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**THE EFFECTS OF ANANDAMIDE, A CB1 AND CB2 AGONIST, ON THE
BEHAVIOR AND NEUROCHEMISTRY ON A RAT MODEL OF POST-
TRAUMATIC STRESS DISORDER**

A thesis submitted to

Regis College

The Honors Program

In partial fulfillment of the requirements

For Graduation with Honors

By

Zachery Koneval

May 2016

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ACKNOWLEDGEMENTS

I would like to thank Dr. Thomas Bowie and Dr. Johnathan Howe who both have served as Director of the Regis College Honors Program for their guidance and support throughout my undergraduate career. I would also like to thank my thesis advisor, Dr. Mark Basham, for his dedication and support, as well as Dr. Johnathan Dimos, my thesis reader, for his unique insight and advice. I would also like to extend thanks to Dr. Ashley Fricks-Gleason for her aid in matters of both my thesis and my health. Finally, I would like to thank both Hailey Benesch and Spencer Bowles who were instrumentally helpful in the completion of my research experiment.

Vocation: A Call to Action

“Vocation is from the Latin vocare, to call, and means the work a person is called to by God.” – Frederick Buechner

Have you ever been awoken by the soft voice of a child who knows they ought to be sleeping, but are kept awake by the nightmares that ensnare their minds to the point they envision monsters hiding in every shadow? Did you happen to notice the helplessness in their eyes, the fear in their trembling voice, and did you not try to do everything in your power to ensure that they were safe, to get them to realize that the visions in their head are not real and as soon as they open their eyes the demons simply disappear. Now I ask, what would you do if you saw that same look in the eyes of a catatonic twenty-something young woman who just happened to catch a glimpse of a man, whose profile is vaguely similar to another’s who is serving fifteen years in prison?

For her the demons are monsters from her past, whose actions have seared themselves into her memory grossly invading her present without so much as a warning. How are we supposed to help someone like her? Simply telling her that it is over is not enough for she has heard that repeated over and over like a song stuck on loop and it is short of impossible to try and console someone who recoils at the slightest touch. The worst about all of this is that I cannot say she is the exception to the rule, that this is a unique experience within our society. All one has to do is look around, at the homeless begging on street corners, to the soldiers in their war torn uniforms, to those who have seen their livelihoods swept away by forces of nature; because if you look closely enough you may be able to see the despair in the eyes of

those who suffer from what is commonly known as post-traumatic stress disorder (PTSD).

Sadly, I happen to know an unfortunately high amount of people to suffer from PTSD. I know what it is like to help someone find their second set of earmuffs so they do not have to re-experience war during 4th of July celebrations, to talk someone back into the present, and honestly I was proud of myself because I believed that I was helping them to the best of my ability. However, entering into my senior year I felt a nagging in the back of a mind that was telling me that, maybe I could do a little bit more.

It all finally clicked for me when a friend happened to mention the astonishingly high amount of veterans with PTSD who are living homeless in our nation's major cities. Sure, I have seen these men and women panhandling on the street and tossed them extra change if I could but I never gave a second thought to the ordeals that they are going through, and I am someone who has seen first-hand how debilitating PTSD to someone's livelihood. Furthermore, at least the people in my life have the support of their family and friends but these people are out fighting this disorder on their own with minimal resources. So I decided to join those whose research has been done in order to serve, not only veterans, but all people who suffer from PTSD. No longer shall I stand idle.

Abstract

The neurological pathways for posttraumatic stress disorder have come under scrutiny in recent years due to emerging research on the endocannabinoid system. Suggested involvement in the fear and stress pathways involve the pre-synaptic modulation of corticosteroid activity upon the consolidation and extinction of emotional memories in the hippocampus or the basolateral amygdala or possibly the GABA, 5-HT, and NE neuronal projections that are centered in the medial pre-frontal cortex that have been implicated in the hyperarousal and negative affect of PTSD. This study will focus on the use of anandamide, a CB1 and CB2 agonist in the treatment of PTSD behavioral symptoms of a rat model, specifically general anxiety, avoidance, generalization, and spatial learning and memory. It will also utilize immunohistochemistry to observe the neurochemical alterations that are facilitated by the onset of the disorder and subsequent treatment in regards to the cannabinoid receptor availability in the CA1 area of the hippocampus and basolateral amygdala. Rats were subjected to moderate unescapable foot shocks, paired with a predatory stressor and a novel stimulus, in conjunction with situational reminders in order to elicit and maintain PTSD symptomology. Anandamide was shown to significantly increase spatial memory functioning and moderately reduce avoidance behaviors, but did not have a significant effect on anxiety or generalization behaviors.

Introduction

Posttraumatic stress disorder (PTSD) is no longer the enigmatic disorder that had puzzled physicians of years past. It is no longer a diagnosis that is given under different names, such as Gulf War Syndrome, shell shock, or post-combat fatigue, but a single diagnosis that will be given to nearly a tenth of the general U.S. population and nearly a third of those who serve in the Armed Forces. However, while now that it is possible to recognize the disorder focus must be shifted towards the discovery of an underlying cause and possible treatments.

The criterion for a PTSD diagnosis been outlined in the latest iteration of the Diagnostic and Statistics Manual (American Psychiatric Association, 2013), which separates them into five distinct categories. The first is that one must experience a trauma, either directly or through a close relation, which can range from combat experience to natural disasters to sexual assaults. While this criterion might seem obvious two meta-studies found that PTSD was found to be comorbid on average 76% of the time with borderline personality disorder in women or dependent personality disorder in men, 66% of whom reported that they had never experienced a trauma (Ford et al., 2015). The second involves symptoms of invasive memories or thoughts which can manifest in ways ranging from vivid nightmares or uncontrolled flashbacks. Third, the patient must show avoidant behaviors which can include social withdrawal or simple everyday behaviors to avoid stimuli that are found to be noxious. The next criterion is that patients with PTSD present with negative affect or cognitions, which can be directed towards oneself or projected. Last is a state of hyper-arousal which can be expressed through the partaking in reckless behavior,

restlessness, or constant anxiety. A patient must display behaviors in all four of these categories for at least six months following exposure to the stressor to receive a diagnosis.

