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THE ANALYSIS OF BEHAVIORAL AND NEUROBIOLOGICAL MARKERS FOLLOWING ADOLESCENT CANNABINOID EXPOSURE

by

Gabriel D. Medley

A thesis submitted to the faculty of Radford University

in partial fulfillment of the requirements for the degree of

Masters of Arts in the Department of Psychology

November 2019

Dayna M Hayes — 12/11/19

Dr. Dayna Hayes, Thesis Advisor — Date

Pamela A Jackson — 12/9/19

Dr. Pamela Jackson, Committee Member — Date

Thomas W. Pierce — 12/11/19

Dr. Thomas Pierce, Committee Member — Date

ABSTRACT

Previous literature has found memory deficits in rats exposed to a synthetic cannabinoid (Robinson, Goonawardena, Pertwee, Hampson, & Riedel, 2007). Adolescent animals tend to suffer the most detrimental effects from consistent exposure to synthetic cannabinoids (O'Shea, Singh, McGregor, & Mallet, 2004). While the conclusion has been drawn that the memory deficits are due to the drug, lack of proper nutrition may be another explanation. Animals exposed to synthetic cannabinoids tend to show a decrease in weight gain when compared to untreated control animals (O'Shea et al., 2004). Another unintended consequence of drug use in general is a decrease in neurogenesis in the hippocampal brain region (Wolf et al., 2010). Research on exposure to synthetic cannabinoids, specifically, has resulted in conflicting data in regards to hippocampal cell proliferation (Abboussi, Tazi, Paizanis, & Ganouni, 2014). The current study investigated the effects of exposure to a synthetic cannabinoid, CP 55, 940, on cell proliferation in the dentate gyrus of the hippocampus and memory deficits on adolescent aged male Long-Evans rats. In order to address the concern of lack of weight gain in drug exposed animals, this study supplemented the rats with Ensure during the injection period. Conditions consisted of a group of free fed untreated control animals with no access to supplement, a drug exposed group with a large Ensure supplement aimed to keep their rate of weight gain similar to the untreated animals, and a group yoked to the drug group in terms of food and supplement while receiving a vehicle injection. The results of this study indicated there were no differences among weight in the conditions during the injection period. Neither object recognition memory of spatial memory was affected in adulthood by condition. Although there was not a main effect of condition on neurogenesis, further analyses revealed that the untreated condition had increased cell proliferation when compared to the yoked condition. The results of the current

study suggested that nutrition may be a key component in some of the deficits observed following synthetic cannabinoid administration rather than the drug alone. Further cannabinoid studies should evaluate the effects of different diets during drug administration and their effects on cell proliferation and memory in both male and female animals.

Gabriel D. Medley, M.A.
Department of Psychology, 2019
Radford University

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CHAPTER 1: INTRODUCTION

In the United States, the use of illicit drugs by individuals aged 12 or older has increased from an estimated 8.3% in 2002 to 9.4% in 2013 (National Institute of Drug Abuse [NIDA], 2015). Marijuana use in particular has increased from 5.8% in 2007 to 7.5% in 2013, while most other drug use has stabilized, or even decreased, over the past decade (NIDA, 2015). Marijuana is often referred to as a "gateway drug," which means that although the drug itself may not be addictive, it is thought to increase the likelihood of use of other illicit substances (Lynskey & Agrawal, 2018).

Marijuana refers to the leaves or flowers from the *Cannabis sativa*, *Cannabis indica*, and *Cannabis ruderalis* plants (Gloss, 2015). Marijuana is composed of psychoactive compounds, such as the main ingredient, delta-9 tetrahydro-cannabinol (THC). Marijuana also contains other active compounds that are not mind-altering, such as cannabidiol (CBD) (Center for Disease Control and Prevention [CDC], 2018). While marijuana was previously viewed as the least potent of the cannabis products, the potency has risen in the past two decades (Alcohol and Drug Abuse Institute [ADAI], 2013; Sevigny, Pacula, & Heaton, 2014). One potential reason for the increase in potency may be the legalization of dispensaries in some states (Sevigny et al., 2014). When marijuana is supplied under a state-sanctioned regime, the product tends to have a higher potency and be of higher quality. This may be due to many factors including, but not limited to, an increase in quality control, efficiency gains in production, and reduced enforcement risks (Sevigny et al., 2014). Although marijuana is legal in some states for recreational and/or medicinal use, it is still illegal at the federal level because it is a schedule I drug.

Marijuana is typically inhaled or ingested, and when inhaled, the effects of marijuana are noticed almost immediately (ADAI, 2013). This is due to THC and other chemicals from the

plant passing from the lungs into the bloodstream, which then quickly distributes the chemicals to the brain (NIDA, 2018). When marijuana is consumed in food or beverages, the effects are delayed and usually noticed between 30 minutes and an hour after administration. Ingestion requires the substance to first pass through the gastrointestinal system, resulting in the delayed effects.

Immediate effects of marijuana can include a pleasant euphoria, relaxation, laughter, drowsiness, decreased nausea, dryness of eyes, throat, or mouth, bloodshot eyes, elevated heart rate, talkativeness, altered perception of time, heightened sensory perceptions, difficulty thinking or problem solving, impaired memory or body movement, or increased appetite (ADAI, 2013; Borràs, Modamio, Lastra, & Mariña, 2011; Lee, Cadigan, & Patrick, 2017; NIDA 2018). Marijuana is sometimes used medicinally to increase appetite, decrease inflammation, alleviate pain, and reduce nausea and vomiting (Bakshi & Barrett, 2018). Haney et al. (2007) discovered that smoking marijuana increased caloric intake, weight gain, and sleep duration in people who were HIV-positive, with no signs of discomfort or impaired cognitive ability. Normally, negative experiences when using the drug are not severe, but using in high doses may result in the individual experiencing anxiety, fear, distrust, panic, hallucinations, delusions, or loss of identity (Hunault et al., 2014; NIDA, 2018). While the effects mentioned thus far are usually temporary, marijuana use may also have long-term consequences.

While some of the immediate effects of marijuana are often viewed as congenial and promote the use of the drug, the drug may also elicit consequences that last well after the “high” is over. One area of functioning that may be affected from consistent marijuana use is cognition. Research suggests that select auditory and visual memory processes are affected negatively in adolescent and adult heavy users and that these consequences may be permanent (Bolla, Brown,

Eldreth, Tate, & Cadet, 2002; Schwartz, Gruenewald, Klitzner, & Fedio, 1989). While most research supports the theory that marijuana use results in cognitive deficits, the domains within cognition that are impaired remain ambiguous. Deficits in attention, information recall, and working memory tend to be consistent in marijuana users. On the other hand, there is speculation regarding the ability of cannabis users to retain information, with research suggesting this is not a problem for users (Bolla et al., 2002; Thames, Arbid, & Sayegh, 2014). Due to performance differences between heavy and light cannabis users on tests of cognition, Bolla et al. (2002) suggested that the effects of marijuana may be dose-dependent, which could explain some of the inconsistencies in the literature. Another explanation for these inconsistencies is that some of these long-term consequences may diminish over time, as evidenced by past users and nonusers performing no differently on tasks of working memory and attention (Thames et al., 2014).

