2010

Effects of CB2 Receptor Activation on Variable Chronic Mild Stress (CMS) Induced Depression and Hippocampal Dependant Spatial Learning

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Effects of CB_2 Receptor Activation on Variable Chronic Mild Stress (CMS) Induced Depression and Hippocampal Dependant Spatial Learning

A Senior Honors Thesis Presented By

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To the Department of Psychology

In Partial Fulfillment of Requirements

For the Degree of Bachelor of Arts

Connecticut College

New London, Connecticut

May 6, 2010

Abstract

Major Depressive Disorder (MDD) accounts for approximately 10% of all diagnosed chronic illness in the United States. The most common treatments for MDD is serotonergic manipulation via pharmacological treatments such as monoamine oxidase inhibitors (MAO-I) or selective serotonin reuptake inhibitors (SSRI). However, not only have these treatments proven to be ineffective for a portion of the population, but they also fail to treat some of the physiological damages that occur in the hippocampus during MDD due to over-activation of inflammatory cells known as microglia. Activation of the CB_2 receptor in the hippocampus has been shown to inhibit microglial cells and promote neuron proliferation. This study investigates the effects of the CB_2 agonist GW405833 on rats in the chronic mild stress model for depression. Results showed significant decrease in depressive-like behavior in the Forced Swim Test, the Open Field Test for anxiety and the Morris Water Maze. Sucrose Preference testing and weight gain did not differ significantly. Immunohistochemical staining for activated microglial cells and neuron nuclei was performed. The data suggest that CB_2 activation could play an important role in MDD treatment for both behavioral and physical changes occurring in the brain.

Acknowledgments

Foremost, I would like thank the Connecticut College Neuroscience department for allowing me to pursue this project. The support, knowledge, and guidance of both Dr. Schroeder and Dr. Grahn made this project possible, and I must express my sincerest thanks. Thanks are also due to Dr. Richard Hunter of the Pfaff/McEwen Laboratories at Rockefeller University for his contributions. I would also like to thank Dr. Garth Whiteside for his aide in finding the pharmacotherapy used, and supplying insight into proper drug delivery. Finally, I must thank Caroline Abbott and Keith Winking for their assistance in experiment. None of this would have been possible without any of the aforementioned support.

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Introduction

1.1 Major Depressive Disorder overview

Major Depressive Disorder (MDD) accounts for approximately 10% of all diagnosed chronic illnesses in the United States. Anti-depressants are commonly found to be among the top 20 drugs prescribed by physicians (National Center for Health Statistics, 2009). Despite this, there is still no complete and clear understanding of depression (Swaab, Bao, & Lucassen, 2005). Due to the lack of knowledge on physiological malfunctions in MDD, current diagnostic criteria focus on behavioral symptoms alone. The Diagnostic and Statistical Manual of Mental Disorders IV (DSM-IV) qualifies a person as "depressed" if they exhibit five of the following symptoms: despairing mood, loss of interest in pleasure or activities previously considered pleasurable, fatigue, weight fluctuations, insomnia or trouble sleeping, psychomotor agitation or retardation, feeling of worthlessness, excessive or inappropriate guilt, diminished ability to concentrate, and, finally, reoccurring or constant thoughts of death/suicide (American Psychiatric Association, 2000). After performing a search in PubMed for *MDD* (or *depress**) and *diagnos**, all results came back with studies that used behavioral scoring methods such as Beck's MDD Index or Hamilton MDD Rating Scale (HDRS). Another search was performed in PubMed for *MDD* and *physiology*. The minimal results from this search showed vague, incomplete molecular theories of MDD involving many systems including: impairments to regional structural plasticity, neurodegeneration, neuroinflammation/activated microglial cells and cytokines, and lowered structure volume/cortical thickness, most notably in the hippocampus and areas of the prefrontal cortex (as summarized by Manji, Drevets, & Charney, 2001). However, this also shows that there is a clear lack of congruity between the behavioral and physiological understandings of MDD.

Prior to the 20th century and Freud, MDD was commonly referred to as "melancholia." This term stems from the ancient Greek understanding of medicine known as humorism. Humorism was a theory developed by Hippocrates that was based on a balance of the four humors within the body: black

bile, yellow bile, phlegm and blood. MDD was seen as an imbalance in the black bile. Melancholia was defined as “a prolonged sensation of despondency and worry” (Hippocrates, *Aphorisms*). In the 1900’s this disorder was renamed as MDD (Lewis, 1934). The current understanding of MDD comes from studies based on a treatment/outcome basis. Initially, these treatments included such things as shock therapy and lobotomies. Doctors would test their theories on patients by using these procedures and then studying the patient in follow-ups. However, these techniques often caused more harm to the patient than good. In the late 1940’s and early 1950's there were two major breakthroughs in the understanding of MDD, and both were accidental. First, tubercular patients being treated with a monoamine oxidase (MAO) inhibitor known as iproniazid were seen to have improved moods. Second, patients treated with reserpine, an anti-hypertensive medication that depletes monoamine stores, were seen to become depressed as a side effect (Owens & Nemeroff, 1994). These discoveries led to the proposition of the catecholamine theory (Schildkraut, 1965). The catecholamine theory suggested that low levels of a class of neurotransmitters called the monoamines caused MDD or MDD-like symptoms. This new insight into MDD led to the development of the main pharmacological treatments for MDD today.

1.2 Serotonin and MDD

Serotonin (5-hydroxytryptamine: 5-HT) is a monoamine (MAO) that was first identified in 1948 as a vasoconstrictor in the heart (Rapport, Green, & Page, 1948c). The name “serotonin” is derived from the Latin word “serum” and the Greek word “tonic.” After further study, 5-HT was found to play major roles in not just the heart, but also in the gastrointestinal tract, lungs, kidneys, platelets, and the brain (Mohammad-Zadeh, Moses, & Gwaltney-Brant, 2008). In 1957, Brodie & Shore proposed that 5-HT be classified as a neurotransmitter after reviewing studies that show localization of 5-HT receptors in the brain (Amin, Crawford, & Gaddum, 1954). The discovery and classification of 5-HT has led to the characterization of other MAO neurotransmitters including epinephrine,

norepinephrine (NE), dopamine (DA).

Epinephrine, NE, and DA all fall under a secondary category of MAO's referred to as catecholamines[1]. The catecholamines differ from serotonin mainly in terms of synthesis. Serotonin is synthesized from L-tryptophan, an essential amino acid not naturally produced by the body. The synthesis of serotonin begins when tryptophan is hydroxlyated by tryptophan hydoxylase to 5-hydroxytrypophan (5-HTP). 5-HTP is then decarboxylated by L-aromatic amino acid decarboxylase (AAAD) to form 5-hydroxytryptamine (5-HT) (Clark, Weissbach, & Udenfriend, 1954). 5-HT synthesis and storage in the central nervous system (CNS) occurs in presynaptic serotonergic neurons located in nine groups of cell bodies found in the pons and midbrain (Dahlstroem & Fuxe, 1964). However, approximately 80-95% of the serotonin in the body is found in the periphery, mainly in enterochromaffin cells in the gut (Tyce, 1990). CNS serotonergic neurons store 5-HT in storage vesicles, specially designed for ease of release as well as protection from metabolism. In the CNS, serotonin is released when a neuron is activated and fires an action potential. When an action potential causes neuron terminal depolarization calcium flows into the cell, allowing storage vesicles to release 5-HT into the synaptic cleft. Serotonin can bind to postsynaptic serotonin receptors or pre-synaptic autoreceptors. Autoreceptors regulate the amount of serotonin being released by providing negative feedback to the pre-synaptic cell (Cerrito & Raiteri, 1979). Most serotonin is then taken back into the pre-synaptic cell via the selective serotonin transporter (SERT). Back inside the neuron, serotonin can either be re-stored in vesicles, ready to be used again, or it can be metabolized by monoamine oxidase. MAO metabolism is the primary metabolic pathway for all serotonin within the CNS (McIsaac & Page, 1959).

Serotonin plays a wide variety of roles in both the CNS and the periphery. In the periphery, 5-HT plays a role in platelet aggregation, vascular tone, hypertension (sometimes hypotension), and

[1] As mentioned previously, Schildkraut's theory was originally known as "the catechole amine theory" but included serotonin. This theory was later updated to be called "the Monoamine theory" to correctly include serotonin, since serotonin is not technically a catecholamine.

motility in the gastrointestinal tract. The effects of 5-HT within the CNS are just as broad. Serotonin is produced by neurons in the brainstem that form the Raphe nucleus. These neurons project to nearly all of the CNS and play a role in the following behaviors: mood, vascular regulation, blood pressure, nausea, anxiety, appetite and pain sensitivity (Mohammad-Zadeh, Moses, & Gwaltney-Brant, 2008).

As mentioned previously, serotonin was seen to be reduced in patients with depressive symptoms. So, initial drug therapies for MDD were created to target the serotonergic system and increase the amount of serotonin in the brain. Three major classes of anti-depressant medications are the monoamine oxidase inhibitors (MAO-Is), the tricyclic antidepressants (TCAs) and the selective serotonin reuptake inhibitors (SSRIs). Inhibiting the MAO with an MAO-I causes a slowed degradation of serotonin, allowing more neurotransmitter to remain in the synapse and act on the post-synaptic cell, eliciting an overall larger response. The second group of antidepressants, the TCAs, can also be referred to as serotonin-norepinephrine reuptake inhibitors (SNRIs)The TCAs were previously the most widely used antidepressant class. However, currently, the most widely used anti-depressant medication is an SSRI known as fluoxetine (Prozac). SSRIs and SNRIs increase the quantity of serotonin in the synapse by blocking the specialized reuptake transporter protein on the pre-synaptic cell. Without the transport mechanism back into the pre-synaptic neuron, the neurotransmitter is left to act on the post-synaptic neuron until it can be broken down. However, SNRIs are non-selective, and modulate norepinephrine along with serotonin. SNRIs have also been found to inhibit L-type calcium channels (Zahradnik, Minarovic, & Zahradnikova, 2008) and inhibit neuronal sodium channels (Pancrazio, Kamatchi, Roscoe, & Lynch, 1998). Both of these extra effects of TCAs have been implicated in the high toxicity and cardiovascular problems associated with TCAs.

Besides the negative side-effects that have been seen with some antidepressants, most antidepressants are criticized for their delay of therapeutic effects. Anti-depressants do not always work in patients and often take 2-4 weeks before changes can be seen in MDD patients, but the exact reason

for this is still unknown (Hamon & Bourgoin, 2006, Posternak & Zimmerman, 2005). Recent studies have also found that effectiveness of anti-depressant treatment increases with the severity of the depression in a person, and, therefore, do not have effects on all patients (Fournier, DeRubeis, Hollon, & al., 2010).

1.3 Hippocampal neurogenesis and MDD

Recent studies have found that although the treatment based on the catecholamine theory provides an inadequate aid for some people suffering from MDD: the treatments do not cause remission in patients (Thase, 2009) and they do nothing to actually address the malfunctions and damage occurring within the brain (Charney, 1998 and Manji, 2000). It is more and more apparent that MDD is not due to any one physiological malfunction, but, instead due to a wide variety of genetic, environmental, autonomic, endocrine, cognitive, and sleep abnormalities (Manji, Drevets, & Charney, 2001). One of the common and most pronounced effects from prolonged exposure to stress, as seen in MDD, is increased hippocampal degeneration via inflammatory cytokines produced by activated microglial cells (Leonard, 2007). In rats, the chronic mild stress (CMS) model of depression has been seen to reduce the number of hippocampal granule cells (Jayatissa, Bisgaard, West, & Wiborg, 2008). Commonly, these damages, or any other damage to the brain, are referred to as brain insults. During a brain insult the brain responds as any part of the body would, and attempts to defend itself using an inflammatory response.

In the brain, microglial cells are the specialized cells responsible for recognizing brain insults and instigating the protective inflammatory response by releasing specialized proteins known as cytokines. These cytokines promote the defending cells to utilize extra energy and promote neuron growth. However, these resources are quickly drained and the initially protective microglial cell response becomes neurotoxic via the release of cytokines such as interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α). TNF-α expression has been shown to cause abnormal phosphorylation of

AMPA receptors, increased glutamate release and decrease of dendritic spines (Rossi, Bernardi, & Centonze, 2010). Prolonged responses from activated microglial cells cause neurons to obliterate themselves (McNally & Bhagwagar, 2008). The cytotoxic molecules from microglial cells have also been shown to decrease functioning of glia such as astrocytes and mylein forming oligodendrocytes. Not only do oligodentrocytes lose function, but the cytotoxins have also been shown to attack the mylein on the neurons themselves (Cramer, Newcombe, Black, Hartle, Cuzner, & Waxman, 2004). Eventually this overactivativty causes hippocampal degeneration and dysfunction has been seen in many psychological ailments including MDD (Sapolsky, 2000).