The narrowing of symptoms to highly definable psychological categories is crucial because it has led to the discovery of the underlying neurological structures and chemicals that lend themselves to the clinical manifestation of the disorder. Through extensive research it has been shown that the four major neurological structures that have been correlated with PTSD and considered to be a part of the fear and stress circuit within the brain are, the basolateral amygdala (BLA), the hippocampal nuclei (CA1), the medial pre-frontal cortex (mPFC), and the hypothalamic-pituitary axis (HPA) (Hauer et al., 2014; Ford et al., 2015). Furthermore, the neurotransmitters involved are, glucocorticoids, GABA, serotonin (5-HT), and noradrenaline (NE) (Ford et al., 2015). However, the most effective pharmacological treatments (SSRI's) that are based off the hypothesis that one or more of these systems are malfunctioning have only proven to be 60% effective at alleviation of initial symptoms and only 20% effective in assisting in full or partial clinical remission of the disorder (Neumeister et al. 2015). This suggests that critical information about the fear circuit. Recent studies (Ganon-Elazar & Akirav, 2013; Hauer et al., 2015; Neumeister, 2013) into how the endocannabinoid system interacts with all of the components of the fear circuit seem to point to endocannabinoids (eCBs) as being the previously missing, neuro-modulatory, component.

To fully understand the complexity and the role of cannabinoids in the regulation of the fear circuit one must look at the neurochemical control they exert in the four main

structures of the circuit. This is best done by examining the effects that eCBs have on the release of the known neurotransmitters (NT).

Glucocorticoids

Glucocorticoids (GCs) are known as the stress hormone as they are released via the HPA axis in response to stressful stimuli. What is unique about the relationship between eCBs and GCs is that it is bidirectional. It begins with the initial release of GCs causing a Ca^{++} cascade that activates the enzymes that convert lipid membrane into eCBs (Hillard, 2000). The eCBs then bind pre-synaptically and act as a feedforward mechanism facilitating the release of GCs, until a point where the buildup of both NTs causes eCBs to act as a feedback inhibitor and prevent any further release of GCs (Ganon-Elazar & Akirav, 2013; Neumeister, 2013; Evanson et al., 2010). Meanwhile GCs are up and down regulating the availability of eCB receptor (CB1 and CB2) availability, specifically in the BLA and hippocampus (Neumeister et al. 2013; Neumeister et al., 2015). Furthermore, a buildup of the GCs precursor, corticotrophin releasing hormone (CRH), activates fatty acid amide hydrolase (FAAH) which breaks down the eCBs in the synapse (Gray et al., 2015). Seemingly, both NT are required to maintain proper control and normal levels of the other (Cota et al., 2007).

While these two NTs might assist each other chemically in regards to behavior, specifically aversive memory consolidation and extinction, they are the antithesis of each other. Synthetically increased levels of GCs in the BLA and hippocampus have

been shown to facilitate the consolidation, hinder extinction, and even re-consolidate conditioned fear responses (Campolongo et al., 2009). The injection of synthetic cannabinoids or inhibition of FAAH on the other hand has been shown to facilitate the extinction of aversive memories, without any psychoactive effects altering initial consolidation or conscious recall (Campolongo et al., 2009; Chhatwal et al., 2005; Bitencourt et al., 2013; Das et al., 2013). Interestingly, this effect is time dependent in that the injection only facilitates extinction if given hours after the exposure to the stressor, if given earlier it seems to have a preventative effect (de Oliveira et al., 2008). This suggests that eCBs are effectively reducing the levels and effects of GCs. This idea is backed by evidence that CB1 receptors in the BLA are down-regulated and those in the hippocampus are up-regulated. These differing regulations would lead to smaller initial but chronic GCs activation in the BLA, which is consistent with the over activation theory of PTSD (Neumeister et al., 2013), and a short but initial flooding of the hippocampus, which could lead to the oxidative and subsequent atrophy seen in a majority of PTSD patients.

It is important to note that while researchers still debate whether or not a reduced hippocampal size is evident of PTSD or if it is simply a risk factor, but they do agree that hippocampal dysfunction is present in patients with PTSD (Neumeister et al., 2015). This is evidenced by the poor performance of PTSD patients and animal models of PTSD on spatial memory tasks. Therefore, it is possible that CBs could prevent or cause this damage depending on the regulation of their receptors. Overall, this evidence suggests that CBs could be used to facilitate the extinction of avoidance behaviors as well as the destruction of hippocampal neurons through the regulation of GCs in the brain.

GABA

Through years of research it has been shown that GABA acts as the main inhibitory NT in the brain. Ford et al. (2015) explains that the main GABAminergic projections in the fear and stress pathway begin in the mPFC and terminate in the BLA. They suggest that the over-activation of the amygdala might be due to a lack of inhibitory signals by the mPFC which can result in the lack of ability to distinguish between a potentially dangerous or harmless stimulus (Lin et al., 2009). Lin et al. (2009) and Ratano et al., (2014) have shown that the activation of novel CB receptors by the synthetic cannabinoid, cannabidiol CBD, on the GABAminergic neurons could potentially assist in the release of the NT and potentiate the inhibition of BLA activation. This suggests that CBs could potentially reduce the amount of generalizing behavior by reducing the amount of BLA activation.

Serotonin & Noradrenaline

While the interconnectedness of the serotonin and noradrenergic and the cannabinoid system has not been as extensively researched as the previous systems, recent discoveries show promising results.

The NE neurons in the mPFC are utilized to activate the bodies sympathetic nervous system (SNS) which controls arousal states (Morilak, 2012). The injection of CBD

into the mPFC has shown to decrease arousal when administered after exposure to a stressor, but increases it if given prior to exposure (Fogaça et al., 2014). This suggests that the eCB modulation of NE could be similar to that of GCs and be used to reduce hyperarousal-induced anxiety.

The 5-HT neurons in the mPFC are related to emotional control, expressly depressive behaviors. Marinho et al., (2015) and Sartim et al., (2016) directly injected CBD into the mPFC of rats, after extended periods of inescapable restraint, and found that it reduced depressive symptoms. Furthermore, they showed that this effect could be blocked via the co-injection of a 5-HT antagonist. This suggests that the pre-synaptic activation of CB receptors could be utilized to reduce negative affect and related negative cognitions.

This study attempts to determine the effectiveness of using cannabinoids as a treatment for PTSD symptoms by targeting the areas of interaction outlined above. It will utilize the eCB, anandamide which is the intrinsic activator of CB1 and CB2 receptors, to provide insight as to whether or not CB are a viable target for future synthetic pharmacological interventions.

This study will induce PTSD in a rat model so that the symptoms developed will be comparable to those laid out for humans in the DSM 5 but still adhering to reactions that can be observed in nature for the selected animal model as presented by Ford et al. (2015). It will also attempt to observe all changes made to all behaviors related to PTSD in regards to the pharmacological interventions.