Many studies have also found that marijuana users have decreased executive functioning, IQ, manual dexterity, and information processing speed compared to nonusers, although some of these differences may diminish with abstinence (Bolla et al., 2002; Fried, Watkinson, James, & Gray, 2002; Thames et al., 2014). Utilizing a gambling task, Whitlow et al. (2004) found chronic marijuana users made more decisions that led to greater, more instantaneous gains despite the costly losses that accompanied the choice when compared to a control group. This suggests that long-term, heavy users may be unable to balance rewards and punishments, which could explain the pattern of continued drug use. Information processing speed is also negatively affected in individuals who use marijuana regularly. Pavisian, Staines, and Feinstein (2015) found that participants who used daily were approaching a significantly slower response time on the Symbol Digit Modalities Test when compared to participants who were cannabis naïve. A deficit in IQ may also be present in users who began in adolescence. Meier et al. (2012) found that over

25 years, beginning at age 13, adolescent marijuana users showed a significantly greater decline in IQ over time than adult onset users. Cessation of marijuana use did not restore neuropsychological functioning (i.e., executive functioning and processing speed) in the users who began in adolescence (Meier et al., 2012). This provides evidence that using marijuana in adolescence may have some irreversible consequences. Abstinence from marijuana can also produce a variety of physical and psychological changes in an individual. Haney, Ward, Comer, Foltin, and Fischman (1999) found that abstinence from marijuana use increased ratings of anxiety, irritability, and stomach pain, but decreased food intake when compared to baseline ratings of the individuals. Kouri, Pope, and Lukas (1999) also found an increase in aggressive behavior on days 3 and 7 of marijuana abstinence compared to participants' pre-withdrawal aggression score. The aggressive responses returned to baseline levels after 28 days of abstinence.

Recent research has found that the long-term effects of marijuana may be more prominent depending on the developmental time period in which the drug use took place. Engaging in marijuana use at a young age may be responsible for altering brain development (Thijssen et al., 2017). The brain is believed to be especially sensitive to damage from drug exposure in individuals under the age of 20. The frontal cortex, which is responsible for planning, judgment, decision-making, and personality, is one of the last brain regions to develop fully and adolescent drug use is thought to damage this brain region. The endocannabinoid system, the neurotransmitter system through which marijuana exerts effects, is also underdeveloped in teens. This system is important for cognition, neurodevelopment, stress responses, emotional control, and modulation of other neurotransmitter systems. Consistent

exposure to marijuana can decrease cellular activity of endocannabinoids in this system (Lewis et al., 2012).

Cannabinoids activate the endocannabinoid system. Endogenous cannabinoids, or endocannabinoids (ECBs), originate inside the body and are naturally produced. Many stimuli can induce the synthesis of ECBs, such as neuronal activity, the expression of glucocorticoids, and the presence of insulin and cytokines (Malcher-Lopes & Buzzi, 2009). ECBs are then secreted throughout the brain and peripheral tissues by many cells in the body. ECBs are synthesized from membrane-derived phospholipids and are retrograde messengers, which means ECBs are made in the postsynaptic cell and travel backwards across the synapse (Wilson & Nicoll, 2002). Endogenous cannabinoids interact with cannabinoid receptors to regulate basic functions such as mood, memory, appetite, pain, and sleep (Carr, Jesch, & Brown, 2008). ECBs, including Arachidonoylethanolamide (anandamide [AEA]) and 2-arachidonoylglycerol (2-AG), are only produced when the body signals that they are needed and are instantly degraded after acting on nearby receptors (Woods, n.d.). AEA and 2-AG are two well-studied ECBs, but there is also evidence of other ECBs (e.g., N-arachidonoyl dopamine, noladin ether). However, the functions and mechanisms of action of the more newly identified ECBs are not fully understood (Carr et al., 2008; Petrocellis & Di Marzo, 2009). Recently, two omega-3 fatty acid ethanolamides, docosahexaenoyl ethanolamide and eicosapentaenoyl ethanolamide, were found to have substantial potency in activating cannabinoid receptors, indicating that these ligands have a role in the ECS (Pastor, Farré, Fitó, Fernandez-Aranda, & Torre, 2014).

Within the endocannabinoid system, there are two known cannabinoid receptors: CB1 and CB2. CB1 was the first identified cannabinoid receptor (Carr et al., 2008). It is a G_i protein-coupled receptor with 473 amino acids (Gui, Tong, Qu, Mao, & Dai, 2015). The inhibitory effect

of CB1 varies depending on the specific cannabinoid compound, and CB1 is more responsive to psychoactive cannabinoids than nonpsychoactive cannabinoids. Due to this, CB1 is expected to be the molecular target through which marijuana produces its effects, although marijuana is capable of binding to both receptors (Suigiura, Kishimoto, Oka, & Gokoh, 2006). This was further supported by evidence suggesting that the CB1 receptor exists predominantly in the central nervous system and is correlated with the motor and rewards systems. A high density of CB1 receptors was found in multiple regions of the brain, such as the cerebral cortex, hippocampus, basal ganglia, and cerebellum (Carr et al., 2008). Compounds that bind and activate CB1 receptors are involved in the regulation of appetite, mood, and anxiety (Carr et al., 2008). CB2 is also a G_i-protein coupled receptor and is mainly found in the peripheral immune system under normal conditions. CB2-preferring agonists and antagonists are not associated with psychoactive effects like the CB1 receptor (Carr et al., 2008). 2-AG and AEA are capable of binding to CB1 and CB2 receptors, but the likelihood of them binding and activating the receptors differ (Crozier & Hurst, 2014). 2-AG is a full agonist of the CB2 receptor while AEA is a partial agonist (Carr et al., 2008; Suigiura et al., 2006). While there is emerging evidence of other cannabinoid receptors, CB1 and CB2 are currently the only cannabinoid receptors that mediate cannabinoids.

The components of marijuana are exogenous cannabinoids and act on the ECS in a similar manner as the endogenous cannabinoids. One difference is exogenous cannabinoids antagonize endogenous cannabinoid signaling and may desensitize cannabinoid responses following prolonged exposure (Mackie, 2008). Exogenous cannabinoids interact with cannabinoid receptors to produce the physical and psychological effects in the body that result from marijuana use. Though legal in some states, research regarding the direct effects of

marijuana use on humans is scarce due to the federal Schedule 1 classification of the drug. Therefore, until very recently, researchers have turned to synthetic drugs, which allows for the determination of causality in some instances. In order to mimic the effects of marijuana, animal research often utilizes synthetic cannabinoids, such as CP 55, 940 or WIN 55, 212-2, which act on many of the same receptors as marijuana. Specifically, CP 55, 940 was shown to affect binding in the brain in a similar manner as rats administered THC (Oviedo, Glowa, & Herkenham, 1993). At the striatal level, Oviedo et al. (1993) found chronic exposure to CP 55, 940 and THC resulted in downregulation of cannabinoid receptors, which led to a decrease in sensitivity to the drugs. Because researchers are not required to have a Schedule 1 license to possess CP 55, 940, this synthetic cannabinoid may be an appropriate alternative to THC when studying the effects of marijuana.

Both natural and synthetic cannabinoids can be administered in many different ways. In regards to animal research, common administration techniques include injecting the animal intravenously or intraperitoneally, utilizing a vapor chamber, inserting an osmotic minipump, or allowing the animal to orally consume the drug (Guhring et al., 2001; Kirschmann, Pollock, Nagarajan, & Torregrossa, 2017; Marchalant, Cerbai, Brothers, & Wenk, 2008; O'Shea et al., 2004). Administration of the drug can occur at different developmental stages of an animal's life and often yields differing results. For example, when comparing the outcome of adolescent versus adult synthetic cannabinoid administration in rat models, adolescents exposed to the drug normally experience more detrimental and longer lasting consequences than adult rats, such as impaired memory and increased anxiety (O'Shea et al., 2004; Quinn et al., 2008).