For many years it was widely speculated that the brain does not produce new neurons after development. However, recent studies have shown that new cells are constantly being produced, especially in the hippocampus, throughout the adult life in a process known as neurogenesis (Eriksson, et al., 1998, Gage, 2002, Ming & Song, 2005). The cause and function of neurogenesis is still widely cogitated (Aimone, Wiles, & Gage, 2006). The hippocampus is involved in many processes in the brain and body, but is mainly associated with learning, memory, and spatial awareness (Kelley, Domesick, & Nauta, 1982, Amaral & Witter, 1989). The most famous case in studying the hippocampus was from a patient known as H.M. (recently revealed as Henry Gustav Molaison) who had a surgical destruction of his hippocampus. H.M.'s surgery demonstrated that damage to the hippocampus can cause severe impairment in forming memories and learning (Squire, 2009). It has also recently been shown that degeneration of the hippocampus caused by a brain insult often causes decreased performance on memory tasks as well as spatial learning tasks and is also associated with onsets of dementia and neurodegenerative diseases such as Parkinson's and Alzheimer's disease (Eisch, 2008, Sahay & Hen, 2007). As Leonard showed in 2007, the inflammatory response produced during depression causes degeneration of neurons in the hippocampus. This leaves hippocampal neurogenesis as a prime target for novel anti-depressant medications not based on the monoamine system alone (Elder, DeGasperi, &

GamaSosa, 2006).

1.4 Cannabinoids

The active ingredient in marijuana or *cannabis sativa* known as Δ-9 tetrahydrocannabinol (THC) was the first classified cannabinoid. This classification led to the discovery of the endogenous cannabinoid system. There are two known receptors and two endogenous ligands known in the endocannabinoid (eCB) system. The two known receptors of the cannabinoid system are known as cannabinoid receptor 1 (CB_1) and cannabinoid receptor 2 (CB_2). Acting on these receptors are endogenous THC-like, lipid molecules known as anandamide (AEA) and 2-arachidonoylglycerol (2-AG) (Ameri, 1999). Endocannabinoids are not stored in the body, but instead synthesized as needed by the body. The exact biosynthetic pathways for eCB's are unclear and multiple pathways have been suggested. The suggested precursor for eCB's is *N*-arachidonoyl phosphatidylethanolamine (NAPE). This molecule has been found to be hydrolyzed by phospholipase D to form AEA, directly. NAPE has also been seen to produce AEA through more indirect mechanisms, not as well classified (Liu, et al., 2008). It is clear the eCB system is far from being completely classified and understood.

CB_1 receptors are most densely expressed in the basal ganglia and the cerebellum, but are also found in other areas such as the pre-frontal cortex. Because of these areas involvement in locomotor activity, high activation of CB_1 receptors in these areas results in spontaneous locomotor activity (Ameri, 1999). It is suggested that this locomotor effect seen from CB_1 activation causes inhibition in the striatonigral GABAergic neurons and the nigrostriatal dopaminergic neurons causing loss of motor control (Romero, Garcia, Cebeira, Zadrozny, Fernandez-Ruiz, & Ramos, 1995). At low activation levels CB_1 receptors have been found to increase firing of serotonergic neurons in the pre-frontal cortex, which project to the raphe nucleus to increase serotonin release. However, at high activation, these effects dissipate drastically and actually inhibit the firing of the same serotonergic neurons. The reason for the differential effects seen from different dose concentrations of cannabinoids is unknown

(Rubino, et al., 2008). Studies implicate the CB1 receptor in mood disorders such as MDD (Hill & Gorzalka, 2005, Witkin, Tzavara, & Nomikos, 2005, Bambico & Gobbi, 2008). These studies have shown that the therapeutic effect seen is mainly due to CB1 activity on serotonergic pathways (McLaughlin, Hill, & Gorzalka, 2009). This receptor has not been seen to affect the inflammation or neurodegeneration caused by MDD (Maresz, et al., 2007). Therefore, CB_1 affects MDD via the same indirect mechanisms of the main pharmaceutical therapies already developed.

CB2 receptors have been implicated in modulating the immune response throughout the body. Until recent, these receptors were thought to be exclusive to the peripheral nervous system (Griffin, et al., 1999, Galiegue, et al., 1995). However, recent discoveries have showed that these receptors can be found in dense populations on microglial cells, especially in the hippocampus (Nunez, et al., 2004, Benito, et al., 2003). CB_2 receptors are seven transmembrane G-protein coupled receptors (GPCR) that work through various intracellular cascade mechanisms including the inhibition the of adenylyl cylcase pathway via the cyclic AMP (cAMP)/protein kinase A (PKA) pathway and the p42/p44 mitogen activated protein kinase (MAPK) (however, the effect on MAPK is concentration-dependant). Cyclic AMP inhibition blocks phosphorylation necessary to activate microglial cells in the brain. Inhibition of MAPK leads to the inhibition of Interleukin-2 (IL-2) which is also necessary for microglial activation in the brain (Demuth & Molleman, 2006). These pathways implicate CB_2 receptors in neuron proliferation and survival as well inhibition of microglial cells and inflammation (Fernandez-Ruiz, Pazos, Garcia-Arencibia, Sagredo, & Ramos, 2008).

This study aims to evaluate the anti-depressant effects of CB_2 activation using the agonist GW405833. Previous studies on CB_2 agonism and MDD have been limited in number and scope. Some studies have evaluated anti-MDD via CB_2 agonism in a pain model (Hu, Doods, Treede, & Ceci, 2009). In this study, Hu et al. attempted to demonstrate a novel model for MDD in rats as well as show anti-depressive effects. The model involved surgery to invoke neuropathic pain to induce MDD-like

behavior. However, when studying inflammation, a model utilizing surgery is not the best option due to the increased inflammation and damage that can occur from the surgery, as increased inflammation could skew the results. CB_2 has also been shown to have anti-nociceptive properties (Hosking & Zajicek, 2008). Therefore any anti-depressive effects seen by CB_2 activation could have been due to its action as a neuropathic pain reducer, meaning that it would not be relevant to most cases of MDD seen in humans (which have more to do with stressors than physical pain). However, this could be beneficial in treating patients suffering from mood disorders related to other diseases such as multiple sclerosis. A more relevant study has shown that CB_2 antagonism is effective at reducing depressive-like behavior after a stressor is removed (Garcia-Gutierrez & Manzanares, 2009). The basis for this study is that antagonism of any receptor causes an upregulation of the receptor. By administering a CB_2 antagonist after the stress period, CB_2 receptors increase in number, allowing for the endogenous cannabinoid system to have increased action. Therefore, this study offers proof that increasing CB_2 activation, via upregulation of the CB_2 receptor, causes anti-depressive effects. However, it is unknown if long-term treatment in this manner would provide the same effect. Obviously there are many possible benefits to this research, but more needs to be done before a pharmacological treatment could be considered.

The synthetic cannabinoid GW405833 will be used as an agonist for CB2 activation in the current study. GW405833 has been pharmacologically and behaviorally characterized by Valenzano et al. (2005). GW405833 has been seen to have high affinity for the CB_2 receptor but not for the CB_1 receptor (1200:1 preference in human CB_2 receptors and 80:1 in rat CB_2 receptors). The drug has also been shown to elicit the general effects of CB_2 activation including blockade of neuropathic and inflammatory pain. Due to the blockade of inflammatory pain, as well as the known anti-inflammatory action of activated CB_2 receptors, other studies have suggested the investigation of specific anti-inflammatory action of GW405833 (Clayton, Marhsall, Bountra, & O'Shaughnessy, 2002). Valenzano et al. (2005) have also show than GW405833, like other CB_2 specific agonists, does not have the

harmful side-effects of ataxia or catalepsy nor is it associated with the psychoactive effects seen from CB_1 activation. However, GW405833 has not been seen to have anxiolytic effects.Therefore, GW405833 shows potential as an anti-depressant compound by via activation of anti-inflammatory and pro proliferation pathways.

1.5 Chronic mild stress (CMS) paradigm and measuring MDD

The current study utilizes unpredictable CMS model as shown by Bekris et al. (2005). CMS has been utilized and shown to be an effective model of MDD in rats (Bekris, Antoniou, Daskas, & Papadopoulou-Daifoti, 2005). Behaviors elicited from CMS animals have been shown to be similar to those of depressed humans, and therefore is one of the better available models (Willner, 2005). CMS is most advantageous because it utilizes techniques from other models in an unpredictable fashion to mimic the variability of stressors that commonly cause human MDD. Each of the stressors used in study were taken from the Bekris et al. protocol, but the schedule was not as sever due to time constraints. Stressors to be used for the current study include individual housing, restraint, predator odor, foreign cage/foreign object, food and/or water deprivation, 30^o cage tilt with no bedding, and wet cage bedding. Behavioral and immunohistochemical (IHC) staining analyses are used in the current study to determine the level of depression-like behavioral and neural effects in the rats.

Behavioral testing is used to determine depressive-like behavior in rats. The three behavioral tests performed were the Forced Swim Test (FST), the Open Field Test (OFT), and the Sucrose Preference (SP) test. The FST was developed in the 1970's by Porsolt et al as an effective animal model of depression (1977). A different set of protocols were developed for using rats in the test opposed to mice (Porsolt et al. 1978b). The animal is given a 15 minute pre-exposure to the apparatus on the day before the actual test. The test looks for three different behaviors in the each rat defined as climbing, swimming and immobility. Porsolt showed that depressed rats display less "active" behaviors such as climbing and swimming when compared to non-depressed animals. Depressed rats also spend more

time performing “passive” behaviors (immobility).

The OFT used in this study was modified from van der Staay et al (2009). This test has been used to measure anxious behavior in rats. Rats are placed on a black, square stage with black walls approximately 30cm tall. The stage is divided into two sections: the center and the surround. The center is the inner square of the stage. The surround is the area of the stage not occupied by the center square. The OFT is used to evaluate anxious-like behavior in rats. This test measures total time of immobility, total time in the center, and total distance travelled as dependent variables. Non-anxious rats spend less time being immobile, more time in the center and exhibit higher locomotor activity (Hiroi & Neumaier, 2009).

SP testing has been performed to evaluate anhedonic behavior in rats. This study uses a modified version of the test from Ying et al. (2009) and Pothion et al. (2004). Anhedonic behavior, or a loss of interest in pleasure, is one of the diagnostic features of MDD (American Psychiatric Association, 2000). Sucrose preference is defined as total volume of sucrose consumed divided by the total volume of liquid consumed. Willner et al (1987) were the first to show the decrease in sucrose preference in depressed rats as a measure of anhedonic behavior.

The Morris Water Maze was developed by Morris et al to demonstrate hippocampal dependant spatial learning in rats (Morris, Garrud, Rawlins, &O’Keef, 1982). This same study showed that rats with hippocampal lesions had decreased performance in the Morris Water Maze. The hippocampus has been shown to be important for spatial learning (Amaral & Witter, 1989). As discussed previously, hippocampal degeneration has been shown to be a component of MDD (Leonard, 2007). The Morris Water Maze will be used in this study as a measure of hippocampal function as affected by chronic mild stress. A white, circular tub is filled with water made opaque by paint. The tub is typically divided into four quadrants and a clear, colorless platform is placed in one of the quadrants and left in the location for the entirety of the trials. Visual cues are placed in the area surrounding the tub to ensure

hippocampal dependence in the task.

Hippocampal function, specifically microglial activation and neuronal cell counts was performed using IHC procedures. Microglial cells are normally found in the brain in their inactive state. Only once the cell is activated will it begin to release cytotoxic and inflammatory mediators (Gehrmann, Bonnekoh, Miyazawa, Hossman, & Kreutzberg, 1992). One of the neurochemicals produced by activated microglial cells is known as CD11b. To ensure accuracy of the first marker, another histological stain was performed using the Ox-42 antibody (Rana, Stebbing, Kompa, Kelly, Krum, & Badoer, 2010). This marker was used to detect morphological changes in microglial cells between groups (Bloss, Hunter, Waters, Munoz, Bernard, & McEwen, 2008, Bulloch, et al., 2008). Finally, a neuronal nuclei (NeuN) will be counted using NeuN antibody. NeuN is a neuronal specific marker for nuclear protein only found within neurons, distinguishing it from glial cells (Kumar & Buckmaster, 2007).