This study suggests that the cannabinoid system act in a neuro-modulatory fashion within the fear and stress pathways and that it is the disruption of this system that is at

the center of PTSD symptomology. Furthermore, the application of cannabinoids should restore the cannabinoid control of glucocorticoid, GABA, NE, and 5-HT release which should reduce the clinical presentation of their related symptomologies.

My hypotheses are that (1) rat behaviors, such as anxiety, avoidance, and generalization, will be increased, while spatial memory will decrease due to the application of the stressors. (2) Any behavioral changes due to the trauma will be alleviated by the application of the pharmacological intervention of anandamide. (3) The regimented administration of the pharmacological intervention. (4) There will a downregulation of CB1 and CB2 receptors in the hippocampus and an upregulation of receptors in the basolateral amygdala with the application of anandamide in comparison to the control.

Methods

All procedures were carried out under strict compliance with ethical principles and guidelines of the NIH Guide for the Care and Use of Laboratory Animals. All treatment and testing procedures were approved by the Institutional Animal Care and Use Committee of Regis University in Denver, Colorado.

2.1. Animals

Adult, male Charlie-River rats will be used throughout the study. The animals were housed singly or in pairs in a cage with a width of 25 cm, a length of 45 cm, and a height of 25cm. The conditions in the animal facility will be stabilized at room temperature. The animals were in an environment with a twelve-hour alternating light/dark cycle with the light being initiated at 6:00 and dark at 18:00. All animals had food and water available *ad libitum*. The cage bedding was changed every three days. All subjects were allowed to adjust to their environment for a week before experimentation began.

2.2. Experimental design

This study consisted of four main experiments, three of which assessed the effectiveness of anandamide as a treatment through the observation of differing behavioral symptoms of post-traumatic stress disorder in an animal model. The fourth was a brain imaging study that utilized immunohistochemistry to analyze the differentiation of cannabinoid receptor availability in the basolateral amygdala and hippocampus between the control and experimental groups. The independent variable is treatment levels. The first facet being that the rats were either controls and given a saline injection or in the experimental groups and given anandamide. The second facet is treatment regimen, where one experimental group received a single, initial injection and the other experimental group received weekly injections in addition to the initial injection post stressor. The dependent variables are the subjects' performance on an elevated plus maze, a modified elevated plus maze, a Morris water maze, and qualitative levels of cannabinoid receptors in the hippocampus and amygdala.

2.3 PTSD Model Induction

Due to the increase in funding for PTSD research since the 1990's there has been multitudes of research protocols developed for the production of

PTSD symptoms in animal models (Campos et al., 2013; Corral-Frias et al., 2013). This experiment integrated the most replicable aspects of each model in order to constructively and consistently induce a rat model of PTSD.

PTSD was induced one and a half weeks after the subjects were randomly assigned to either of the two experimental groups or the control group, allowing them to acclimate to the possible stressors of being handled in the animal facility. For the experimental application of stress, the rats were first placed in a bright white chamber and allowed to acclimate to it for 5 minutes. This chamber served as the situational reminder, which has shown to increase the duration of behavioral symptoms of PTSD under experimental conditions without causing extinction of behavior through placing the rat in the operant chamber without shocks (Korem & Akirav, 2014; Louvart et al., 2005; Pynoos et al., 1996). The rat was then placed in a darkened standard operant chamber and allowed to acclimate for thirty seconds. Then an unavoidable foot shock was applied (1.5 mA intensity, 0.5 sec duration) randomly every 30 seconds for a total of 10 minutes in combination with a predatory stressor (Korem & Akirav, 2014). Exposure to a novel object, a yellow soft-flight golf ball, was included during the stressing environment and was later utilized as an aversive stimulus in the modified elevated plus maze.

All subjects will receive treatment of either anandamide (5mg/kg) or an equivalent amount of 9% saline solution via i.p. injections two hours after the initial stressor event. Additionally, one of the experimental groups received weekly injections of anandamide (5mg/kg) starting one day following situational reminder testing.

2.3.1 Situational Reminder

All subjects were placed back in the original bright white chamber, for one minute, 3 and 5 days after the initial stressor which acted as a situational reminder. The rats were not exposed to either the stress chamber or the novel object, soft-flight golf ball, in order to prevent situational extinction of the aversive memory (Korem & Akirav, 2014; Louvart et al., 2005; Pynoos et al., 1996). Their activity within the chamber was observed by the experimenter but not recorded.

2.4. Chemicals

Anandamide is one of two known neurotransmitters that is lipid in nature and therefore cannot be directly injected or directly dissolved in saline

solution. Therefore, the anandamide (5mg/kg) was first emulsified in DMSO (10g/mL) and then mixed with enough 0.9% saline solution, which acted as a vehicle, to achieve $\pm 5\%$ concentrated dose which was given intraperitoneally at a volume of 1.5mL per kg of weight. The control rats were given a similar dose of just the vehicle, 0.9% saline solution, as it has been previously shown that DMSO has no behavioral effects on rats in a PTSD model up to concentrations well above 10% (cite) and it reduces the chance of accidentally using a contaminated chemical. Due to the nature of the synthesis the anandamide was emulsified and placed in the vehicle the day of the initial stressor and was covered and refrigerated in between injections, and allowed to reach room temperature before being injected.

2.5. Behavioral Testing

In order to analyze the behavioral symptoms of general anxiety, avoidance, and generalization the subjects were tested on their performance on the elevated-plus maze and modified elevated plus maze, 7 and 28 days and 14 and 28 days respectively, after the initial stressor event. The subjects' spatial learning performance on the Morris Water maze was tested 10 days following the last situational reminder trial, and a probe trial to examine

spatial memory was conducted 10 days following the last spatial learning trial. All observations and recordings were made by the primary investigator.

2.5.1. Elevated Plus Maze

The Elevated Plus Maze (EPM) (Figure 1) is a common behavioral test utilized to assess the subjects' general anxiety levels following a stressor and in this study subsequent situational reminders (Gobira et al., 2013). The ideology behind the EPM is that it contrasts the subjects' natural exploratory behavior against its desire to protect itself and that the more anxious the subject is, the more it will want to be protected and therefore the more time it will spend within the closed arms of the maze. The EPM consists of four arms with a length of 80 cm and a width of 6 cm that are suspended 60 cm off of the ground. Two of the arms are enclosed on the sides and end, were darkened using black construction paper, and given the designation of closed and the others were not enclosed at all and designated as open. There was also a depressed area of the maze, 6 cm x 6 cm, in the center where all four arms connected which was considered as a neutral space. At the beginning of testing all subjects were placed in the neutral space facing the open arm away from the experimenter and allowed to roam freely throughout the maze and

the time spent in the arms is recorded. The subject was considered to be located on an arm when all four legs had crossed the line that separated the arm from the neutral space. The time that was spent in the neutral space, when all four legs were within the separatory boundaries, was recorded as its own location rather than being in either a closed or open leg. The subjects' anxiety level was then recorded as a ratio determined by the amount of time spent in the closed arms of the maze in relation to the total time spent in the maze.