Drug administration is often paired with behavioral testing to examine the drug effects in animals. These investigations can occur at any stage of development and often involve

assessments of emotionality (e.g., anxiety, sociability) and memory. Decades of research have validated the use of specific tasks to identify emotionality, in addition to memory. Memory, both short and long term, is often one area that is diminished following consistent drug use. Memory is a complex construct and is assessed via many different behavioral tasks. Some of the well-known spatial tasks, which require a functional hippocampal region, include object location recognition and object recognition. In object location recognition, the animal is evaluated to determine if it is aware an object has switched to a new location. Object recognition entails observing whether the animals are aware of a novel object on the maze (O'Shea et al., 2004; Quinn et al., 2008). Memory deficits after marijuana use are subject to varying outcomes. O'Shea et al. (2004) found that exposure to CP 55, 940 led to a long-lasting deficiency of working memory in adolescent, but not adult, rats. Robinson et al. (2007) found deficits in spatial learning when utilizing a reference memory task. On the other hand, studies show rats that were trained on a memory task before receiving the drug did not differ from vehicle animals in regards to spatial memory (Robinson et al., 2007). Deficits in memory are not always the result of marijuana use. Pamplona, Prediger, Pandolfo, and Takahashi (2006) found rats exposed to WIN 55, 212-2 performed significantly better than control animals on the water maze reversal task, which is indicative of proficient spatial memory. Research has also found that cannabinoids administered to rats in late adolescence may reverse memory deficits in adulthood that resulted from early life stress (Alteba, Korem, & Akirav, 2016).

Since memory tasks are hippocampal dependent, it is necessary to investigate the integrity of the hippocampal structure and its resultant function. The hippocampus is one of the two regions of the brain where neurogenesis occurs throughout the lifespan (Gonçalves, Schafer, & Gage, 2016). Neurogenesis in the hippocampus is involved in neuronal plasticity, specifically

in regards to the formation of new memories and learning (Koehl & Abrous, 2011). Neurogenesis consists of the birth of new and functional neurons and this process is affected by drug use. In mammals, this occurs in two areas of the central nervous system: the subventricular zone and the subgranular zone of the dentate gyrus in the hippocampus (Singh, Mishra, Srivastava, & Shukla, 2017). Neurogenesis is a multistage process including proliferation, migration, differentiation, and integration of mature neurons into the pre-existing circuitry (Galvan & Jin, 2007). The first stage of neurogenesis is proliferation and consists of the production of new cells. Migration follows this step and involves these new cells traveling to specific areas in the brain (Braun & Jessberger, 2013). The third phase of neurogenesis is differentiation, which can be a multistep process producing different cell types, such as neurons, astrocytes, and oligodendrocytes (Braun & Jessberger, 2013; Gage, 2000). Functional neurons that undergo these phases will then be integrated into a pre-existing circuit. In young adult rats, there are approximately 9,000 new cells generated each day and a majority of them differentiate into new neurons, but only about half of these new neurons survive after a few weeks (Cameron & McKay, 2001; Cameron, Woolley, McEwen, & Gould, 1993; Galvan & Jin, 2007; Hastings & Gould, 1999). Neurogenesis can be modulated by many different factors including, but not limited to, neurotransmitters, transcription factors, stress, learning, neurodegenerative disorders, and/or an enriched environment (Singh et al., 2017).

Few studies have observed the effect of THC and synthetic cannabinoids on neuronal cell proliferation in animals, with the available data displaying conflicting results. Following cannabinoid exposure, cell proliferation is generally altered within the hippocampal region of the brain. However, whether increasing or decreasing, this stage of neurogenesis seems to depend upon the specific cannabinoid administered, the dose, and the method and duration of

administration (Prenderville, Kelly, & Downer, 2015). In regards to dosage, acute administration of synthetic cannabinoids has been shown to have no effect on cell proliferation, while chronic administration has different outcomes. Jiang et al. (2005) found that the synthetic cannabinoid HU201 promoted neurogenesis in adult rats in the dentate gyrus of the hippocampus, which may have led to anxiolytic and antidepressant-like effects. To the contrary, Abboussi et al. (2014) found that adolescent rats exposed to a different synthetic cannabinoid, WIN55, 212-2, had cognitive deficits, which were positively correlated with a decrease in the number of newly generated neurons in the dorsal hippocampus when observed during the adolescence period. CBD and THC administered to female mice in food reduced precursor cell proliferation, but CBD increased cell survival while THC did not (Wolf et al., 2010). A possible variable to consider when viewing the results of Wolf et al. (2010) may be the route of administration utilized. Consumption of marijuana may produce different outcomes than inhalation or injections, which both result in similar substance absorption (Turner, Brabb, Pekow, & Vasbinder, 2011). An important caveat about the data is that research has demonstrated a self-imposed calorie restriction when animals and humans are exposed to the drug for a consistent time period (O'Shea et al., 2004). Hemb, Cammarota, and Nunes (2010) also found that in rats, early malnutrition decreases body weight and brain weight. Spatial memory is also negatively affected following exposure to early malnutrition (Hemb et al., 2010). This leads some researchers to believe that malnutrition, and not the drug alone, may be a possible explanation for the memory deficits observed. Although research has found that a high fat diet impairs neurogenesis, this concept has not been explored with animals treated with synthetic cannabinoids to eliminate lack of weight gain (Lindqvist et al., 2006). Diet and nutritional factors affect neurogenesis and the current study aimed to evaluate the effects of supplementation to

maintain an animal's weight gain during synthetic cannabinoid exposure. The effect of cannabinoids on cell proliferation is a fairly new field of research and the outcome is still inconclusive and based on a multitude of factors.

The current study involved injecting adolescent-aged rats (beginning on Post Natal Day [PND] 35) intraperitoneally for a 2-week period while receiving supplementation. Behavioral testing began on PND 78, in order to observe whether there are long-term effects of using the drug during adolescence and whether these effects are still present with supplementation aimed to eliminate nutritional deficits of drug exposed animals. This study employed object location recognition and object recognition tasks in a small open-field apparatus that assessed memory. Brain tissue was collected from all animals following behavioral testing and was sectioned, stained, and quantified to investigate the neurogenic indices of the groups. It was hypothesized that adolescent male rats exposed to the synthetic cannabinoid, CP 55, 940, would display an increase in neurogenesis and perform similarly on the memory tasks when compared to controls in adulthood.

CHAPTER 2: METHODS

Subjects

Thirty male Long-Evans rats were selected for the study. Long-Evans rats were bred in-house from animals purchased from Charles River Laboratory (Raleigh, NC). Litters were culled to 12 animals within 48 hours of birth, and weaning occurred on postnatal day (PND) 22. At weaning, animals were separated by sex. All rats were maintained on a 12-hour light:dark cycle in a temperature and humidity controlled environment. Drug administration and behavioral testing were conducted during the light phase of the cycle. Animals were provided unlimited access to water and food, except during the 2-week injection period where rats in some conditions received restricted access to food and/or a 50% solution of Vanilla Ensure™ (Abbott Laboratories). This study was approved by the Institutional Animal Care and Use Committee at Radford University, and all procedures were consistent with regulations established by the NIH Guide for the Care and Use of Laboratory Animals.