Animals were exposed to a chronic mild stress paradigm for 21 days and injected with GW405833, daily. After CMS, anhedonia, anxious-like, and depressive-like behavior were assessed along with spatial learning capabilities, microglial activation and neuronal nuclei in the hippocampus. The purpose of this study is to evaluate the potential antidepressant and neuroprotective effects of chronic CB_2 agonism during stress.

Methods

2.1 Animals

Thirty-two male Sprague Dawley rats were used for the experiment. The rats weighed, on average, 190g at the start of the experiment and 330g by the end of the experiment. The rats were randomly placed into one of four groups: 1) saline control (non-stress), 2) saline stress, 3) CB_2 control (non-stress), and 4) CB_2 stress. Rats 1-8 were assigned to the saline control group and housed in pairs for the duration of the experiment. Rats 9-16 were assigned to the saline stress group and housed singly for the experiment. Rats 17-24 were assigned to the CB_2 control group and were housed in pairs. Rats 25-32 were assigned to the CB_2 stress group and were housed singly. The rats were approximately 45 days old at the time of arrival at the facility. The rats were all housed in plastic cages with wire frame tops and alpine woodchip bedding. All rats in the facility receive a transparent, red, plastic enclosure to use as additional housing within the cage. Two days after arrival, the red enclosures were removed from the stress rat cages. Control rats were given access to food and water at all times and kept on a light cycle with the rest of the facility (12h light/12 dark). Stress rats were placed in the same conditions unless stated otherwise. Procedures were approved by the Connecticut College Institutional Animal Care and Use (IACUC) before commencement of the experiment.

2.2 Drug

Rats in the saline-control (group 1) and the saline-stress (group 2) received daily intraperitoneal injections of saline (1 ml/kg of body weight). Rats in the CB_2-control (group 3) and the CB_2-stress (group 4) received daily intraperitoneal injections of CB_2 agonist GW405833 (1 mg/kg of body weight). GW405833HCl is lipophilic and therefore was first dissolved in hydroxypropyl-β-cyclodextrin (HPBCD) which was diluted to a final concentration of 25% HPBCD in distilled water. The final concentration of injectable GW405833 was 1 mg/ml. The drug was measured out and prepared daily

via sonication in 45^{o}C water for 45 minutes. For the stress groups, injections were given prior to the first stressor of the day, for 21 days, unless otherwise stated. The non-stress groups received injections at approximately the same time.

2.3 Unpredictable Chronic Mild Stress

Rats in the stress only and experimental groups were exposed to the CMS protocol outlined in the appendix (see Table 2). This protocol has been modified from Bekris, Antoniou, Daskas, & Papadopoulou-Daifoti (2005). Chronic mild stress took place over the course of 25 days. Rats in the stress paradigm were not given the full amount of time to acclimate to their new living situation. Animals in the stress groups began injections two days after arriving to the animal facility whereas rats in the control group began injections seven days after arriving. The various stressors included behavioral testing, 30^{o} cage tilt, restraint, food and/or water deprivation, wet cage bedding, predator odor, constant light, and foreign cage/foreign object. Stress rats received a daily stressor and an overnight stressor. Daily stressors were administered for one to two hours during the day (exact duration of stressors detailed below). Overnight stressors were terminated the following morning between 9am and 11 am.

For 30^{o} cage tilt bedding was removed and one end of the cage was titled 30^{o} above the horizontal plane. This stressor lasted two hours as a daily stressor. During restraint rats were placed in plastic restraint tubes for one hour (7in length, 3 in diameter). Restraint was only used as a daily stressor. Food and/or water deprivation was used as primarily as an overnight stressor, but was also used as a 24 hour stressor during other periods. During these periods, food and/or water were removed from the rats' cages. Wet cage bedding was an overnight stressor only. This involved 150ml of tepid tap water being poured over the bedding of the cage. For predator odor, 10 or 20 µl (see appendix 1) of trimethylthiazoline (TMT) was pipetted onto a small piece of filter paper and placed on top of each

cage. Water was removed from the cages at the start of the stressor and replaced after the stressor was over. Rat cages were placed under a fume hood to minimize odor dissipating to other lab areas. After the hour exposure, rats were put into new cages with fresh bedding to avoid contamination of the housing facility with TMT odor. During constant lighting stress, rats were put in a separate room from the other animals, and exposed to constant light for either 36 or 60 hours. For foreign cage/foreign object, rats were placed in separate wire-frame cages without bedding. The cages were a completely new environment for the rats upon their first exposure. Inside the cage was a round glass fishbowl (1 gallon) containing a large rubber snake. The object was used mainly to take up extra space in the cage. These stressors were varied over a period of 3 weeks as seen in the timeline (appendix 1). After 3 weeks, injections stopped, but overnight stressors continued for the duration of the testing.

2.4 Behavioral Testing

Behavioral testing was performed both before and after the injection period. Sucrose preference testing occurred on the night before the first day and the penultimate night of injections. Open field testing took place on the first day of stress and the last day of stress (see timeline).

Sucrose Preference

Testing began on the stress rats on the third afternoon after their arrival at the facility with sucrose preference testing (testing began 5 days later for control rats). Water bottles were filled with approximately 125mL of 1% sucrose in tap water. A second water bottle was filled with tap water and placed in each cage. Both bottles were weighed immediately prior and immediately after exposure. For testing on control animals (which were housed in pairs), one animal of each pair was placed in a separate cage for the night to determine individual behaviors. After 16 hours, both the bottles were removed from the each rat cage and weighed to measure fluid intake. At this time, control rats were put back into their normal paired cages.

Open Field Testing

Open field testing was based on the procedure by van der Staay, et al (2009). Following the end of sucrose preference testing, open field testing was performed. A 3m x 3m black plastic arena with 1m walls on all sides (originally used for Cincinnati Maze testing) was set up under a web camera (Fire-i firewire digital camera, Unibrain). The webcam was attached to a pole directly above the apparatus. The arena was divided into two regions defined as center and surround. The center square was defined as the inner area of the arena with the perimeters of the region 20cm from the closest side of the whole arena. Rats were placed individually in the apparatus for 5 minutes. During the 5 minute period, the experimenter left the room, so as to not influence the behavior of the animal in any way. Video recordings of each animal were analyzed using Any-maze video tracking software. Measures included time spent immobile in the center and surround, as well as overall time in the center square. The apparatus was cleaned between subjects. After the first open field testing period the animals were weighed and received their first injection.

Forced Swim Test

A white bucket (40cm high) with round top (30.5cm) was used for the test. The apparatus was filled to a depth of 30cm using 29-33^{o}C water. The day before the testing, the animals were given a 15 minute assimilation swim period in the apparatus. The animals were dried off with towels immediately after being removed from the water. During the testing period the animals were recorded using a Fire-i firewire digital camera located on a tripod directly above the apparatus. Animals were individually brought into the testing room and immediately put into the water for five minutes. The experimenter left the room immediately after placing the animal in the water, but observed the animal via the webcam. After five minutes, the animal was removed from the water and dried off using a towel before being put back into the cage. Animals were scored for immobility, swimming, and climbing by using a sampling technique to rate the predominant behavior over a 5 second interval (60 total counts over 5

minutes). Immobility is defined as absence of all movement except motions required to keep the head above the water. Climbing is defined as thrashing movements along the sides of the water tank while swimming behavior consists of horizontal motion moving from one quadrant of the water tank to another. After completion of the 5 minute trial, animals were dried off and returned to their cage. Video recordings were analyzed by a blind, unbiased experimenter.

Morris Water Maze

The Morris Water Maze procedure (Morris, 1984) was conducted over the course of days 22-25 for all animals. Animals in stress conditions (groups 2 and 4) continued to be exposed to variable mild stressors at night during the Morris Water Maze testing period (see appendix). A small pool (diameter of 4 ft, divided into four quadrants: north, south, east, west) with a platform in the northeast quadrant was filled to a depth of 13 inches with water (22-25^{o}C) so the water covered the clear, colorless, plastic platform in the northeast quadrant by 1 inch. Three pieces of paper, with distinct patterns and colors, were placed on the north, east, and west walls around the maze. The water was then made opaque by adding one full bottle of white tempura paint. A webcam (Fire-i firewire digital camera) was set up directly above the apparatus to record the animals. Prior to being placed in the pool, rats were placed into plastic cages with no bedding and were put back in these cages for the duration of each four trial period. Rats were measured for their latency to find the platform. Rats were tested four times per day for four consecutive days. For each animal, the latency to find the platform was compared over each trial. After each trial was completed, animals were hand dried using a towel, and then put back into the no-bedding cages. Following the 16th acquisition trial (4th trial on day 4 of testing), expression of spatial memory was assessed by removing the platform and recording the time spent in the platform quadrant. After the final trial of each day, the animals were put back into their normal cages with bedding.

2.5 Sacrifice

The day after the Morris Water Maze trials were completed, animals were individually euthanized in a carbon dioxide chamber. Each animal was transcardially perfused with 0.1M phosphate buffered saline followed by 4% paraformaldehyde. Brains were removed and placed in 4% paraformaldehyde solution. After three weeks, brains were transferred to 30% sucrose with sodium azide solution.

2.6 Histology

Forty micron, coronal hippocampal sections (approximately 3.14mm posterior to Bregma, Paxinos & Watson, 1998) were processed for IHC analysis for activated microglial cells and neuronal cells. Mouse anti- NeuN (Millipore, Temecula, CA) IHC was performed using diaminobenzidine (DAB) at a dilution of 1:1000 and a biotinylated goat anti-mouse secondary (Millipore, Temecula, CA) antibody with a dilution of 1:300. Mouse anti-Ox-42 (Seroec, Oxford, UK) IHC was performed, also with DAB, using a primary antibody dilution of 1:5000 and a biotinylated goat anti mouse secondary (Millipore, Temecula, CA) with a dilution 1:800.

Tissue sections were prepared for IHC by being washed three times for ten minutes each time in 0.01M phosphate buffer saline (PBS). The sections were then transferred to a blocking solution for 30 minutes. The blocking solution was made of 0.5% Bovine Serum Albumin (BSA) in 0.01M PBS. Tissue was then transferred to primary antibody solutions. Each primary antibody was added to solution with the dilution mentioned above. Primary antibody solution also contained 0.1% BSA and 0.25% of 30% Triton-X. Tissue soaked in this solution overnight on a shaker.

Approximately 24 hours later, tissue was washed again in 0.01M PBS three times, for ten minutes each time. Tissue was then transferred to secondary antibody solution. Biontinylated goat-anti mouse secondary antibodies were added to the solution with the dilutions mentioned above. The

secondary solution also contained 0.1% BSA in 0.01M PBS. After 30 minutes in secondary solution, tissue was transferred to 0.01M PBS for 3 more 10 minute washes. At this point, ABC solution was made using 2 drops of Avidin-biotin and 2 drops of horseradish peroxidase to every 10 mL of 0.01M PBS. The ABC solution mixed on the shaker for 30 minutes prior to use. The ABC detection step was then performed on the tissue. After 30 minutes, the tissue was then transferred to 0.01M PBS for 3 more washes for 10 minutes each.

3, 3-Diaminobenzidine (DAB) was used for the final reaction step. DAB tablets were placed in 50mL of 0.01M PBS with 25µL glucose oxidase and sonicated for 10 minutes. Twenty-five milligrams of D-glucose was then added to the solution. The tissue treated with NeuN was incubated in the solution for approximately 10 minutes whereas the tissue treated with Ox-42 was incubated for approximately 25 minutes. The tissue was washed again in 0.01M PBS 3 times for 2 minutes each time, then washed with 0.1M PB 3 times for 2 minutes each time.

Sections were placed onto slides and left to dry overnight. The slides were then dehydrated and coverslipped for microscope evaluation. Ox-42 treated tissue was analyzed for morphological changes and NeuN tissue neuron staining was quantified using IPLab 3.6. Sections of the CA3 region of the hippocampus (between 1-3mm lateral from the midline, Paxinos & Watson, 1998) were viewed using the Olympus BX41 Microscope under 10X magnification. Activated microglial cells were evaluated as having large, dark cell bodies. Neuronal nuclei were counted as dark round bodies and measured using the intensity of color in each image (the darker the area, the more intensity it would have).

2.7 Statistical Analysis

One-way analysis of variance (ANOVA) with Tukey Post-Hoc tests were performed on all dependent measures using SPSS. A P value of <0.05 was considered to be significant.