2.5.2. Modified Elevated Plus Maze

The Modified Elevated Plus Maze (MEPM) is identical to the EPM in all dimensional aspects except that in the corner of one of the closed arms is the yellow soft-flight golf ball that was presented during the stressor period as the paired stimulus, and in the opposite arm is another soft-flight golf ball that is similar in all aspects except that it is orange, as an unpaired stimulus (Figure 2). The object placement This test assesses the subjects' avoidance behavior in regards to an aversive stimuli and to see of the stressor was strong enough to cause the generalizability of aversive stimuli often seen in subjects of classical conditioning. While previous studies (Kniss, 2012) utilized ball burying behavior to assess avoidance behaviors, it has been pointed out

that this behavior has only been intermittently observed under strictly experimental conditions and is not believed to consist of any natural behaviors.

The MEPM on the other hand allows for the subjects' to physically avoid the paired stimulus by escaping to an arm of the maze that does not contain the object. As in the EPM, all subjects were started by being placed in the neutral area of the maze facing the open arm opposite of the experimenter and allowed to roam freely for 5 minutes. However, time spent in the closed arm with the paired object was recorded separately from the time spent in the closed arm with the unpaired object.

The subjects' avoidance behavior will be assessed via an avoidance ratio which will analyze the amount of time that they spend outside of the arm that contains the paired object in regards to the total amount of time that is spent in the maze. Furthermore, if the subjects' attempt to avoid both the paired object and the unpaired object then one may assume that the stressor event lead to the generalization of the paired object, which could be instrumental in understanding the symptomology of PTSD. The subjects' generalization behavior will be assessed via a generalization ratio which will analyze the amount of time spent on either the open arms or neutral space in regards to the total time that is spent in the maze.

2.5.3 Morris Water Maze

Ford et al. (2015) have shown that damage or alterations to the hippocampus caused by stressing events have also been shown to affect scores on spatial learning and memory. The Morris Water Maze (MWM) was used to assess the subjects' spatial learning and memory. The maze is a black circular tub that is 165 cm in diameter and was filled to a depth of 80 cm. Yarn was then suspended above the maze to section it into four separate quadrants which were then arbitrarily given designations that correspond to locations upon a compass for use of reference throughout the entirety of the experiment (Figure 3). Novel pictures of differing shapes and colors were then placed on specific points throughout the experimentation room so that they could possibly be used as location references. Finally, a stand was placed in the East quadrant so that it was submerged about 1 cm beneath the water's surface and concealed by making the water slightly opaque with powdered milk. Testing began by placing the subject in a quadrant about 3cm from and facing the wall and allowing them to freely explore the maze. The time it took the rat to find the platform was recorded, and if they could not find it within a minute they were gently guided to the platform and allowed to stand on it for

ten seconds. All subjects were started in each of the four quadrants per day, starting in the West and rotating clockwise, and were tested over four days resulting in a total of 16 trials per rat. 10 days following the last acquisition trial, a probe trial was run to assess the subjects' spatial memory. Like the learning trials the subjects were started 3cm from and facing the outer wall, however they were only tested from the West quadrant and the platform had been removed. The amount of time each rat had spent in the target quadrant, which was determined by the whole body, excluding the tail, crossing the dividing lines, was then recorded.

2.6. Immunohistochemistry

After experimentation was completed all subjects were euthanized via i.p. injection of euthasol (150 mg/kg) and their brains allowed to fix in 9% para-formaldehyde for 24 hours. The brains were then transferred into a 25% sucrose solution in PBS. The brains were then placed in a cryostat and sliced 30 microns thick and then placed and allowed to freely float in 2 mls wells. For labeling of CB receptors slices were first rinsed with 0.1 M PBS and later in combination with 0.2% Triton-X, between which blocks for endogenous peroxidases and non-specific blocks were applied. They were then placed into a solution containing 2% NGS, and the primary antibodies (Millipore, 209550-100U1 Anti-Cannabinoid Receptor CB1 (1-77), Lot D00175686) at a 1:1000

dilution and then allowed to incubate overnight at 4°C. After incubation and rinse, the slices were incubated in the secondary antibodies (Vector, BS 1000 Biotinylated Goat Anti-Rabbit, Lot C 12 03) at a dilution of 1:200. They were then immersed in ABC solution (ABC Elite Kit, Vector, PK-6100) for amplification and then rinsed. Finally, they were placed in DAB (DAB Peroxidase Substrate Kit, Vector, SK-4100) until visibly stained. Once rinsed slices were then placed on slides and allowed to dry before characterization.

Results

All data were evaluated for skewness and kurtosis to identify whether or not the data were normalized, which is an intrinsic assumption made by the SPSS software. If the data were not normal box plots were used to identify outliers for removal, after which skewness and kurtosis were ran again until population was normalized to ensure more accurate interpretation of the data.

3.1. Elevated Plus Maze

The elevated plus maze assessed the degree of anxiety exhibited by the subjects' post-stressor. The anxiety ratios were analyzed for both the effects of treatment, treatment or no treatment and treatment regimen, and time.

Using a repeated measures generalized linear model, this study found no main effects of treatment or time on the anxiety ratios. The mean anxiety ratios at 7 days post-stressor were, $.407 \pm .322$ for the control, $.744 \pm .116$ for the initial injection group, and $.692 \pm .133$ for the multiple injection group (Figure 4). The mean anxiety ratios at 28 days post-stressor were, $.683 \pm .421$ for the control group, $.690 \pm .373$ for the initial injection group, and $.772 \pm .233$ for the multiple injection group (Figure 4). The main effect of time was not significant on the anxiety ratios recorded, $F(1,20) = 1.888$, $p = .185$. The main

effect of treatment was not significant, $F(2,20) = 1.505, p=.246$. There was no significant interaction between time and treatment on anxiety, $F(2,20) = 1.493, p = .249$.