Drug Manipulation

For the first two cohorts, adolescent rats were semi-randomly assigned to a condition. Beginning with the third cohort, and every other cohort thereafter, rats were assigned to a condition by counterbalancing for weight. Animals were assigned to one of three groups: drug supplement, yoked supplement, and untreated control. During the injection period, PND 35-48, adolescent rats were housed in pairs in plastic barrier tubs with a perforated divider used to separate the two animals. All conditions, except the untreated group, received daily injections intraperitoneally at approximately the same time during the 2-week injection period. Animals were injected with a vehicle solution or the synthetic cannabinoid full agonist, CP 55, 940, at 0.35 mg/kg, which was used to model chronic cannabinoid exposure during adolescence. The

drug solution was made by mixing 3.5 mg of CP 55, 940 and 75.0 μl of Tween 80 (polyoxyethylene sorbitan monoleate), and 0.3 mL of ethanol. After thorough mixing, the ethanol was evaporated using compressed oxygen and the rest of the solution was mixed with 9.925 mL of saline (O'Shea et al., 2004). All animals had unlimited access to water. During this period, the untreated animals had unrestricted access to food. The drug supplement animals received unrestricted food access, and researchers manipulated the Ensure supplement in order to attempt to maintain the weight gain of this group similar to the untreated condition. The amount of Ensure was increased by no more than 10 mL daily if the animal was lacking in weight gain compared to the untreated condition. The yoked supplement group received the same amount of food and Ensure that the drug supplement animal consumed in the previous 24 hours. Following the injection period, animals were group housed with their experimental cohort until PND 68, where the animals were then single housed prior to behavioral testing.

Behavioral Testing

All animals were subjected to a battery of behavioral tests beginning on PND 78 with an Elevated Plus Maze. A social interaction task was also employed in this study on PND 84 and consisted of one day of habituation to the apparatus preceding test day. Object location recognition and novel object recognition tasks were the focus of this project due to an interest in the hippocampus brain region.

Object Location Recognition

Behavioral testing for object location recognition began on PND 89±1. Habituation to the apparatus consisted of four, 10-minute trials, occurring consecutively. Data from the first habituation trial was used to obtain information on activity and anxiety levels. On PND 93±1, animals were exposed to two, 5-minute trials (sample and test phase), which were separated by a

10-minute inter-trial interval (ITI). In trial 1, the sample phase, two identical objects were placed in different corners of the open-field apparatus. The second trial, the test phase, involved the movement of one of the objects to a different location. Contact with the object was coded and defined as the animal being approximately one inch from the object. Object layout was counterbalanced across conditions. For all trials, animals were placed in the center of the apparatus facing the wall opposite the objects.

Apparatus. The open-field arena was located in a small room that contained one table, one door, two bookshelves, two cameras hanging from the ceiling, a sink, a large open-field apparatus placed against one wall for storage purposes, and four posters on two of the walls. The open-field arena was made of wood and painted white (61 cm x 61 cm x 36 cm). Objects utilized in the object location recognition task were two clear cylinder jars (8 cm x 7 cm) with multicolored marbles inside in order to prevent the animal from knocking the object over. Each object was attached to the floor of the open field via Velcro. Each session was recorded via video camera, and saved on a DVR. AnyMaze tracking software was utilized to track the movement of the rats within the open field and in relation to the objects.

Procedure. All animals were weighed before habituation and testing and placed in a plastic holding cage for transfer. The apparatus and objects were cleaned with a 10% vinegar solution before and after each trial. White noise was utilized for all behavioral tasks. Before testing, animals had four consecutive days of habituation to the apparatus that consisted of 10-minute trials. During the habituation period, animals had a 5-minute acclimation period before being placed in the open field to explore. Placement of the animals varied each day of habituation, with the animal facing a different cardinal direction for each of the four days. Following the completion of a habituation trial, animals were returned to their cage in the colony

room. On testing day, subjects were brought to the room containing the apparatus in pairs. After a 5-minute acclimation period, one animal was placed in the open field containing the objects for 5 minutes. Following this animal's first trial, the other rat was placed in the apparatus for the sample phase. Animals had a 10-minute ITI before the second trial, or the testing phase. For the second trial, animals were placed back in the field to examine the displaced and familiar objects. Researchers monitored object exploration in an adjacent room via DVR and AnyMaze. Procedures for the object location recognition task were modeled after Mateos et al. (2011) and Abush and Akirav (2012).

Novel Object Recognition

Testing for the object recognition task began four days after the object location recognition task on PND 98±1. Habituation to the apparatus occurred on two consecutive days and consisted of two, 5-minute trials. On PND 100±1, two 5-minute trials, consisting of a sample and test phase, were performed. The trials were separated by a 30-minute ITI. For the first trial, two identical objects were placed in different corners in the apparatus. During the second trial, one of the objects was replaced with a novel object. Object contact for this task was recorded in the same manner as the object location recognition task.

Apparatus. The object recognition task was conducted in the same room using the same apparatus as the object location recognition task. The objects for the object recognition task were different from the object location recognition task. The objects for this task included two plastic toothbrush holders (6 cm x 13 cm) for the first trials and the novel object for trial 2 was a gray painted, wooden object (5 cm x 12 cm). Each session was recorded via DVR and AnyMaze software.

Procedure. The procedures on test day were similar to the object location recognition task. For habituation, animals were placed in the apparatus facing opposite walls for each day and allowed to explore for 10 minutes. On testing day, subjects were acclimated to the room for 5 minutes before being placed on the apparatus. There were two trials separated by a 30-minute ITI, where the animals were returned to their home cage until time for the second trial. In the first trial, animals had 5 minutes to explore the two identical toothbrush holders. Following this sample phase, one of the objects was replaced with the wooden novel object, and the animals were placed on the apparatus for 5 minutes to explore. Researchers monitored object exploration in an adjacent room via DVR and AnyMaze. Procedures for the object recognition task were modeled after Mateos et al. (2011), Abush and Akirav (2012), and O'Shea et al. (2004).

Brain Tissue Collection and Preparation

Twenty-four hours after the last behavioral task was completed (PND 100), rats were anesthetized using sodium pentobarbital (Fatal Plus, Vortech Pharmaceuticals) and then transcardial perfusion was performed. Perfusions consisted of flushing the animals with a 0.1M phosphate buffered saline (PBS) solution followed by 4% paraformaldehyde (PFA). Then, brains were extracted and placed in a 4% PFA solution at 4°C for approximately 24 hours before being transferred to a 0.1M PBS solution for storage. Brain tissue was collected in 40 μm coronal sections in a 1:8 series from approximately bregma 1.70 mm to bregma -6.30 mm (Paxinos & Watson, 1986), using a vibrating microtome. After slicing, the tissue was stored in ethylene glycol plus glycerol cyroprotectant solution at -20°C until staining. Brains from all three conditions, resulting in a total of 30 brains, were analyzed further.

Immunohistochemistry

A single well of brain tissue from animals in all treatment conditions was stained using Ki67 antibodies to examine cell proliferation. For this protocol, the tissue was washed in TBS to remove any remaining cryoprotectant on the slices. Then, tissue was incubated in 0.6% hydrogen peroxide to block endogenous peroxidases. Following this step, Ki67 tissue was exposed to a sodium citrate solution at 65°C for antigen retrieval. The tissue was then blocked in a solution of 3% normal horse serum plus 0.1% tritonX-100 in TBS. After this, the tissue was incubated in monocolonal mouse anti-rat Ki67 primary antibodies (Dako Labs, Denmark) at a concentration of 1:100 for four days at 4°C.

After the four days of incubation, a blocking solution was used to wash the tissue. Next, the tissue was incubated in secondary antibody plus specie-specific serum solution at room temperature (biotinylated horse anti-mouse secondary for Ki-67 tissue [Vector Laboratory Inc.]) and then incubated in ABC solution for an hour (Vectastain ABC Kit, Vector Laboratory Inc.). The final step of the staining procedure consisted of incubation of the tissue in 3,3'-diaminobenzidine tetrahydrochloride (DAB; Polysciences Inc.) substrate for approximately five minutes. Once the Ki67 tissue was mounted, it was counterstained with cresyl violet to produce the appearance of anatomical landmarks, which aids in more efficient quantification.