Results

3.1 Reward-seeking behavior

Sucrose preference was calculated by dividing the total volume of sucrose consumed by the total volume of liquid consumed (Figure 1). There was no significant effect seen between groups when comparing pre-CMS to post-CMS scores ($F[7,51] = 1.077$, $p = .392$). However, significant effects were seen when comparing the percent change in sucrose consumption between groups ($F[3,24] = 4.109$, $p < .05$, see Figure 2), but no significant differences were seen between groups for total water consumption ($F[3,25] = 2.752$, $p = .064$). Tukey Post-Hoc comparisons revealed the saline stress group as having a significant decrease in sucrose drinking when compared to the CB_2 stress and the saline control groups.

3.2 Anxious Behavior

In the Open Field Test, animals were scored before and after the CMS period on total distance travelled, total time in the center of the maze, and total entries in the center. The difference in scores was calculated by subtracting the scores before the CMS period from the scores after the CMS period. The change in total distance travelled (Figure 3) showed no significant effects ($F[3,28] = 1.016$, $p = 0.400$). However, significant effects were seen for change in the number of center entries ($F[3,28] = 7.522$, $p < .05$, Figure 4) and in change in time spent in the center of the maze ($F[3,28] = 4.268$, $p < .05$, Figure 5). The saline control group was found to enter the center fewer times than the saline stress group and the CB_2 stress group. The saline stress group was also seen to spend significantly more time in the center than the CB_2 control group.

3.3 Depressive behavior

All raw data from the Forced Swim Test was converted to a percentage score by dividing the

initial score by the total number of time intervals the animal spent in the apparatus. Since each time trial of 5 minutes was divided into 5 second intervals, each trial consisted of 60 intervals. Because standard deviations were extraordinarily high for each group (Table 3), only one ANOVA was run per behavior and scores were not analyzed further (see Figure 6).

Climbing

There was a significant effect seen between groups ($F[3,28] = 6.934$, $p < .05$). Post-Hoc Tukey tests showed that the saline control group had significantly higher scores than the CB_2 control group. The saline stress group also had significantly higher percentage than the CB_2 control and CB_2 stress groups.

Swimming

Significant differences were seen between groups for the percentage of time spent swimming ($F[3,28] = 2.980$, $p < .05$). Tukey tests revealed that differences were only seen between the CB_2 stress group and the CB_2 control group with the CB_2 stress group having a higher percentage of time spent swimming than the CB_2 control group.

Immobility

There was a significant effect between groups for percentage of time spent immobile ($F[3,28] = 5.527$, $p < .05$). Tukey Post-Hoc tests demonstrated that CB_2 control animals spent significantly more time immobile than both the saline control and saline stress groups.

3.4 Spatial Learning and Memory

Latencies

Morris Water Maze testing was performed with four trials per day over the course of four days (Figure 7). The latency scores of each day were averaged to a mean latency per day (see Figure 8). When comparing the averages of latencies each day, ANOVA testing showed significant differences

between groups on day 3 (F[3,28] = 5.346, $p < .05$) and day 4 (F[3,28] = 10.208, p < .05). Tukey Post-Hoc comparisons showed that the saline stress group latencies were significantly higher than the CB_2 stress group and the saline control group on Day 3 trials, and higher than all the groups on Day 4 trials. The groups were also compared by the last trial latencies of each day to reveal a significant effect on the last trial of Day 4 only (F[3,28] = 4.185, $p < .05$). Tukey tests revealed that the saline stress rats had significantly higher latencies than the CB_2 control rats and the saline control rats, but not the CB_2 stress rats.

No Platform trial

In the final trial of the Morris Water Maze, the platform was removed from the North East quadrant and rats were given one minute to explore the maze (Figure 9). ANOVA comparisons revealed no significant differences between groups when comparing the time spent in the North East quadrant of the maze (F[3,28] = 1.44, $p = .253$).

3.5 Immunohistochemistry

Cell counts were performed in the tail end of the CA3 region of the hippocampus between groups after staining with a NeuN marker (Figure 10). ANOVA statistical analysis revealed significant effects between groups (F[3,53] = 15.170, $p < .05$). Tukey Post-Hoc tests showed that the saline control group had significantly higher cell counts than all three of the other groups. However, the saline stress group was seen to also have significantly lower counts than both the CB_2 stress and CB_2 control groups (see Figure 11). Ox-42 sections revealed no microglial cells or other stained bodies upon microscopy analysis and were therefore not used. This result was most likely due to an improper dilution of either primary or secondary antibody.

Discussion

The goal of this project was to investigate the effects of CB_2 agonism on depressive-like behaviors and hippocampal neurodegeneration in rats. This is one of the first experiments to investigate the CB_2 receptor as a possible target for neuroprotection from chronic stress. Research has shown that chronic stress causes hippocampal neurodegeneration via activation of microglial cells (Leonard, 2007). More recent studies have revealed that CB_2 receptors are not only present in the brain (Galiegue, et al., 1995), but are also responsible for inhibition of the microglial cells and promotion of neural growth (Fernandez-Ruiz, Pazos, Garcia-Arencibia, Sagredo, & Ramos, 2008). The functions of the CB_2 receptor implicate it as a possible mediator of neuroprotection and treatment for depressive symptoms.

Overview of Results

Since one focus of this project was to assess the CB_2 receptor in treating depressive-like behaviors, it must first be determined whether the animals displayed depressive behavior. The sucrose preference test for anhedonia showed no effect between groups. This suggests that no group of rats displayed reward-seeking behavior more or less than another group. The open field test for anxiety also showed no significant differences between groups when looking at features such as center time, distance travelled, and total immobility time. Once again, this suggests that no group showed more anxious behavior than another group. Finally, the Forced Swim test, one of the most commonly used tests for depressive-like behaviors in rats, did display significant differences. However, these differences were obscured by large standard deviation scores and seemingly random scores for each group. In order to fully understand the implications of these results, the validities of each model must be assessed.

The Sucrose Preference Test

The sucrose preference test is not a widely used or evaluated procedure. A PubMed search for *sucrose preference* and *depress** came back with no reviews, and limited information on the test itself. Most papers simply use a modified procedure from a previous study, yet there seems to be no complete method for procedures. One failing of this study when compared to other studies methods for this test is in the pre-exposure to sucrose solution. Other studies generally give the rats an acquisition period to two water bottles present in the cage (Pothion, Bizot, Trovero, & Belzung, 2004). Whether this pre-exposure plays a role in the results from this test is uncertain, but it could play a role in the insignificant results from this experiment. However, this study had time constraints that did not allow for additional pre-exposures to the test. Another inconsistency between experimental methods is in the exposure time to the sucrose. Some experiments only administer sucrose test one time, for anywhere between 1 and 24 hours, whereas other experiments administer the sucrose test multiple times a week over many weeks (Li, et al., 2009).

The final inconsistency is in the scoring method. The most commonly used method for scoring is the same as the one used in this study, the calculation for sucrose preference. This calculation simply divides the volume of sucrose consumed by the total volume of liquid consumed (water and sucrose). However, other studies have qualified sucrose preference as percentage change compared either to another group to a previous exposure from the same group (Saenz, Villagra, & Trias, 2006). If this method was used for calculating sucrose preference, then it could be determined that all groups greatly increased sucrose drinking habits from pre-CMS to post-CMS by approximately 40% or greater except the saline stress group, which drank more than 50% less sucrose on the second exposure. By this methodology, the sucrose preference test did come back with significant results that showed extreme differences between the saline stress group and the stress group treated with the CB_2 agonist GW405833 as well as the saline control group. However, as seen by Figure 2, standard deviations were much larger for this calculation than for the original sucrose preference calculation. This must be taken

into account for future studies as the effect could be changed with larger populations. Therefore, more investigation must be done to determine whether sucrose preference or sucrose intake are affected by CB_2 agonism when using CMS.

The Open Field Test

In this experiment, only two of the three parameters in the open field test showed significant effects. The effects that were seen seemed to show that the saline stress and CB_2 stress groups both had lower anxious-like behavior than the control groups. Due to the data, it is impossible to draw any conclusions about the effects of CB_2 agonism on anxious behavior. This poses a few potential conclusions: 1) the model has low validity, 2) the dependant variables were not effective at evaluating anxious behavior, or 3) the stressors allowed the stress animals to habituate to anxiety-producing situations 4) another variable was not accounted for. Each of these offers potential explanations for the outcomes of the test.

One major problem with this test is the inconsistency between studies in terms of actual apparatus set-up, behaviors monitored, and length of time in the maze. Previous studies have reported immobilization time and center entries as means of determining anxious-like behaviors (Staay, Schuurman, Reenen, & Korte, 2009, Hiroi & Neumaier, 2009, Zhang, Zhang, Sun, Cao, & Zhang, 2009). However, these studies determined that decreased center entries and increased immobilization time was indicative of anxious behavior. The present study showed nearly the opposite effect that was expected. A possible explanation for the effects in this study could be seen in the housing of the rats. Control rats in this study were housed in pairs, whereas stress rats were housed individually. In the studies cited previously, all rats were housed in pairs in all conditions. The individual housing was used as an additional stressor for stress rats, but this could have played a role in the change seen in anxious-like behavior.

In terms of CB_2 agonism and anxious behavior, it is important to note that no study has implicated the effects of CB_2 and the attenuation of anxious behavior. Regardless of the findings on CB_2 and anxious behavior, GW405833 has been found to not affect anxious behavior (Valenzano, et al., 2005). This does not speak for all CB_2 agonism, just for the drug used in this experiment, as agonists with differing affinities for the receptor could cause different effects.

The Forced Swim Test

Forced Swim test data showed seemingly random effects between groups, but did nothing to demonstrate that animals were displaying any depressive-like symptoms. Generally, depressed animals have been seen to display less immobile like behavior when treated with standard antidepressants (Porsolt, Le Pichon, & Jalfre, 1977). However, many researchers have questioned the validity of this test (Lopez-Rubalcava & Lucki, 2000). Lopez-Rubalcava and Lucki (2000) showed that differences in rat strains cause large changes in the Forced Swim test behaviors. In the 2000 study, Sprague-Dawley rats were seen to elicit minimal differences in behaviors when comparing depressed to non-depressed animals, especially when compared to Wistar-Kyoto rats. Sprague-Dawley rats were also seen to have higher baseline climbing rats than Wistar-Kyoto rats.

The forced swim test was developed as a model for antidepressant treatment and a model for depressive-like behaviors. However, these are extremely different purposes that could point to a major flaw in the design of the model. For example, many researchers use the test to only demonstrate the effectiveness of antidepressants. One problem with this is that many antidepressants take weeks to take action in humans, yet the protocol for most experiments only requires antidepressant administration in an acute manner. This could point to vast differences in the serotonergic systems between the human and rat brain, yet no studies have been done to investigate this claim. Many studies looking for antidepressant efficacy only use the forced swim test to elicit depressive-like behaviors, whereas this

study used CMS for this purpose, and the FST to evaluate the depression. The FST model was developed to elicit depressive-like features on its own, and not to evaluate animals that were already experiencing depression. If the purpose of the test is to cause depressive-like behaviors, then it requires separate evaluation as a means of diagnosing depressive-like behavior. Minimal studies have demonstrated that the forced swim test appropriately diagnoses depressive-like behavior in rats. Instead, studies tend to focus on whether non-depressed rats have lessened depressive-like symptoms when pre-treated with an antidepressant.

The final problem with this test is that no research has showed whether the forced swim test is primed for the standard treatments for depression or the test is sensitive to other types of treatments. The current, standard treatments for MDD all utilize direct increases of monoamine neurotransmitters. This test may be sensitive to serotonergic manipulation, but not to other types of manipulation that could alleviate depressive-like symptoms. Because of these confounds, the forced swim test is not effective enough on its own to be a complete test of diagnosing depression in rats. With the results seen from the sucrose preference test and the open field test, this test cannot be used as the only means for determining depression, and the other factors must be taken into consideration.

The Morris Water Maze

The Morris Water Maze tests for hippocampal dependant spatial learning. In the present study, the data from the Morris Water Maze demonstrated the ability of GW-405833 to diminish impairments in hippocampal dependant learning to rats exposed to chronic stress. Spatial Learning has been shown to be linked almost exclusively to hippocampal function (Amaral & Witter, 1989). Hippocampal function has also been seen to be interrupted in depression (Leonard, 2007). This study managed to replicate these previous findings by showing the decreased performance of saline stress rats in the maze when compared to a control group. Normal, healthy rats show a learning curve over the course of the

trials in which latency decreases per trial. However, the saline stress rats did not exhibit any normal learning pattern. Unlike the saline stress rats, the CB_2 stress rats showed a normal learning curve with no significant differences from the control rats. This effect demonstrates a difference in hippocampal dependant learning between the CB_2 stress group and the saline stress group.