3.2. Modified Elevated Plus Maze

The modified elevated plus maze assessed the avoidance and generalization behaviors exhibited by the subjects' post-stressor. The avoidance and generalization ratios were analyzed for both the effects of treatment, no treatment or treatment and treatment regimen, and time. Using a repeated measures general linear model, this study found marginal main effects of treatment and time on avoidance ratios, but no significant interaction between the two.

The mean avoidance ratios at 14 days post-stressor were, $.900 \pm .10494$ for the controls, $.758 \pm .180$ for the initial injection group, and $.752 \pm .099$ for the multiple injection group (Figure 5). The mean avoidance ratios at 28 days post-stressor were, $.767 \pm .309$ for the control group, $.728 \pm .270$ for the initial injection group, and $.495 \pm .282$ for the multiple injection group (Figure 5). There was a marginal main effect of time on avoidance ratios, $F(1,19) = 3.720, p = .069$. A post hoc Bonferroni analysis showed that avoidance ratios were .140 lower at 28 days post-stressor than they were at 14 days. There was also a marginal main effect of treatment on avoidance ratios, $F(2,19) = 3.010, p = .073$.

There was, however, not a significant interaction between time and treatment on avoidance ratios, $F(2,19) = 1.160, p = .335$.

Using a repeated measures generalized linear model this study found a marginal main effect of time, but no main effect of treatment on generalizing ratios. The mean generalization ratios at 14 days post-stressor were, $.370 \pm .432$ for controls, $.415 \pm .337$ for the initial injection group, and $.1877 \pm .091$ for the multiple injection group (Figure 6). The mean generalization ratios at 28 days post-stressor were, $.2134 \pm .180$ for controls, $.308 \pm .341$ for the initial injection group, and $.130 \pm .103$ for the multiple injection group. There was a marginal main effect of time on generalizing ratios, $F(1,18) = 4.201, p = .055$. A post hoc Bonferroni analysis showed that the generalization ratios at 28 days post stressor were .107 lower than those 14 days. There was no main effect of treatment on generalizing ratios, $F(2,22) = 1.285, p = .301$. There was also no significant effect of the interaction between treatment and time on the ratios, $F(2,18) = .254, p = .778$.

3.3. Morris Water Maze

The Morris water maze consisted of two separate testing periods, the first of which assessed the subjects' spatial learning, and the second of which assessed their spatial memory. Acquisition training scores were analyzed for

effects of both time and treatment, and the probe trial was analyzed for effects of treatment.

Using a repeated measures generalized linear model, this study found a significant main effect of time but did not find a main effect of treatment on spatial learning. There was a significant main effect of time on spatial learning trials $F(15,330) = 10.649, p < .001$. After establishing significance, a post-hoc Bonferroni test was applied which showed that all subjects took significantly longer to find the platform on trial 1, 20.597 sec, then all other tests except for trial 2, $p < .010$ and $p = .582$, respectively. It also showed that the subjects took significantly longer times on trial 2 than on trials 8, 11, 15, and 16, $p < 0.028$. There were no significant differences between all other trials, $p = 1.000$. There was no main effect of treatment on acquisition training $F(2,22) = 1.000, p = .384$. There was no effect of the interaction of between treatment and time on the acquisition trials $F(2,22) = 1.549, p = .257$.

Using a one-way ANOVA, this study found a significant main effect of treatment on spatial memory. The mean times that the subjects spent in the target quadrant were, 20.48 ± 4.23 for controls, 23.51 ± 2.71 for the initial injection groups, and 29.93 ± 7.42 for the multiple injection group (Figure 7). There was a significant main effect of treatment on the probe trial, $F(2,22) = 6.291, p = .007$. A post-hoc Bonferroni test showed that the multiple injection

group stayed in the target quadrant significantly longer than both the control, $p=.012$, and initial injection groups, $p=.041$, 9.45sec and 3.03sec respectively.

There was not a significant difference between the control and initial injection groups, $p=.940$.

Discussion

4.1. Elevated Plus Maze

The main results of the Elevated Plus Maze suggest that the administration of cannabinoids do not reduce the general anxiety levels of rats who have been exposed to stressors. Furthermore, results show that time also does not produce any significant change in anxiety levels.

While these results seem to conflict with some of the results of current literature that show that cannabinoids should be anxiolytic the discrepancy between them could be explained in two different ways. The first being that the waiting period between the trauma and the initial injection was not long enough and accidentally ended up producing the anxiogenic effects that were seen in Fogaça et al., (2014). Since none of the other behavioral tests seemed to be affected by this reversal of expectations, it might be possible that the cannabinoid modulation of the NE receptors in the mPFC is through a novel mechanism and not the proposed regulation of glucocorticoids. This undefined mechanism might be related to uncharacterized cannabinoid receptors in the brain that have higher affinity binding for synthetic cannabinoids like CBD which are not direct CB1 agonists.

The second explanation correlates to the structures involved in the activation of the SNS. While studies have shown that cannabinoids have an

anxiolytic effect, the majority of them have centered on eCB activity in the mPFC and not ones in the locus coeruleus. It is possible that the anandamide was not able to fully activate the GABA inhibition of the amygdala, which would have led to an increase in arousal even if anandamide was modulating the transmission of NE from the mPFC.

Experimentally testing these two theories would actually be quite simple. The first experiment would consist of varying the time of the initial injection from beginning just after the exposure to a stressor to more than 24 hrs following it. This would allow one to see if the presentation of anxiety is dependent upon the time of injection. For the second experiment one would have to inhibit the locus coeruleus while applying the cannabinoids and if there is a anxiolytic effect than you could assume that it was due to activation in the mPFC and if not you could say that it might not be the activation of CB1 receptors that result in the modulation of anxiety, but rather some other cannabinoid receptor.

4.2. Modified Elevated Plus Maze

The main findings of the Modified Elevated Plus Maze are that time has marginal main effects on avoidance and generalization behavior whereas

cannabinoids only have a marginal main effect on avoidance behaviors. This suggests that avoidance and generalization behavior might have a cognitive component to it that allows for the behaviors to be reduced over time.

However, it also shows that the injection of cannabinoids does facilitate in the reduction of avoidance behavior.

The reduction in the avoidance behavior can be due to three different mechanisms (Neumeister et al., 2015). The first is that the cannabinoids act directly upon the BLA, reducing the levels of GC's facilitating in the complete extinction of the traumatic memory. Secondly, the cannabinoid could be activating the GABAminergic neurons in the brain bolstering the suppression of BLA activity resulting in the conscious differentiation between the stimuli. Lastly, the cannabinoid could be acting upon the hippocampus and through GCs control, allowing for the correct contextual information about the traumatic event to be projected to the mPFC.