Quantification

Ki-67 expression was examined using profile cell counting methodology in the subgranular zone of the dentate gyrus in the hippocampus under 1000x magnification using an Olympus BX-43 microscope.

Statistical Analysis

A repeated measures analysis of variance (ANOVA) was conducted to analyze weight differences among the conditions (drug, vehicle, untreated) over the injection period. A repeated measures ANOVA was also conducted to compare group differences of percent time and actual time (sec) spent interacting with the novel object and unfamiliar object location in the second trial to the original object and original location in the first trial. A one-way ANOVA was conducted to evaluate the relationship between the experimental condition and the cell proliferation in the dentate gyrus of the hippocampus. LSD post hoc tests were conducted on significant effects and effects approaching significance.

CHAPTER 3: RESULTS

Due to the lack of weight gain normally observed in animals injected with a synthetic cannabinoid, weight was recorded daily during the injection/supplementation period. All analyses included the treatment (drug, yoked, or untreated) as the independent variable. Two animals were excluded from all analyses, one from the untreated condition and one from the drug condition, due to one being an outlier and one being deemed a backup untreated animal that did not participate in all of the behavioral testing.

Weight

A 3 (treatment) x 16 (days) repeated measures ANOVA was conducted on body weights during the injection/supplementation period (PND 34-49). There was a significant main effect for day, $F(15, 375)=904.47$, $p<.001$, $\eta p^2=.973$. These results show that weight increased significantly during the injection/supplementation period regardless of condition. There was also a significant interaction between treatment and day, $F(30, 375)=5.94$, $p<.001$, $\eta p^2=.322$. There was no main effect of treatment, $F(2, 25)=.455$, $p=.640$, $\eta p^2=.035$. See Figure 1.

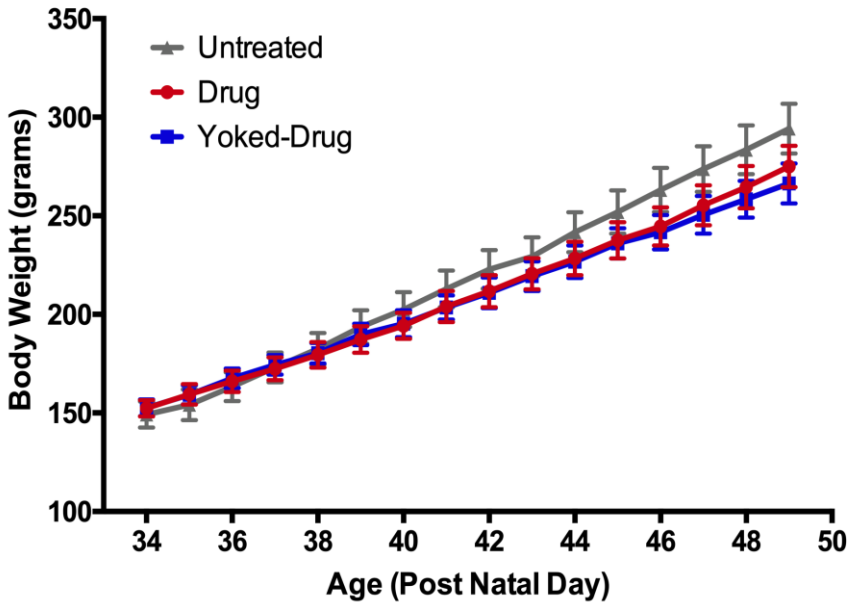

Figure 1. Mean body weight (g) for treatment groups during the injection period (PND 34-49). All conditions increased in weight over the injection period. A significant interaction of day by treatment was also observed. Further analyses were conducted to ensure there were no differences between treatment groups for each day of the injection period and results revealed no significant differences. There was no main effect of treatment.

Object Location Recognition

Two 2 (trial) x 3 (treatment) repeated measures ANOVA were conducted to determine if there were any differences between time spent exploring two identical objects in an open-field maze on trial 1 and on trial 2. In trial 2, the location of one of the identical objects changes. Good spatial memory would consist of the animal spending more time on trial 2 investigating the object in the novel location.

The first analysis of this task consisted of the percent time spent in interaction on trial 1 with the object that will be moved and the percent time spent in interaction on trial 2 with the moved object. There was no significant interaction of treatment by trial, $F(2, 25) = .201$, $p = .819$, $\eta p^2 = .016$. There was no significant main effect of trial, $F(1, 25) = .294$, $p = .592$, $\eta p^2 = .012$. All treatment groups spent the same percent of time exploring the target object on trials 1 and 2. There was no main effect of condition on time spent exploring the objects, $F(2, 25) = .078$, $p = .925$, $\eta p^2 = .006$. This means all animals, regardless of condition, spent the same percent of time exploring the target object in trial 1 and 2. See Figure 2.

This task was also analyzed utilizing actual time, in seconds, exploring the target object on trial 1 and trial 2. There was no significant interaction of treatment by trial, $F(2, 25) = .678$, $p = .517$, $\eta p^2 = .051$. There was a significant main effect of trial, $F(1, 25) = 14.554$, $p = .001$, $\eta p^2 = .368$. Animals spent significantly more time exploring the target object in trial 1 ($M = 57.23$, $SD = 15.01$) when compared to trial 2 ($M = 46.58$, $SD = 17.69$). There was no main effect of treatment on time spent exploring the objects, $F(2, 25) = .684$, $p = .514$, $\eta p^2 = .052$. This means all animals, regardless of condition, spent the same time exploring the target object in trial 1 and 2. See Figure 3.

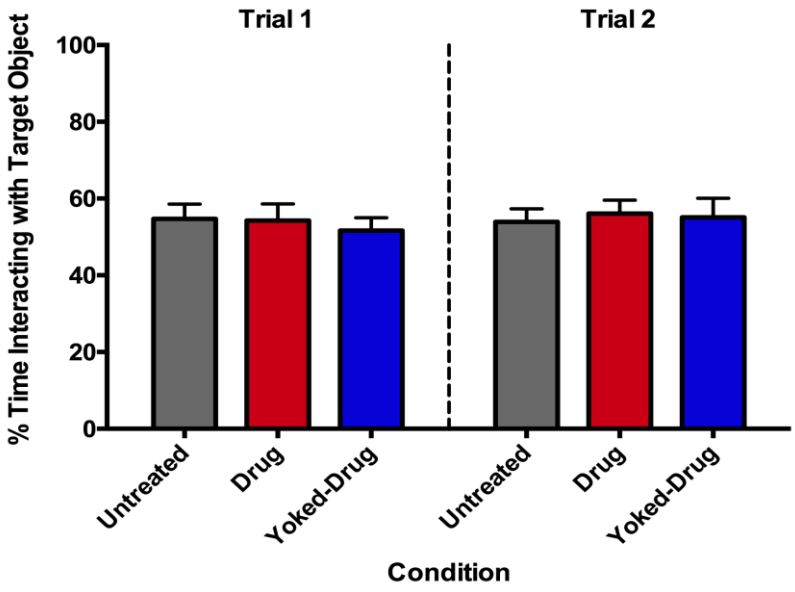

Figure 2. Percent time interacting with the target object on trial 1 and trial 2 for all treatment groups on the Object Location Recognition task. There were no differences in percent time in interaction when comparing trials 1 and 2. There were also no differences between the conditions in either trial.