However, when the platform was removed from the maze and rats were allowed to explore the space for a full minute, all groups spent nearly equivalent time in the north east quadrant (where the platform was placed for the trials). Without the platform, hippocampal dependant memory (not learning, as assessed previously) was evaluated. This shows a completely different effect from the learning curve seen in the Morris Water Maze. However, the data do show a trend that the saline stress group did spent less time in the north east quadrant than the other groups. Therefore, two separate conclusions can be drawn from the Morris Water maze: 1) CB_2 agonism alleviated hippocampal dependant learning impairments caused by CMS, but 2) CMS did not show to have a significant effect on hippocampal dependant memory.

To further evaluate the results seen from the maze, a follow-up test could be performed in future studies. After the first 16 trials, the platform could be moved to a different quadrant for another 16 trials. This addition would allow for the interpretation of both memory and learning in a hippocampal dependant task. It would also be a good follow-up in experiments that did not show significant differences between initial Morris Water maze tested animals. This would have been useful for this test as the animals did not show differences in memory function, but great differences in learning. This follow-up would evaluate the animals' ability to combine these two functions by evaluating the ability to adapt to the new location of the platform.

Immunohistochemistry for neuronal nuclei

Neuronal nuclei stains were performed to determine if CMS resulted in hippocampal neuronal cell lost that could be correlated with deficits seen in spatial learning. A significant effect was seen, showing that neuronal nuclei were less in number in the CA3 region of the hippocampus in the saline stress group when compared to the three other groups (see Figure 10). However, it was also seen that the saline control group had significantly higher neuronal nuclei counts than both the CB_2 groups. While this must be taken into consideration, the most important piece of data is that the saline stress rats had significantly lower neuronal nuclei counts than the three other groups. However, after careful review, previous studies have not shown that the CA3 region of the hippocampus is the source of neuron proliferation, but instead, where the projections of new neurons end. The subgranular zone of the dentate gyrus is the only place in the hippocampus known to have neurogenesis (Eisch, Cameron, Encinas, Meltzer, Ming, & Overstreet-Wadiche, 2008). The projections of the neurons refer to the synapse connections that occur with old neurons in the hippocampus. Therefore, the differences seen in cell counts demonstrate only the potential for neurodegeneration and do not account for the potential of CB_2 agonism to induce neural proliferation.

This data could be analyzed in a few ways: 1) the cell counts could be considered for each group, or 2) each stress condition could be compared to its corresponding control condition. In this analysis, the significance is even more pronounced with the saline rats showing a large difference between control and stress (difference in mean values = 46.15) compared to the difference between CB_2 control and CB_2 stress groups (difference in mean values = 5.73). Either way, a significant effect is seen, giving further evidence that CB_2 agonism attenuates neurodegeneration caused by stress.

This process, however, is easily skewed by experimenter bias, and must be scrutinized fully before being considered a legitimate method of counting neurons. The largest problem with this is in the analysis itself. The IHC process revealed large amounts of background staining of cell bodies without neuronal nuclei. The cell bodies appeared as light circles with an unfilled or partially filled

center. The excess noise in the hippocampal regions of interest made physical counting nearly impossible and easily skewed the detection in the IPLab3.6 analysis. This background posed a problem in that it was difficult to determine whether consistency in intensity and background cancellation was held throughout the counting process. Another extremely important variable was the experimenter bias that could have very easily given rise to the results seen. During the analysis of the hippocampal sections, the experimenter was completely aware of the condition of each animal. This was another unfortunate result of time constraints. The program is highly sensitive to and easily skewed by slight changes in degree of labeling as well as intensity of DAB staining. Therefore, it requires decisions by the experimenter on each brain as to how much intensity should be considered for each image. This provided the opportunity for the experimenter manipulate the image, which immediately added bias into the count. Because of this hypersensitive system, the neuronal counts must be evaluated further, with more precise and unbiased measurements. Without the confine of time, this could have been accomplished within the present study.

Future implications

This study provides enough meaningful information to merit the continuation of research on the cannabinoid 2 receptor and its function in stress and neuroprotection in the hippocampus. However, many areas could be improved upon in this study and more areas could be considered for future research. One extreme limiting factor of this experiment was the limitations of time. Experimentation was on an extremely sensitive time scale, leaving behavioral and immunohistochemical tests to a minimum. In addition to limiting the testing, the CMS procedure has been used for anywhere between 3 weeks (as used in this experiment) and 7 weeks (Bekris, Antoniou, Daskas, & Papadopoulou-Daifoti, 2005, Bielajew, et al., 2003). Animals under a longer stress regime could display more depressive-like symptoms which would provide better models for this experiment. The previously mentioned studies

have also shown that stress animals tend to weigh less at the conclusion of the experiment (Table 1). Unfortunately, due to time restraints, the rats in this experiment were not the same age at the time of CMS-period commencement and therefore had varying start/end weights ad could therefore not be used as a measure in this experiment.

Originally this study planned to investigate morphological changes to microglial cells to determine activation. However, problems with the IHC prevented this marker from working effectively. Future studies should look at activated microglial cells in comparison with neuronal markers to further the understanding of the CB_2 system in inhibiting activated microglial cells, as well as classifying activated microglial cells in depression. Animals should also be assessed for cytokines in their CSF such as TNF-α, IL-1, or apoptotic markers to determine cell death, such as NF-κB. All of these markers are known to be increased by microglial cells and inhibited by CB_2 agonism. Another interesting focus of future research could be investigating the effects on microglial cells with chronic inhibition. No study has published research on the actual effects on microglial function after long-term inhibition.

The current study could be modified to further classify the role of CB_2 in depression and stress. Genetic CB_2 knock-out, or, preferably, knock-down animals could be used to assess depressive behavior without chronic administration of a CB_2 agonist. Knock-out animals have the protein or receptor of interest removed from the genetic material so the animal does not have any of the protein of interest transcribed. CB_2 knock-out mice have already been developed (Gong, et al., 2005). Knock-down animals serve the same purpose, except in these creatures, the removal of the gene does not occur until later in development. Knock-down animals develop completely normally, allowing any experimental procedure to account for the protein of interest's role in development. Either knock-down or knock-out animals would allow for a fuller determination of the CB_2 receptor's role in stress and hippocampal function.

The endocannabinoid system must be better classified in terms of its role in the regulation of hippocampal function. As of now, research has focused mainly on the endocannabinoid ligands and their interplay with the CB_1 receptor, with very little focus on endocannabinoids and the CB_2 receptor. For the system to be completely understood, focus must shift from one receptor in the system to classifying all aspects of the endocannabinoids. Unfortunately, there is not much known on the mechanism of action of both AEA and 2-AG (the known endocannabinoid ligands). In fact, it is not completely understood how these molecules are even synthesized (Liu, et al., 2008). With all these unknowns, and the vast potential for the involvement of the cannabinoid system in multiple disorders, it is imperative that the study of the cannabinoid system continue.

Conclusions

Despite the limited information on the topic, the data provided thus far has shown that the cannabinoid system could prove to play an integral role in hippocampal function and depression. Pharmacological focus must shift from the current, stale theories on mood disorders to include neuroprotective therapies that are currently minimally investigated. Inflammation is becoming a larger focus of research on disorders from depression to Alzheimer's disease. With the inhibitory effects of cannabinoids on the inflammatory system, the CB_2 receptor should be a perfect candidate for future research in the study of neurological disorders. The current study showed that activation of CB_2 receptors during chronic stress diminishes degeneration in the hippocampus. This study provides a solid foundation for the further focus on cannabinoid receptor 2 in mood disorders. The continuation of research along this path could lead to novel insights into many degenerative diseases as well as unravel mysteries of hippocampal function and the development of memory.

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Tables and Figures

Table 1.

Animal Distribution

Drug Condition Weights(g)	*Number of Animals*	*Start Weights (g)*	*End*
Saline Control	8	196.4	373.2
Saline Stress	8	147.3	287.4
CB_2 Control	8	228.2	359.9
CB2 Stress	8	190.9	294.9

Table 2.

Variable Chronic Mild Stress Schedule

Day 1	**Day 2**	**Day 3**	**Day 4**	**Day 5**	**Day 6**	**Day 7**
End sucrose preference test Open field testing Start stress animal injections Overnight 30° cage tilt	1 hour restraint Begin food and water deprivation Overnight wet cage bedding	1 hours predator odor (10uL) End food and water deprivation Begin constant light	2 hours 30° cage tilt *Control: sucrose preference*	End constant light *Control: Open Field Testing* *Start control animal injections* Overnight food deprivation	1 hour restraint Overnight water deprivation	1 hour predator odor (10uL) Overnight foreign object/foreign cage
Day 8	**Day 9**	**Day 10**	**Day 11**	**Day 12**	**Day 13**	**Day 14**
1 hour restraint Overnight 30° cage tilt	Begin food and water deprivation Overnight wet cage bedding	1 hour predator odor (20uL) End food and water deprivation Overnight foreign cage/object	2 hours 30° cage tilt Overnight food deprivation	1 hour restraint Begin constant light	1 hour predator odor (20 uL) Overnight water deprivation	End constant light Overnight wet cage bedding

Day 15	**Day 16**	**Day 17**	**Day 18**	**Day 19**	**Day 20**	**Day 21**
1 hour restraint Begin food and water deprivation until next day	End food and water deprivation	1 hour predator odor (20uL)	2 hours 30° cage tilt Begin constant light	1 hour restraint	End constant light	Open field testing, forced swim test induction (15min)
Overnight 30° cage tilt	Overnight wet cage bedding	Overnight foreign cage/object		Overnight food and water deprivation	Sucrose preference testing	Overnight food deprivation
Day 22	**Day 23**	**Day 24**	**Day 25**	**Day 26**	**Day 27-31**	
FST (5min) Morris Water Maze Day 1	Morris Water Maze Day 2	Morris Water Maze Day 3	Morris Water Maze Day 4 *Control: Sucrose preference*	Sacrifice stress animals *Control: open field test, pre-forced swim test exposure*	*Control: Morris Water Maze day 1-4* *On Day 31: sacrifice control animals*	
Overnight cage tilt	Overnight light	Overnight wet cage	Overnight food/water deprivation			

*The night prior to **Day 1**, sucrose preference testing began for stress animals

**Protocol for non-stress (control) animals is written in italics. No protocol for stress animal is italicized.

Figure 1.
There were no significant differences seen between groups in terms of sucrose preference behavior, before or after the CMS period.