Since the injection of anandamide did not have a significant effect upon the generalization behavior of the subjects one could assume that they are not acting within the mPFC to facilitate conscious differentiation. However, if one wanted to pinpoint the precise area of action they would most likely need to utilize micro dialysis to detect which cortical area has lower levels of GCs or through the cortical injection of synthetic cannabinoids.

4.3. Morris Water Maze

The main findings of the Morris Water Maze are that cannabinoids increase spatial memory as evidenced by increased time in the target quadrant. This finding is substantial because atrophy and dysfunction in the hippocampus is a hallmark of PTSD and if cannabinoids are able to have any preventative measures against that, they could be considered as good candidates for clinical use. This candidacy is bolstered by the data that shows that cannabinoids do not effect spatial learning, a negative side effect that is flaunted by public media and had it been proven could have dangerous consequences (Das et al., 2013; Goodman & Packard, 2015; Varvel& Lichtman, 2002). Furthermore, since the increase in spatial memory is fairly conclusive of eCB activity within the hippocampus, there is more evidence that the marginal significance that was found in the MEPM related to avoidance behavior might be due to increase contextual information being able to be projected from the hippocampus.

Conclusion

Overall, the data significantly or marginally supports the hypothesis that exposure to stressors cause worsening PTSD like symptoms in animal models, and that the systemic application of cannabinoids helps to reduce those symptoms. It does not support the idea that any level of cannabinoid would help in alleviating the symptoms of PTSD as in many of the tests there was no difference between the control and initial injection groups. So, taking it at face value the data is evidence in support of the idea that the dysregulation of the cannabinoid system might be at the center of PTSD symptomology, and that the administration of cannabinoids might be the answer to providing full clinical remission to all of its sufferers.

However, interesting questions are not the ones that the data answers but the ones they ask. While this study has shown that cannabinoids do work at alleviating PTSD the focus should now be shifted onto how/why they work. This study has laid the groundwork, the big picture if you will, for going back and looking at sections of the interaction between cannabinoids and the fear circuit piece by piece in order to more clearly define the pathways in which cannabinoids could be used to reduce PTSD symptomology or even lead to its prevention.

Going forward, research should include video analysis of all behavioral testing for greater accuracy to prevent larger than desired

confidence intervals and prevent researcher fatigue. Future research should also add a behavioral test for depression so that negative mood and cognition may be observed in conjunction with the other symptoms.

In conclusion, while this study barely scratched the surface of what could be the cannabinoid theory of PTSD, it provides some evidence that cannabinoids could be a viable candidate for clinical purposes and contributes to the ever expanding knowledge of the neuroscience and PTSD research fields.

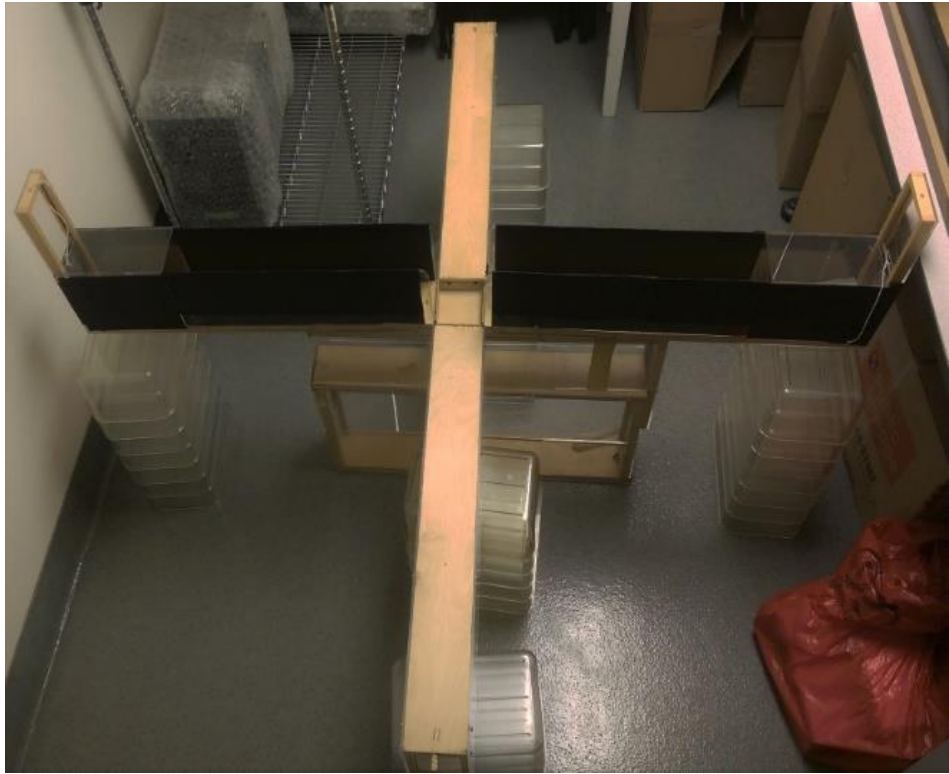


Figure 1.-Aerial view of the Elevated Plus Maze. Note that the apparent curvature is due to the construction paper and not an altered flooring angle