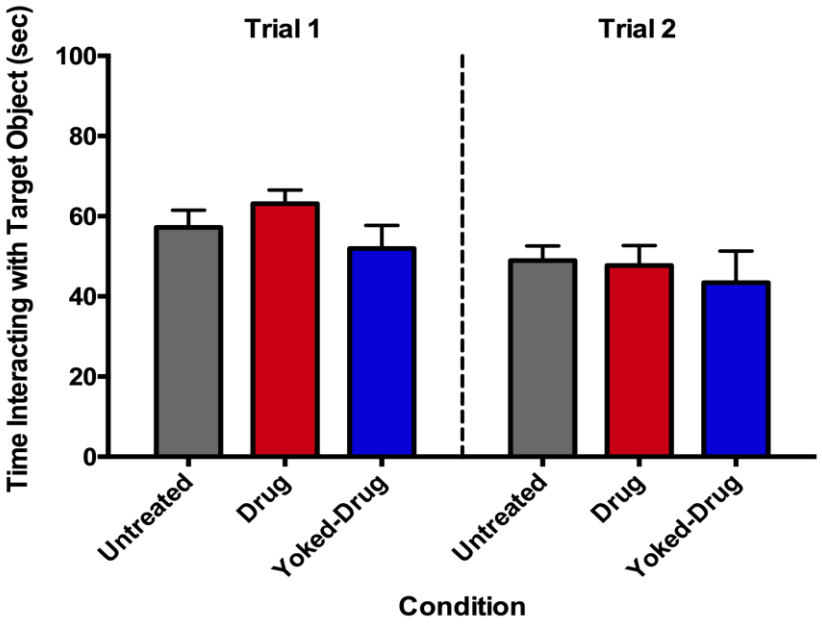

Figure 3. Actual time, in seconds, interacting with the target object on trial 1 and trial 2 for all treatment groups on the Object Location Recognition task. All conditions spent significantly less time interacting with the target object in trial 2 when compared to trial 1. There were no differences between the groups in either trial.

Object Recognition

Two 2 (trial) x 3 (treatment) repeated measures ANOVA were conducted to determine if there were any differences in time spent exploring a novel object on trial 2. Trial 1 consisted of two identical objects and one of the identical objects was replaced with a novel object on trial 2 while the other remained as a constant object. The location of the object did not change in this task. Good memory would consist of the animal spending more time investigating the novel object when compared to the constant object on trial 2.

The first analysis of this task consisted of the percent time spent in interaction on trial 1 with the object that will be replaced with the novel object and the percent time spent in interaction on trial 2 with the novel object. There was no significant interaction of treatment by trial, $F(2, 25) = .108$, $p = .898$, $\eta p^2 = .009$. There was a significant main effect of trial, $F(1, 25) = 80.966$, $p < .001$, $\eta p^2 = .764$. The percent time spent in interaction with the target object on trial 2 ($M = 71.59$, $SD = 11.71$) was significantly more than the percent time spent in interaction with the target object on trial 1 ($M = 47.48$, $SD = 11.58$) for all treatment groups. There was no main effect of treatment on percent time spent exploring the objects, $F(2, 25) = 1.106$, $p = .347$, $\eta p^2 = .081$. This means all animals, regardless of condition, spent the same percent of time exploring the target object in trial 1 and 2. See Figure 4.

This task was also analyzed utilizing actual time, in seconds, exploring the target object on trial 1 and trial 2. There was no significant interaction of treatment by trial, $F(2, 25) = .333$, $p = .720$, $\eta p^2 = .026$. There was a significant main effect of trial, $F(1, 25) = 27.485$, $p < .001$, $\eta p^2 = .524$. Animals spent significantly more time exploring the target object in trial 2 ($M = 68.61$, $SD = 33.73$) when compared to trial 1 ($M = 34.75$, $SD = 15.14$). There was no main effect of treatment on time spent exploring the objects, $F(2, 25) = 1.092$, $p = .351$, $\eta p^2 = .080$. This means

all animals, regardless of condition, spent the same time exploring the target object in trial 1 and 2. See Figure 5.

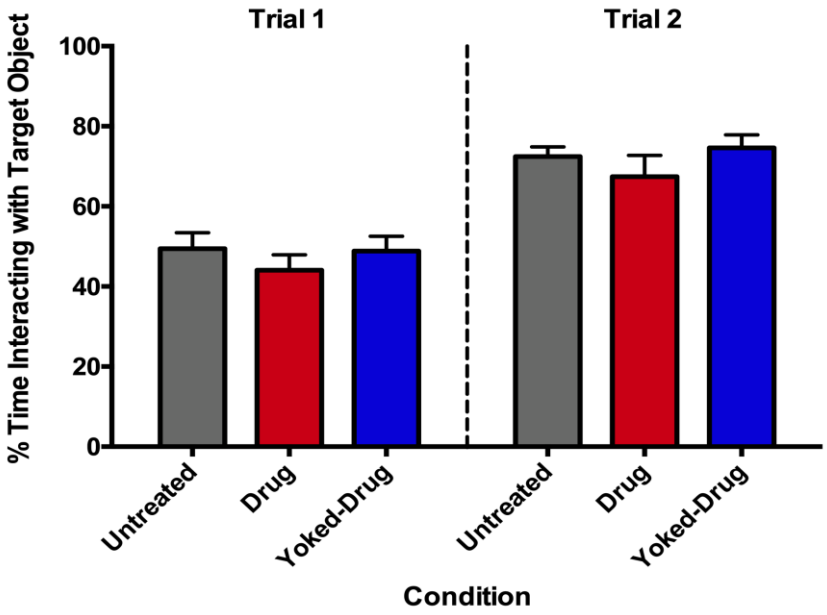

Figure 4. Percent time interacting with the target object on trial 1 and trial 2 for all treatment groups on the Object Recognition task. Results revealed all conditions spent more time interacting with the target object in trial 2 when compared to trial 1. There were no differences between the conditions in either trial.

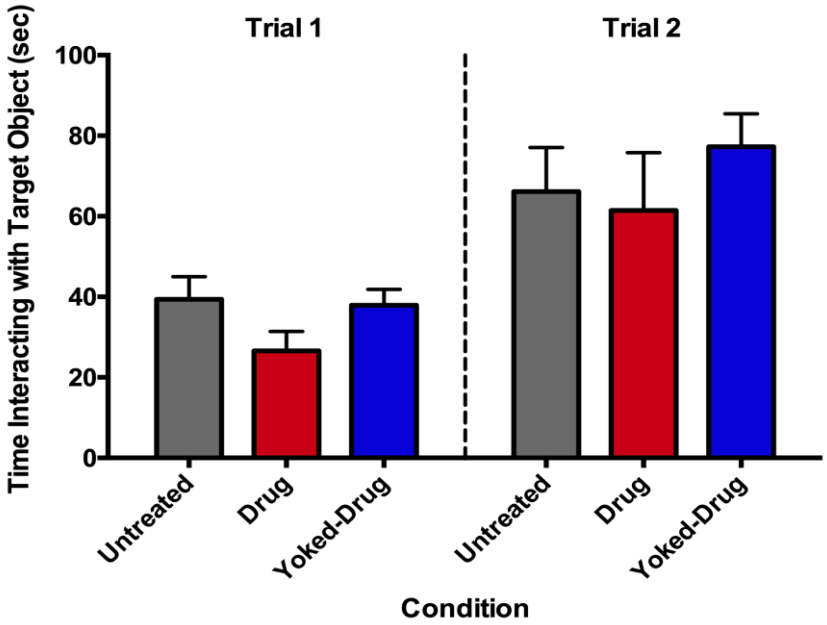

Figure 5. Actual time, in seconds, interacting with the target object on trial 1 and trial 2 for all treatment groups on the Object Recognition task. Results revealed all conditions spent more time interacting with the target object in trial 2 when compared to trial 1. There were no differences between the conditions in either trial.