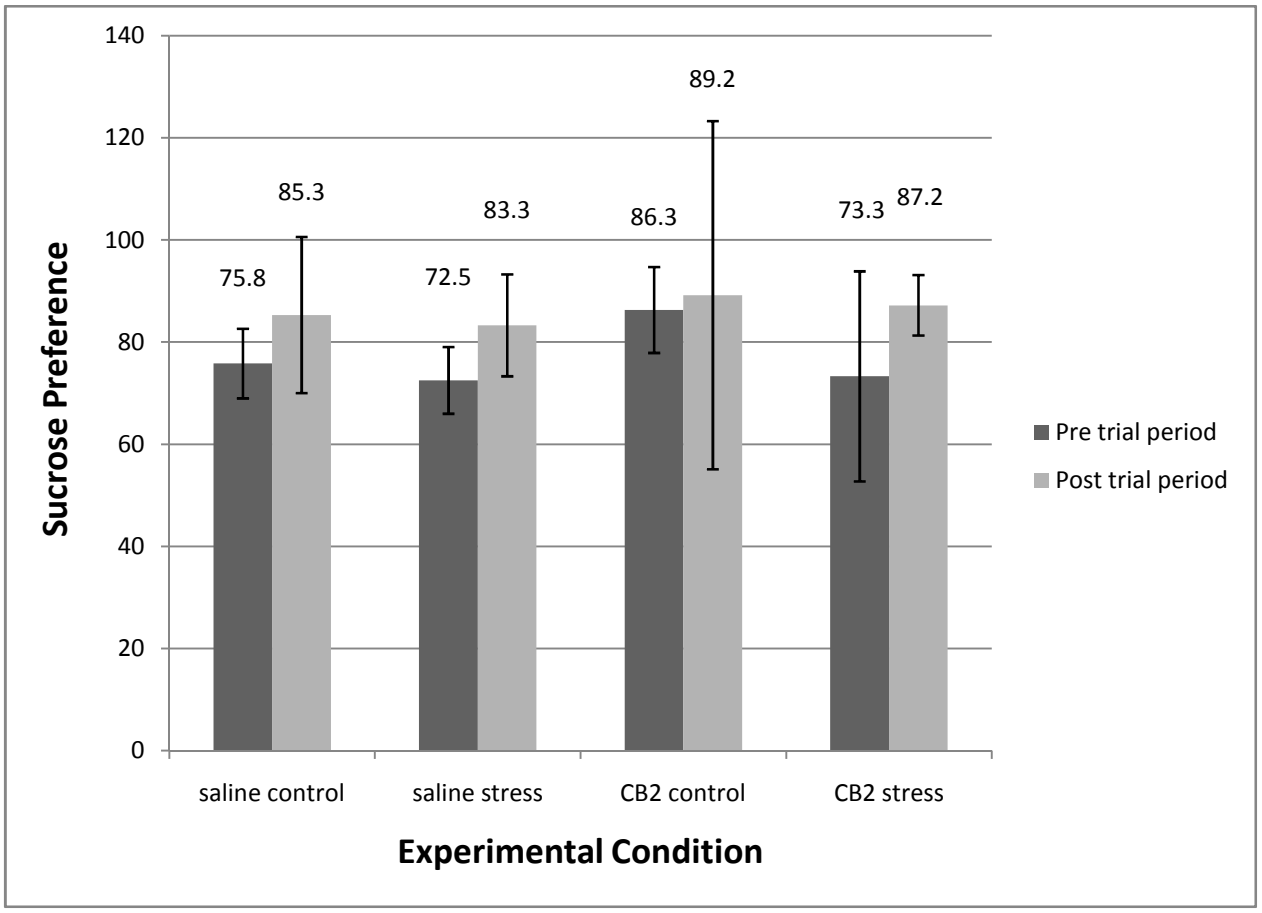
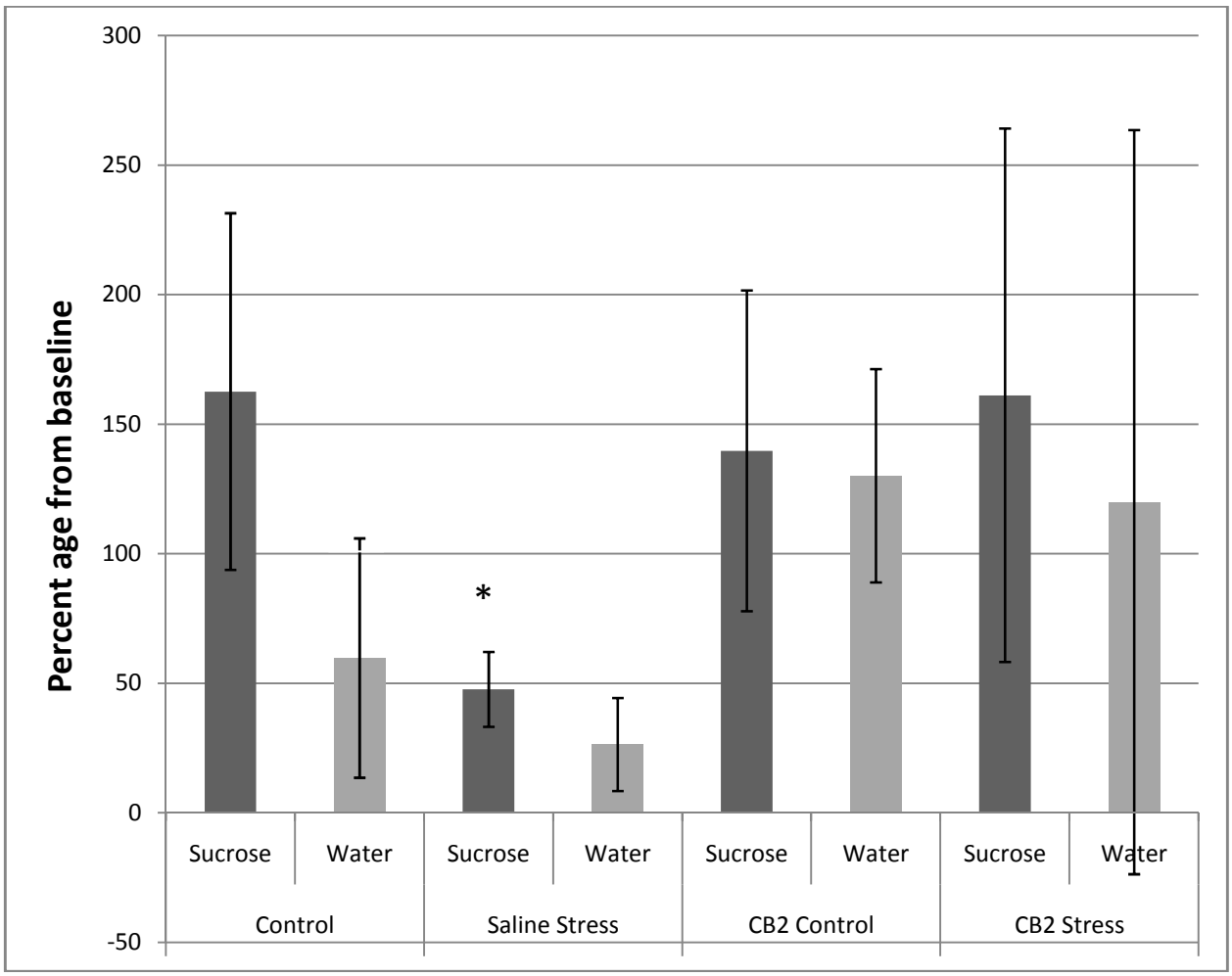


Figure 2.
The effect of drug and stress conditions on sucrose and water intake during the sucrose preference test when comparing the change in drinking behavior from before injection period to after injection period. A score of 100 would determine no change from baseline. Saline stress rats had significantly decreased sucrose intake when compared to Saline Control and CB_2 Stress groups. Percent water intake change revealed no significant differences. High standard deviations must be taken into consideration, but it is important to note the small deviations for most groups in terms of sucrose intake.



* $p < 0.05$

Figure 3.
The effect of drug and stress on change in total distance travelled in the Open Field maze. No significant effects were seen between groups.

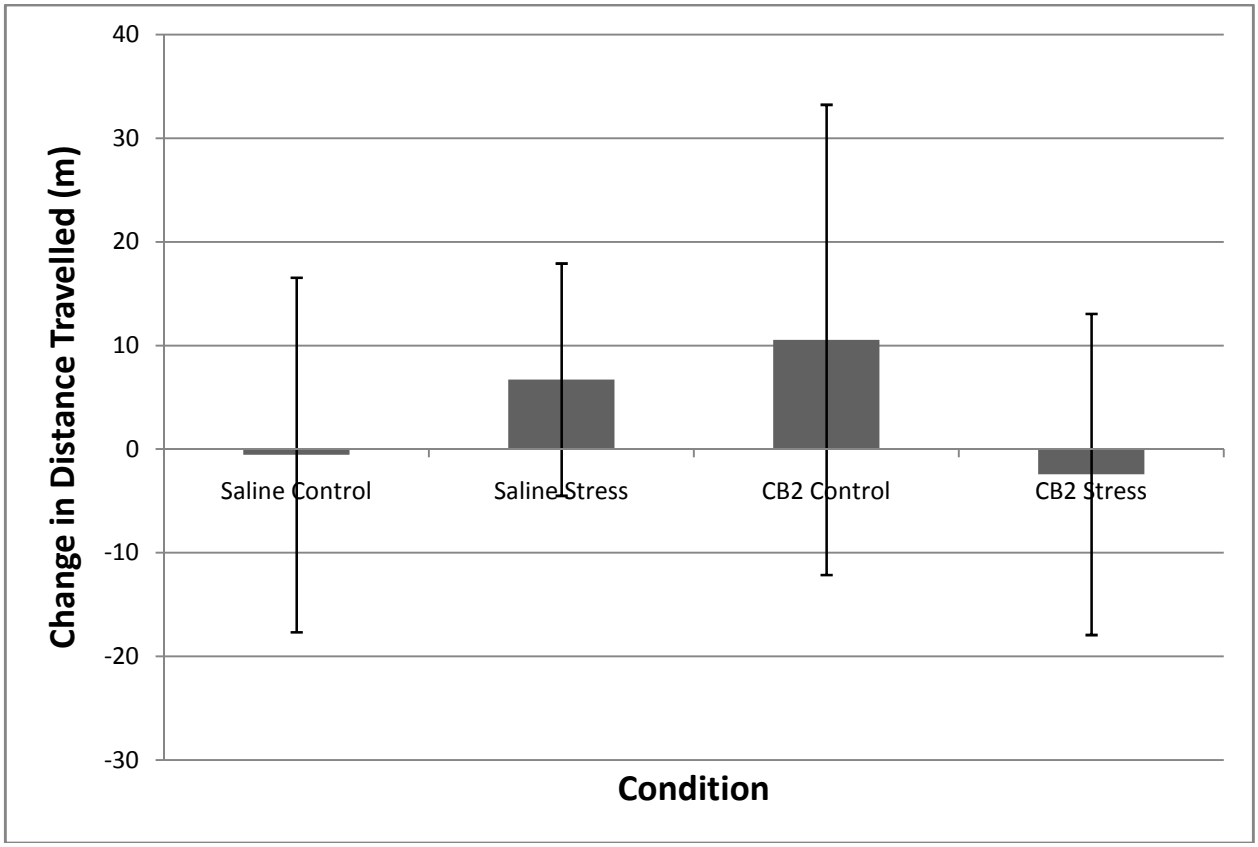


Figure 4.

The effect of drug and stress on total time spent in the center of the Open Field maze. The saline stress group differed significantly from the CB_2 control group only.

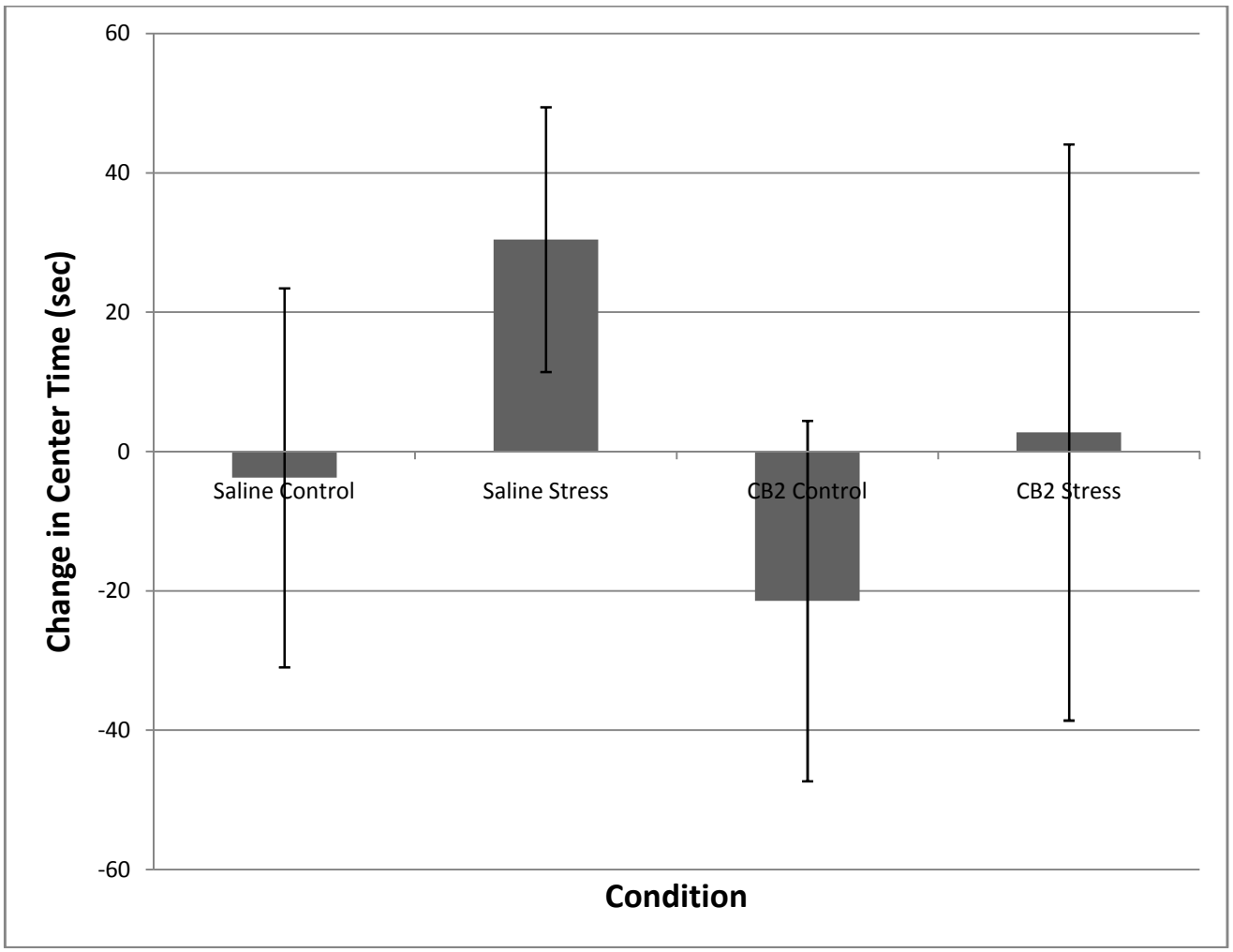


Figure 5.
The effects of drug and stress on change in number of entries into the center of the open field maze. The saline control group was seen to have a smaller change in center entries than the saline stress and CB_2 stress groups.

