert to their environments, aiding their learning of the new location of the platform faster than the control rats due to heightened senses from increased levels of anxiety.

Problems and Limitations

In this study, the behavioral task order was designed to try and avoid causing pre/post test anxiety in the tasks, such as measuring the rats on the anxiety tasks after introducing

them to a highly anxiogenic task such as the Morris water maze. In a further detailed study, it would be preferential to see if the order of the tasks causes any changes in the reports, as it is possible that anxiety measures on the open field may have been affected by the previous exposure to the elevated plus maze four days prior. Potentially changing the order of the tasks to measure memory before and after exposure to anxiogenic tasks like the elevated plus maze and the Morris water maze could also be enlightening in other senses. Logically, the order that was used in this project was probably the most appropriate as the anxiety tasks were measured without being in highly anxiogenic environments prior to testing, however it is possible that the two anxiety tests occurring prior to the first memory task could have introduced a level of anxiety into the results.

The lesser food intake on the yoked control rats took a toll on a few rats. During the experiment, three male rats (one from each condition) were removed due to severe skin lesions, leading to the inclusion of an extra group of males. A few other experimental rats, often yoked, also experienced minor skin lesions that were determined to not be severe enough to remove them from the study. It should be noted the skin lesions were not solely present in the yoked rats, and many of the lesions were only present during the injection periods where the rats were isolated for extended periods of time. The rats were treated with a veterinary-prescribed skin treatment that often resolved any problems within a day or two. Other studies have found that isolation in rats commonly causes increased stress levels, as rats are typically social creatures (e.g., Beery & Kaufer, 2014). One instance during the study did occur in which a male rat's severe skin lesions continued after the injection period isolation. They were not healing during his time with his brothers. This prevented him from

running behavioral tasks at all, but due to the group-housing factor of the experiment, he was kept in the group cage with his brothers and treated throughout the course of the experiment.

Measures of the estrous cycle were not considered during the creation of this design. There is a substantial amount of research that indicates that the estrous cycle could alter the results of both anxiety and memory measures. The design of this study however was based strictly on the ages of the rats, where at best the estrous cycle could have been used as a covariate had the appropriate tests been conducted. However, measurement of the estrous cycle while comparisons were being made to the males may have introduced an additional stressor that would be absent in the males.

During the course of the estrous cycle, fluctuations of sex steroids in the body can cause changes in the brain; however, the brain is typically capable of adapting to these shifts in neuroactive sex steroids in humans (Melcangi, Panzica, & Garcia-Segura, 2011). This point is expanded upon in Lovick (2012), where measures of progesterone were monitored over the estrous cycle. The late dioestrous point of the estrous cycle in rodents caused a dramatic drop in progesterone. The late dioestrous drop of progesterone caused an anxiogenic effect in rats, which could have the potential to alter results of anxiety tasks.

Human models have been developed to determine the level of anxiety in relation to estrous cycle, measuring the levels of stress differences between those who were more sensitive to anxiety and those who were not on points of their estrous cycles. It was discovered that those who were more sensitive to anxiety disorders had said issues at different points in their cycles, specifically the premenstrual period (Sigmon et al., 2000).

Additional measures of the cortisol levels of the rats would have been beneficial to the results of this study, however at the time of the design of this project, obtaining and using

a corticosteroid enzyme linked immunosorbant assay (ELISA) was not possible. The limited time of this project also made it difficult to add other measures to the overall design, as the entirety of this project took place over the span of eleven months. Additionally, with stress as a constant concern, the collection of blood samples from the rats when monitoring a behavioral task could cause further changes in the data overall.

Further studies

In further cannabinoid studies, it would be recommended to put a more heavy focus on the estrous cycle of the rats, as is expected of the next study planned (Rigdon et al., in preparation). A study designed around all females would help to avoid the added stressor of testing the female rats' estrous cycle phase that would not be present in their male counterparts.

A measure of the estrous cycle will also allow us to see if it is possible that the females (specifically the yoked) may have had delayed or hindered estrous hormone release during the beginning of their sexual maturity. Due to the stress of isolation and diminished food intake, it is possible that this delay may have occurred as stress can affect hormones pretty heavily if the conditions are right.

The brains of half of the rats run for the experiment were collected to perform analyses of tissue structure, determining if any effects could be seen in the hippocampi of the rats. Another future project may also look into the sex differences in the interaction of the food manipulations and the drug on neurogenesis.

Another issue that occurred with this experiment was that the yoked rats were fed based upon the food intake of the drug rats, but with a one-day delay behind the drug rats. In order to follow up with this yoking, a formula could be developed based upon previous data

to give an estimate of the amount of food that should be given based on the weight of the rat and its age. This formula did not fall within the scope of this thesis, however, the data collected within could be used in the development of said formula.

Additional data regarding the environment in the elevated plus maze task would also be beneficial to the process. As many of the effects of the elevated plus maze were not very strong or null, as they have been across multiple studies in the Radford lab, some external variables are probably responsible. Identification of this variable would help explain the frequent lack of results that have occurred, whether it be lighting or the apparatus, adjustments could be made to create a more effective task. The process would require inducing stress and having a control anxiolytic group while manipulating external variables to determine if perhaps too much lighting or external stimulation (posters on the walls) could be causing a change in the behavior of the rats that has led to measuring a different behavior with anxiety (such as explorative or food-seeking behavior).

In conclusion, the current study is an indicator that the effects of nutrition during pre-adult cannabinoid exposure are one of the most important variables often overlooked that must be considered by all researchers involved in the field to assure any effects found are not effects of nutrition. Comparatively, it is also very important that we as scientists ethically research and inform the public of the true hazards of newly legalizing cannabis and cannabinoids could have on children and adolescents, as very real changes in emotion and memory deficits can occur.

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