Hippocampal Neurogenesis

A one-way ANOVA revealed that there were no significant differences in hippocampal cell proliferation based on drug or supplement treatment, $F(2, 27) = 2.830$, $p = .078$, $\eta p^2 = .185$. However, due to this approaching significance, LSD post hoc analyses were used to further investigate potential differences between groups. Results revealed no differences between the drug supplement ($M = 2.77$, $SD = 2.69$) and yoked drug ($M = 2.17$, $SD = 1.44$) treatment groups ($p = .794$). The untreated ($M = 7.15$, $SD = 8.11$) and drug supplement group were also not statistically different from each other ($p = .070$). On the other hand, the untreated animals were statistically different than the yoked drug animals ($p = .037$). See Figure 6.

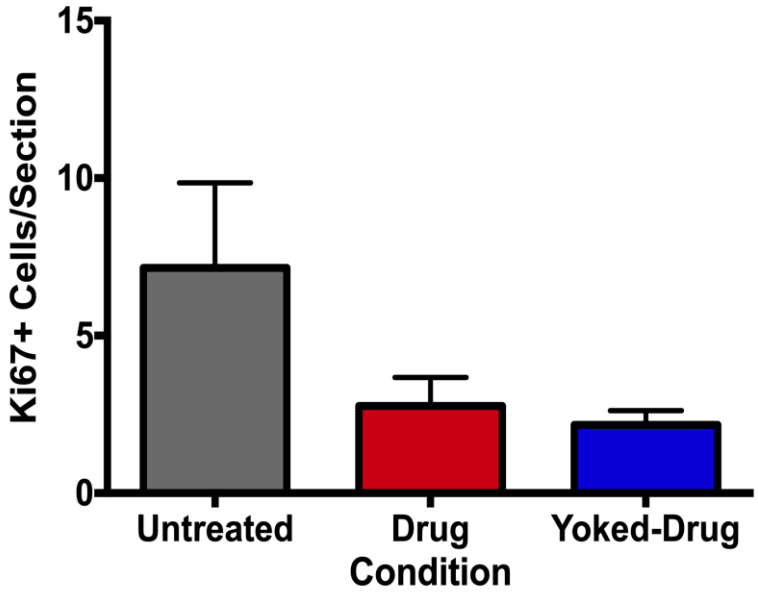

Figure 6. The number of Ki67+ cells per hippocampal section for all treatment groups. Results revealed no significant differences between the groups. LSD post hoc analyses showed that untreated animals had statistically more Ki67+ cells per section than yoked drug animals, but there were no differences between the remaining treatments.

CHAPTER 4: DISCUSSION

The current study investigated the effects of the administration of a synthetic cannabinoid, CP 55, 940, during adolescence on weight, memory, and cell proliferation in the hippocampus of rats. This study also aimed to eliminate the lack of weight gain that is often observed in the drug condition when compared to the untreated control by providing the drug animals with a nutritionally complete dietary supplement.

In this study, there were no differences in percent weight gain during the injection period among the conditions. In previous studies, a lack of weight gain due to a decrease in food consumption was observed in animals receiving a synthetic cannabinoid (Dalton, Wang, & Zavitsanou, 2009). We believe the current study eliminated these differences by providing the drug and yoked animals with a nutritional supplement over the course of the injection period. Both memory tasks, object location recognition and object recognition, were analyzed similarly. Actual time, in seconds, and percent spent exploring the target objects served as dependent variables for the analyses. Results revealed that animals in the three conditions performed the same across both memory tasks, regardless of the dependent variable. Due to the analysis of neurogenesis approaching significance, LSD post hoc analyses were conducted. Results revealed that the yoked drug condition had fewer Ki67+ cells in the hippocampal region than the untreated animals. Although the drug supplement condition was not statistically different than the untreated condition, the mean number of Ki67+ cells in the hippocampal region of the drug supplement condition was similar to that of the yoked drug animals.

Some studies have observed decreased weight gain in animals exposed to a synthetic cannabinoid when compared to untreated animals (Dalton et al., 2009; Mateos et al., 2011). The current study attempted to alleviate the weight differences by providing drug and yoked animals

with a nutritional supplement, in addition to standard rat chow, during the injection period. There were no differences in weight between the conditions during the injection period in this study. This suggests that the nutritional supplement provided to the drug and yoked animals may be responsible for maintaining appropriate weight gain in these conditions. While the weight differences were not evidenced in this study, the reason for these differences should be explored further along with other methods for maintaining appropriate weight gain in these drug-exposed animals. One possible explanation for the lack of weight gain in animals exposed to the drug may be the route of administration. The current study, and most studies that observe the weight differences between the drug conditions and untreated conditions, administer the drug via intraperitoneal injections. Studies that involve oral administration of cannabinoids in early adolescence have not observed these weight differences (Dow-Edwards & Zhao, 2008). This may be due to the slow absorption rate of cannabinoids when the drug is administered orally. The slow absorption rate may minimize the psychoactive effects that are often present following injections, which have a faster absorption rate (Dow-Edwards & Zhao, 2008). Another possible explanation for the weight differences normally observed may be the dose of the drug administered. While all drug doses (small, moderate, large) seem to result in decreased body weight in the first few days of injections, all animals eventually start gaining weight, although the animals injected with the moderate and high doses do not fully recover by the end of the injection period when compared to control animals (Dalton et al., 2009). Animals exposed to a high dose of the drug often suffer from more severe and long-lasting weight differences when compared to animals receiving a lower dose of the drug (Dalton et al., 2009). Since previous research has shown that all animals, regardless of dose, tend to have decreased body weight in the first few days of injections, and the current study did not observe this phenomena, it supports

the conclusion that our nutritional manipulation was effective. The current study also utilized male rats, and males tend to be less receptive to the cannabinoid and its effects. Research has shown male rats still display these weight differences when compared to untreated animals, but the weight difference between females and untreated animals is more pronounced (Mateos et al., 2011). Future research should observe weight fluctuations in female and male rats when provided a nutritional dietary supplement during different routes and dosages of cannabinoid administration. A comparison group that receives the drug, but no supplement should also be included in the future.

For object location recognition, all conditions spent less time, in seconds, exploring the target object on trial 2 when compared to trial 1. For this task, the percent time exploring the target object did not differ between trials 1 and 2. This may suggest that none of the conditions learned the task since all animals spent more time, in seconds, in object exploration with the target object on trial 1, whereas good spatial memory is defined as spending more time in object exploration with the target object on trial 2. Many studies have found that exposure to synthetic cannabinoids impairs memory, especially in adolescent-aged animals (O'Shea et al., 2004; Robinson et al., 2007). A variable to consider when interpreting the results of this task is the strain of rat used. Renard, Krebs, Jay, and Le Pen (2012) found that two strains of rats treated with the same synthetic cannabinoid as the current study, CP 55, 940, performed differently from each other on a similar object location recognition task, with only one of the strains exhibiting poor spatial memory following adolescent cannabinoid exposure. This suggests that spatial working memory following cannabinoid exposure can vary with strain. For the object recognition task, all conditions spent more time, in seconds and percent, exploring the target object in trial 2 when compared to trial 1. This suggests that all animals, regardless of drug and

supplementation, displayed adequate recognition memory on this task. Adequate performance on this task also suggests the animals did not have other deficits, such as deficits in visual perception or lack of interest in the novel objects. Most studies consistently observe a deficit in recognition memory following adolescent cannabinoid exposure (O'Shea et al., 2004; Renard et al., 2012). While strain was a variable to consider for the spatial memory task following cannabinoid exposure, the previous literature indicated that strain may not affect recognition memory (Renard et al., 2012).