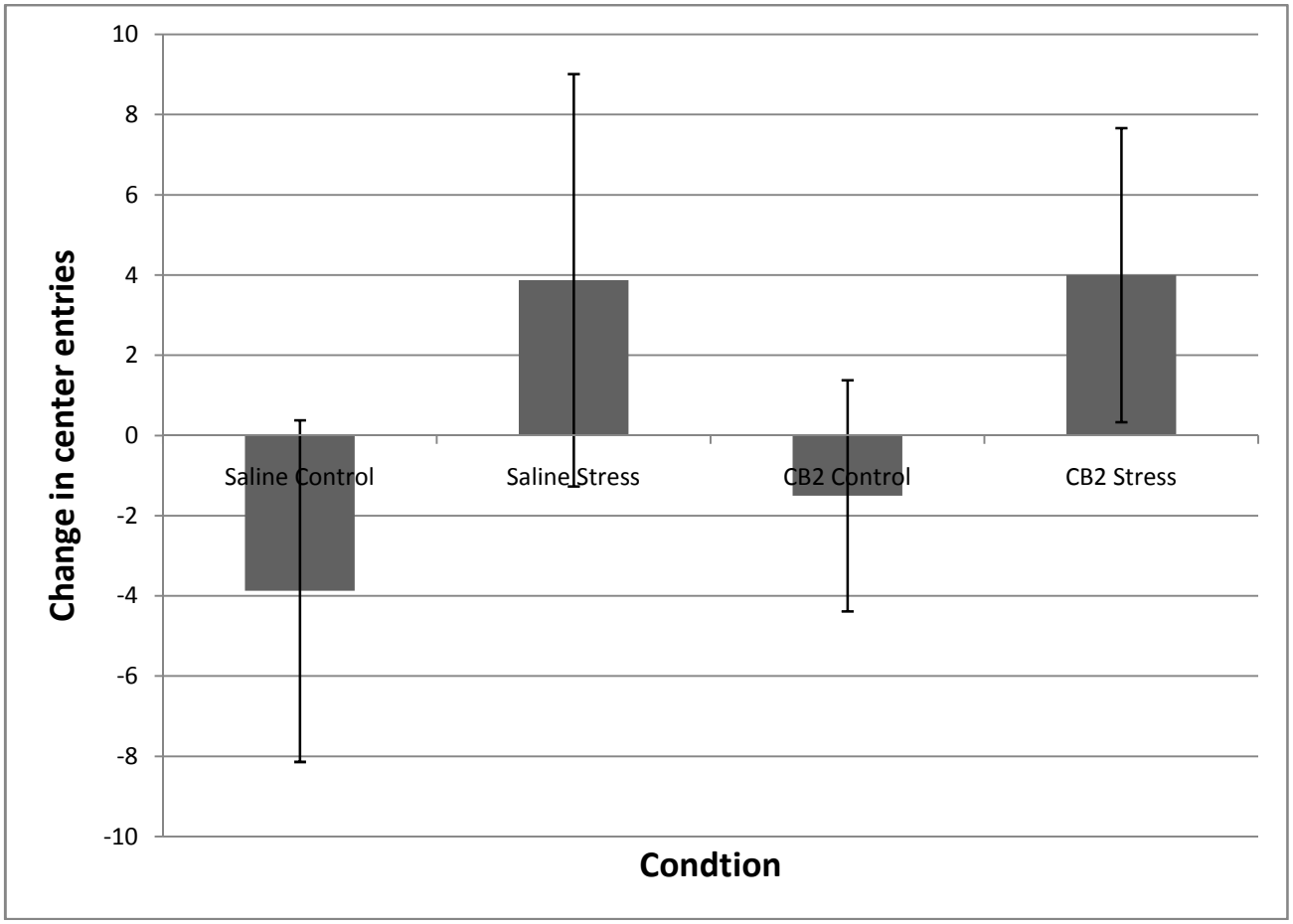


Figure 6.
T he effect of drug and stress on the percentage of time spent climbing, swimming, and immobility in the Forced Swim Test. Significant effects were seen in climbing between saline control and CB_2 control as well as saline stress and both CB_2 groups. For swimming, significant differences were seen between CB_2 stress and CB_2 control. Finally, in floating, CB_2 control animals were shown to be significantly different from both saline groups.

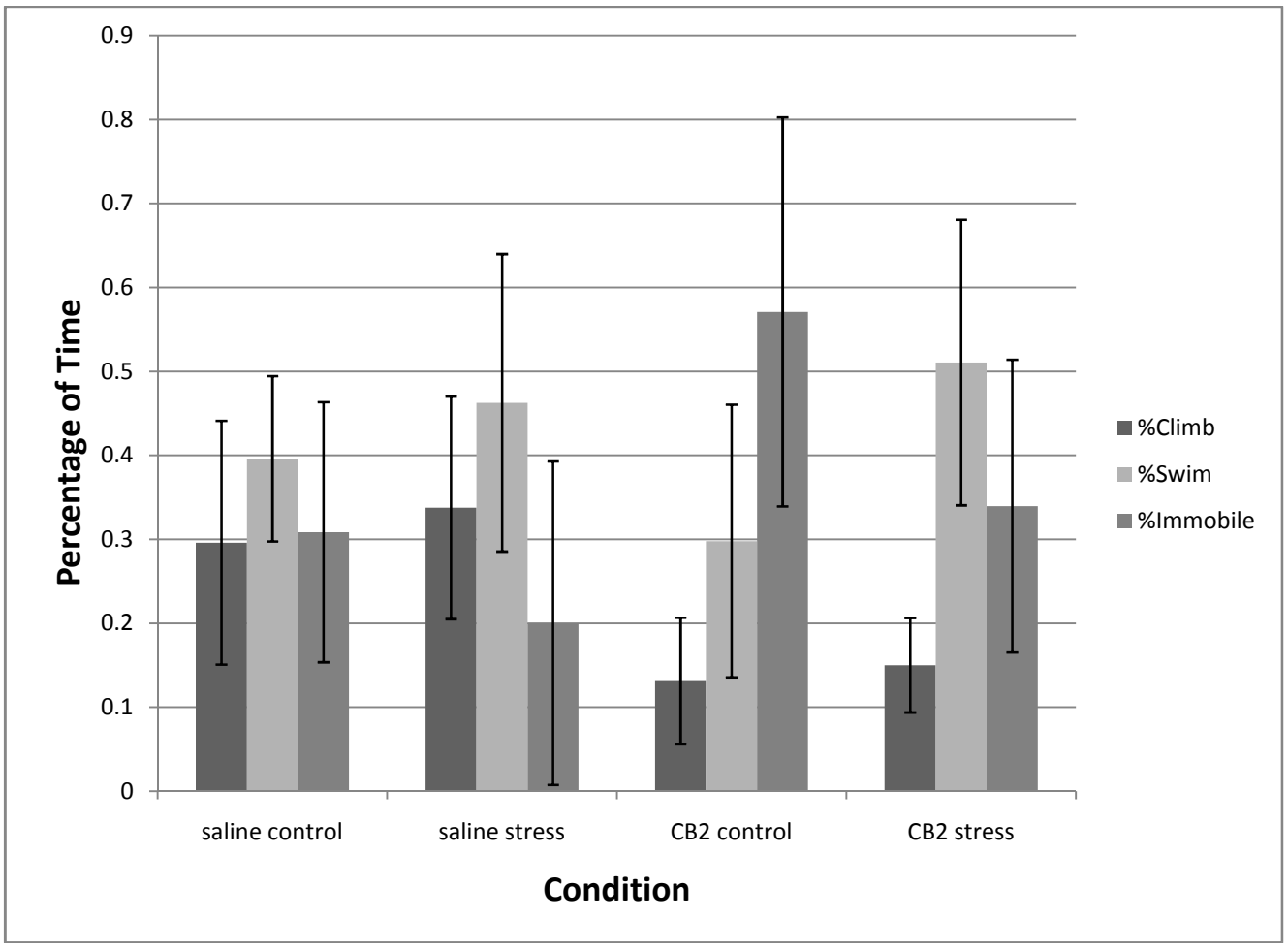


Table 3.
Standard deviations for each group and each measured activity in the Forced Swim Test.

Condition	*SD %Climbing*	*SD %Swimming*	*SD %Immobility*
Control	0.145	0.098	0.155
Saline Stress	0.133	0.177	0.193
CB_2 Control	0.075	0.162	0.231
CB_2 Stress	0.056	0.170	0.174

Figure 7.
The effects of drug and stress on latencies to find the platform in the Morris Water maze as seen by each trial. Saline stress rats showed decreased performance on trials 9-16.