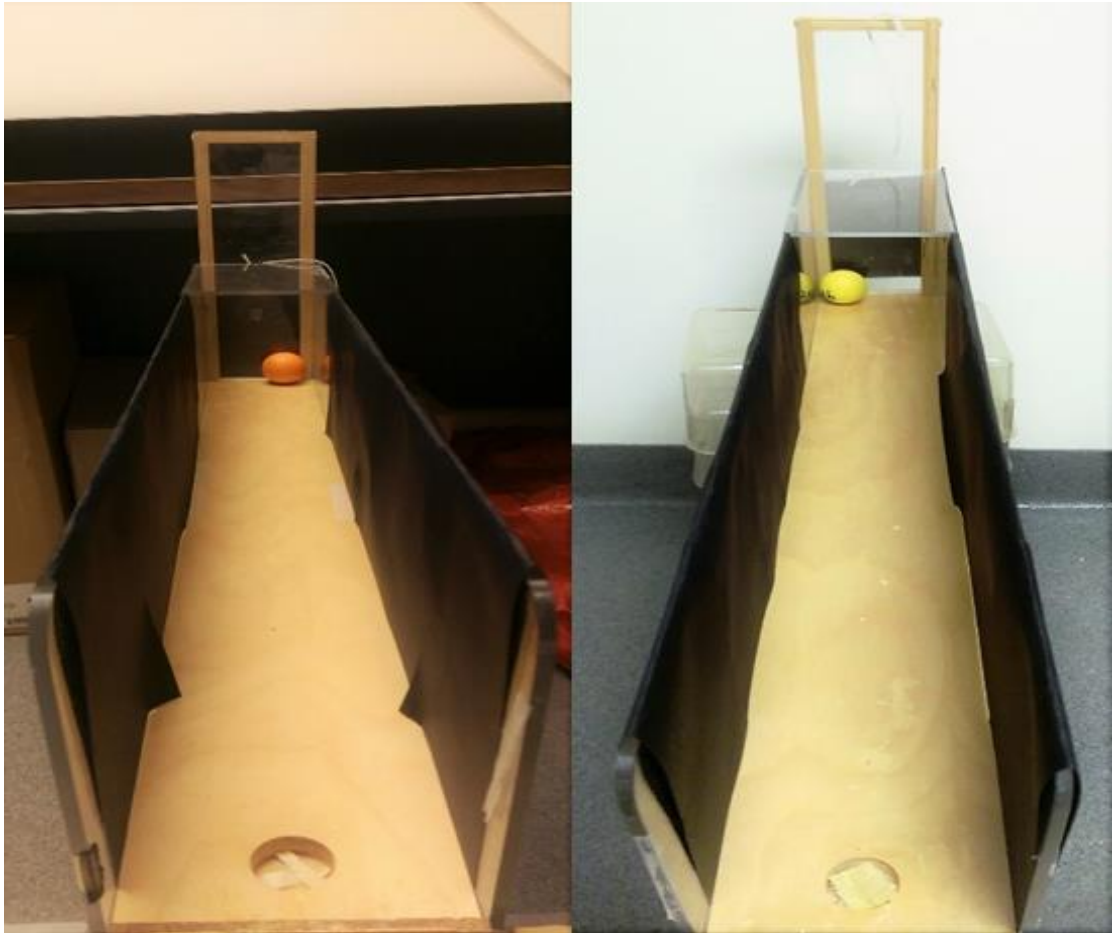


Figure 2.– Detailed images of an example orientation and location of stimuli in the Modified Elevated Plus Maze



Figure 3.– Image of the Morris Water Maze with gridlines and subject for reference

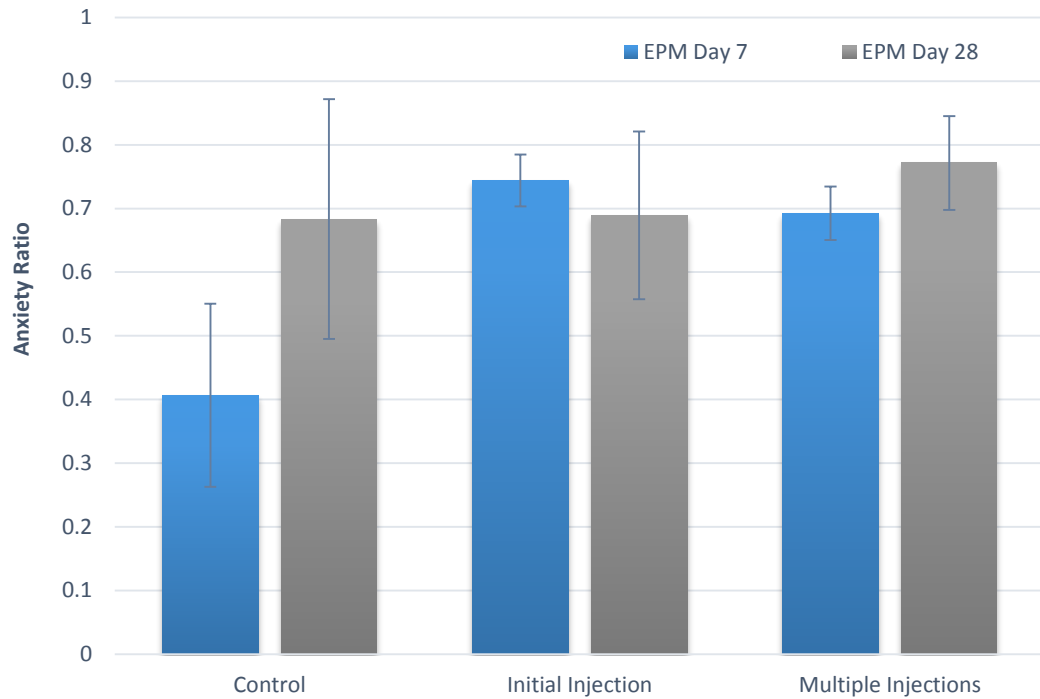


Figure 4. - This figure illustrates the mean anxiety ratios for all treatment levels on the Elevated Plus Maze at 7 and 28 days post-stressor. There is no significant main effect of treatment level, $F(2,20) = 1.505, p = .246$, or time $F(1,20) = 1.888, p = .185$.