There are some aspects that should be considered when interpreting the results for both memory tasks. A possible explanation for the discrepancies between the current study and previous studies may be the lack of nutrition animals received during the exposure period. If animals are not receiving proper nutrition, this may be responsible for some of the observed memory deficits in previous studies. Malnutrition in early life was shown to negatively affect some areas of memory, and in our study the nutritional supplementation is believed to have protected against these memory deficits (Hemb et al., 2010). The type of memory that is assessed should also be taken into consideration. If the cannabinoid does produce memory deficits, it may not affect memory as a whole and may only impair a specific area of memory. For example, Acheson, Moore, Kuhn, Wilson, and Swartzwelder (2011) found no spatial memory deficits in adult or adolescent animals exposed to a synthetic cannabinoid, while O'Shea et al. (2004) found deficits in recognition memory following exposure. The type of cannabinoid administered also seems to play a role in the area of memory affected. Male and female adolescent-aged rats treated with THC showed impairments in object recognition memory, while only male adolescent animals treated with CP 55, 940 showed impairments in recognition memory when compared to controls (Mateos et al., 2011; Quinn et al., 2008). Mateos et al. (2011) also observed

gender differences related to memory impairments in animals treated with a synthetic cannabinoid. For the recognition task, females performed significantly better than males, whereas in a spatial memory task, the males exhibited better performance than females following adolescent exposure to CP 55, 940 (Mateos et al., 2011). Research suggests the area of memory impaired following drug exposure is dependent on multiple variables, including, but not limited to, nutrition, the cannabinoid administered, strain, and gender.

The hypothesis that adolescent male rats chronically exposed to the synthetic cannabinoid would display an increase in cell proliferation when compared to untreated control animals was not supported. Due to this ANOVA approaching significance with a large effect size, it was warranted to do further investigation. LSD post hoc analyses revealed that untreated animals had statistically more Ki67+ cells per section than the yoked drug animals, and the drug supplement group was approaching being significantly different than the untreated control. The current findings agree with some prior research on cell proliferation following cannabinoid exposure, which observed a decrease in the number of new neurons in the hippocampal region following drug exposure (Abboussi et al., 2014; Lee, Wainwright, Hill, Galea, & Gorzalka, 2013). The primary difference between the current study and previous studies is the nutritional supplementation provided. Our results suggest that nutrition may play an important role in cell proliferation in the hippocampal brain region following adolescent cannabinoid exposure. The role of nutrition in cell proliferation should be investigated further to include a variety of nutritional diets. The current study utilized a high fat dietary supplement to maintain appropriate weight gain in drug exposed animals, but Lindqvist et al. (2006) found that a diet that is high in fat decreased hippocampal neurogenesis in male rats. This may be a possible explanation for the results of the current study. Our yoked drug treatment was statistically different from the

untreated condition, and the drug supplement animals were approaching being significantly different than the untreated condition. One difference between these treatments is that the drug supplement and yoked drug animals received similar quantities of supplementation during the injection period, whereas the untreated animals received no supplementation. This suggests that cannabinoids may not be responsible for the decreased cell proliferation observed in this study, but rather the high fat supplementation. Other diets with varying nutritional components and consumption periods should be evaluated and compared in the future.

Another factor to consider is that the animals with lower Ki67+ cells both received injections, and although the drug condition was not statistically different than the untreated animals, the drug animals had a similar mean to the yoked drug condition. Both the yoked drug and drug supplement treatments received injections and injections have been found to cause stress in rats (Deutsch-Feldman, Picetti, Seip-Cammack, Zhou, & Kreek, 2015). Stress is also related to a decrease in cell proliferation, which could be another possible explanation for the results found in this study (Heine, Maslam, Zareno, Joëls, & Lucassen, 2004). In order to decrease the stress commonly experienced following injections, it may be beneficial to include a handling period with the researchers who will be injecting the animals prior to the injection period (Deutsch-Feldman et al., 2015). Route of drug administration may also be altered in the future to eliminate the stress that accompanies injections, such as utilizing vapor chambers.

The current study investigated cell proliferation, but future research should also investigate other stages of neurogenesis following cannabinoid exposure. It was found that proliferation of cells decreased with age, but the survival of cells increased with age (Amrein, Isler, & Lipp, 2011). Our study observed cell proliferation in the hippocampus approximately five weeks following the injection period and new cells have about a 28-day timeline to survival.

This means that the current study is observing the long-term effects of the drug on cell proliferation, instead of the more immediate effects. Due to these factors, future studies should investigate the more immediate effects of cannabinoid use on cell proliferation, along with cell survival. The decrease of neurogenesis in adult animals that has been observed in some studies following cannabinoid exposure could be more related to the effects of increasing age on neurogenesis than the drug itself, and these results should be interpreted with caution. Another explanation for the discrepancy between the current study and previous studies may be due to a possible floor effect. The untreated animals in the current study had a very low number of cells in the hippocampus, with the average being approximately seven, whereas previous studies have found approximately 15 as the average number of positive Ki67 cells in the same region of untreated animals (Yoo et al., 2012). The low number of Ki67 cells may have caused the effect of the drug to be overlooked. This finding also supports the idea that future research should examine long-term cell survival, rather than cell proliferation.

Study Limitations

There were a few limiting factors in the current study that should be considered. One possible confound for this task is some animals began chewing on one corner of the wooden maze during testing beginning with cohort 7. In order to control for this confound, the human coding, instead of the AnyMaze software coding, was utilized for the analyses. Human coders were instructed to code for the animals' contact with the object only, based on predetermined guidelines, whereas the computer coded based on the location of the animals in the apparatus. Another limitation to consider is that some of the cohorts were injected during different times of the day. Although this study attempted to keep this difference minimal, it is still something to consider. Some cohorts were administered the drug in the morning, while some received the drug

in the early evening due to unavailability of researchers throughout the year. Animals within a cohort were injected at approximately the same time every day to eliminate possible differences within the cohort. Time of day of administration may result in differing drug effects due to the circadian rhythm cycles. Sensitization of the drug is greater during the day cycle than the night cycle for the animal, meaning the short-term effects of the drug may be more prominent when given during the day (McClung, 2007). On the other hand, when evaluating long-term sensitization following drug exposure, sensitization was greater when the drug was administered at the onset of the dark phase (McClung, 2007). The current study injected animals in the light phase and, due to this, it is possible that the effects of the drug were not as strong and may have been overlooked due to the decreased sensitization. The time of day that memory tasks were carried out may also be a limiting factor with it effecting the sleep cycle of the animal and also due to the time of the task varying by cohort in some instances. Research has shown that time of day does affect cognitive performance and age of the animal also contributes to this. The performance of young rats has been shown to be better in the PM while adult rats performed significantly better when tested in the AM (Winocur & Hasher, 2004). The current study involved testing animals in the morning, as well as the early afternoon, due to availability of researchers. This could lead to significant differences between the cohorts and results should be interpreted with caution.

Conclusions and Directions for Future Research

The results of the current cannabinoid study do not support previous literature showing deficits in memory and cell proliferation. The current study also shows the importance of nutrition in cannabinoid research, with nutritional deficits possibly affecting both cell proliferation and memory in animals exposed to cannabinoids. Future research should investigate

other possible diets, instead of the high-fat diet utilized in this study, and the effects on both dependent variables mentioned. Also, future research should aim to focus on different types of memory affected by cannabinoids and if these varying areas produce similar results when animals are provided with supplementation during drug exposure. Different stages of neurogenesis should be investigated following adolescent cannabinoid exposure because it is a very limited area of literature and factors such as the possible floor effect and adult age may be contributing to low levels of cells observed. The primary purpose of the current study was to highlight the importance of nutrition during adolescent cannabinoid exposure and attempt to explain the variability in the literature in regards to synthetic cannabinoid exposure, memory, and cell proliferation.

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