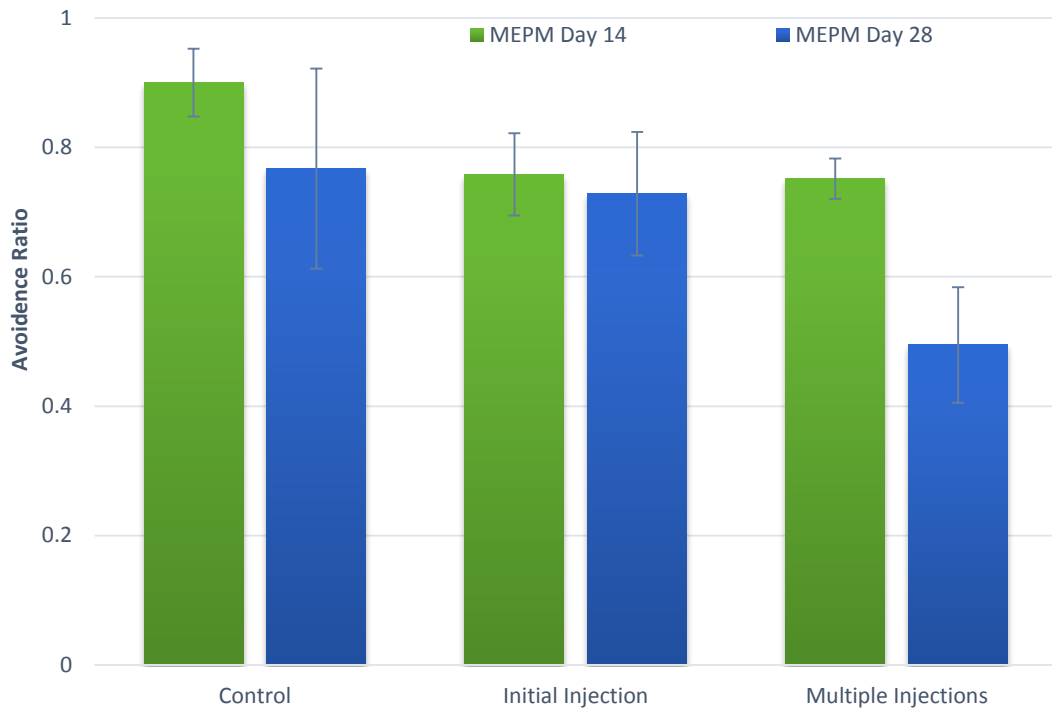


Figure 5. This figure illustrates the mean avoidance ratios for all treatment conditions at 14 and 28 days post-stressor. There was a marginal main effect of treatment, $F(2,19) = 3.010, p = .071, \eta^2 = .241$. There was also a marginal main effect of time, $F(1,19) = 3.720, p = .069, \eta^2 = .164$.

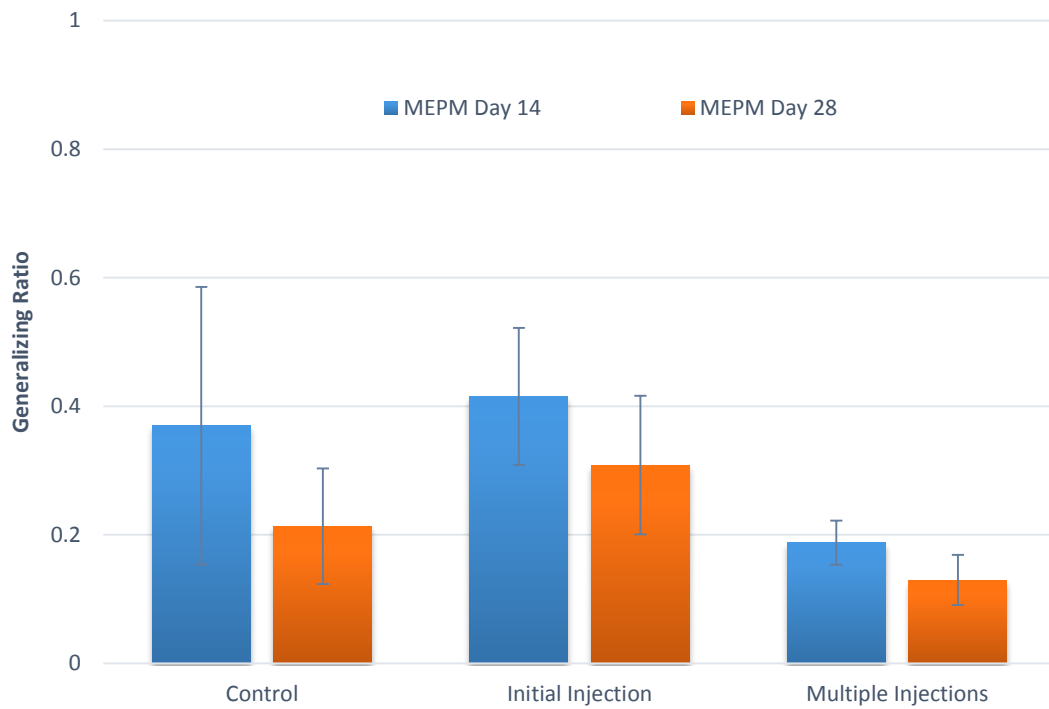


Figure 6.- This figure illustrates the mean generalizing ratios for all treatment condition on the Modified Elevated Plus Maze at 14 and 28 days post-stressor. This study found a marginally significant main effect of time, $F(1,18) = 4.201, p = .055$. This study found no main effect of treatment, $F(1,285) = 1.285, p = .301$

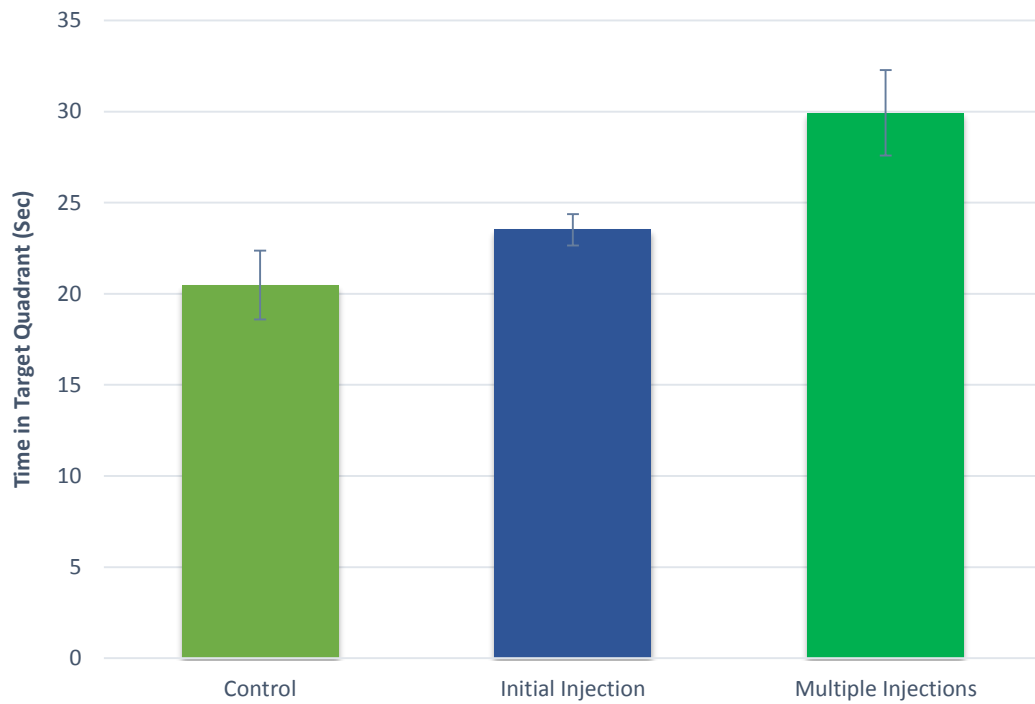


Figure 7.- This figure illustrates the mean time that the subjects in each of the treatment groups spend in the target quadrant of the Morris Water Maze during the probe trial. This study found a significant main effect of treatment, $F(2,22) = 6.291$, $p = .007$

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