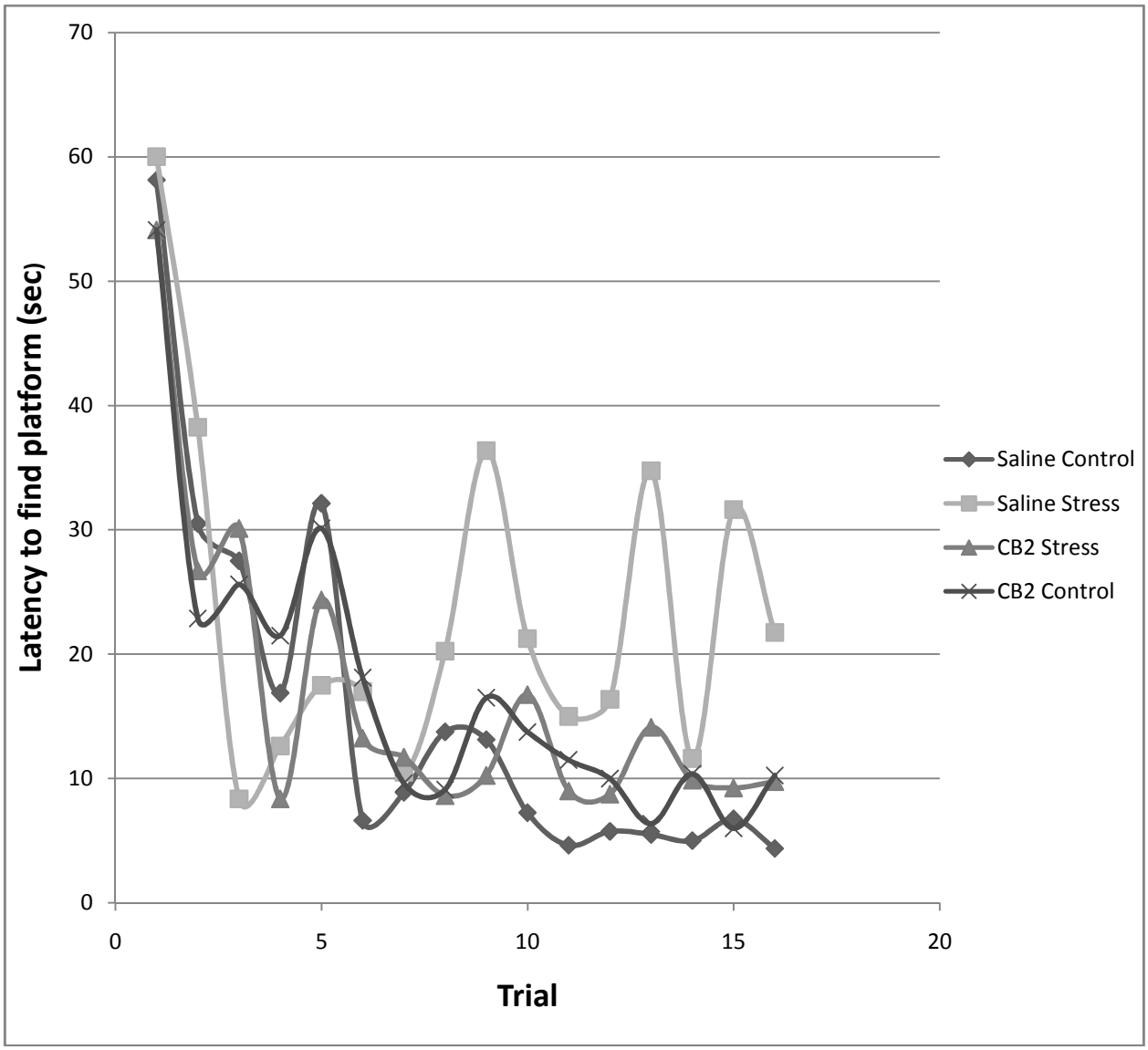
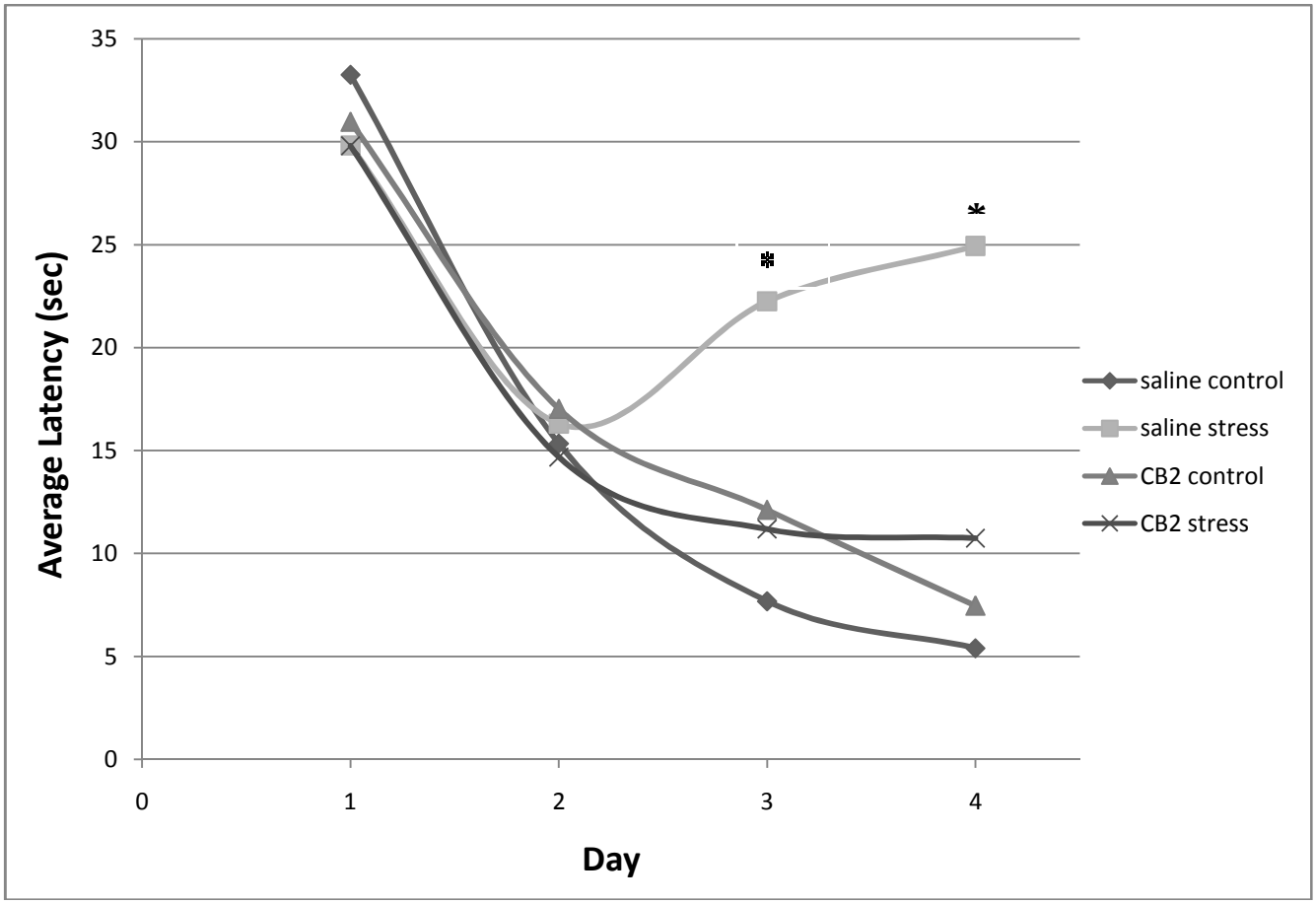


Figure 8.
The effects of drug and stress on the latencies to the platform in the Morris Water Maze averaged per day. Significant differences are seen between the saline stress group and the three other groups on day 3 and day 4.



*$p < 0.05$

Figure 9.
The effect of drug and stress on the amount of time spent in the northeast quadrant of the Morris Water Maze after the platform was removed from the maze. There were no significant effects between groups.

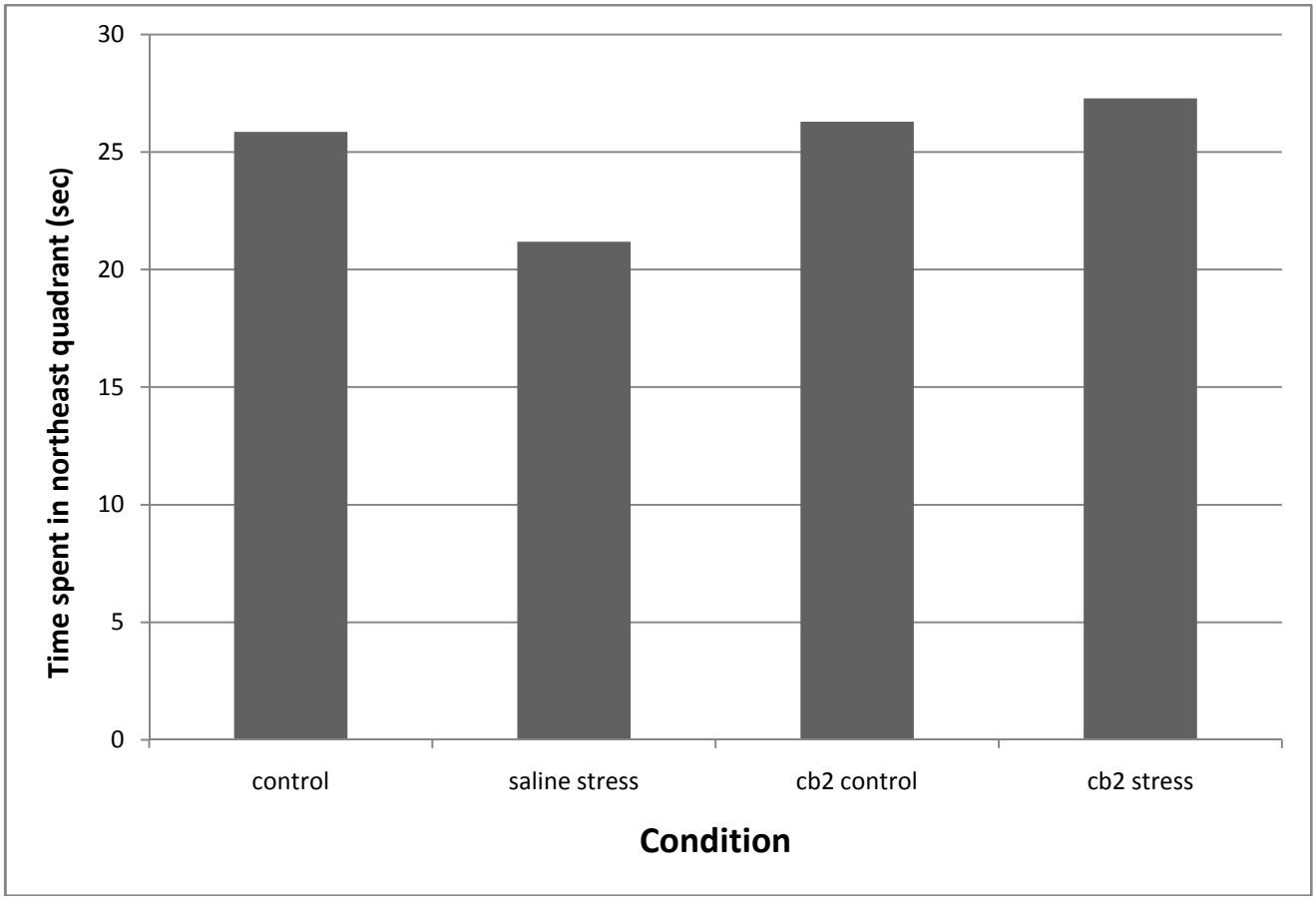


Figure 10.
Examples of hippocampal sections stained with NeuN antibody. NeuN cells appear as darkly colored, completely filled circles. Background staining from cell bodies occurred. The background cells appear as lightly colored, none filled in circles and were not counted in the NeuN count. These images were taken under 5X magnification, but cell counts were performed under 10X magnification. Saline stress rats show significantly less NeuN cells than the three other groups.

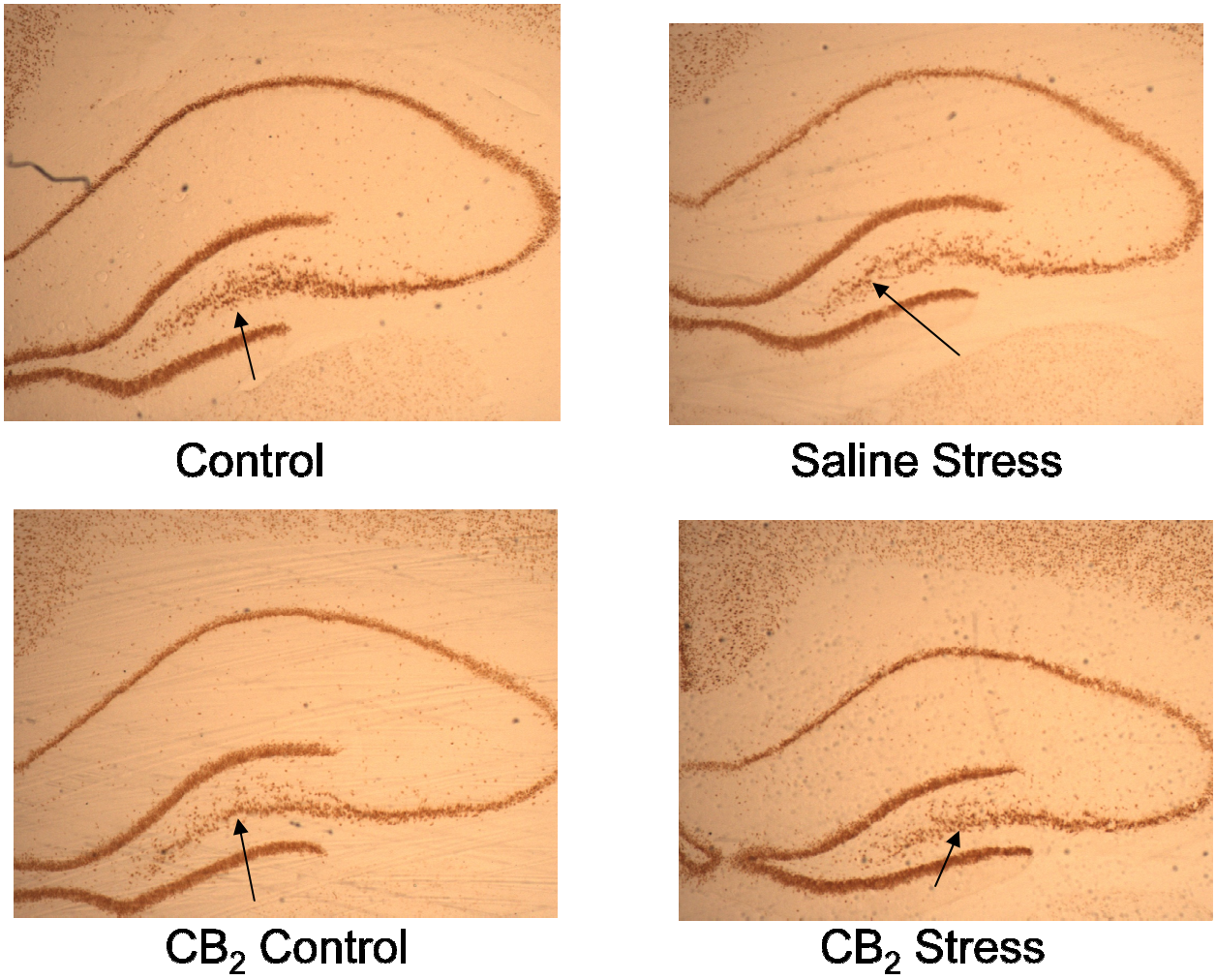
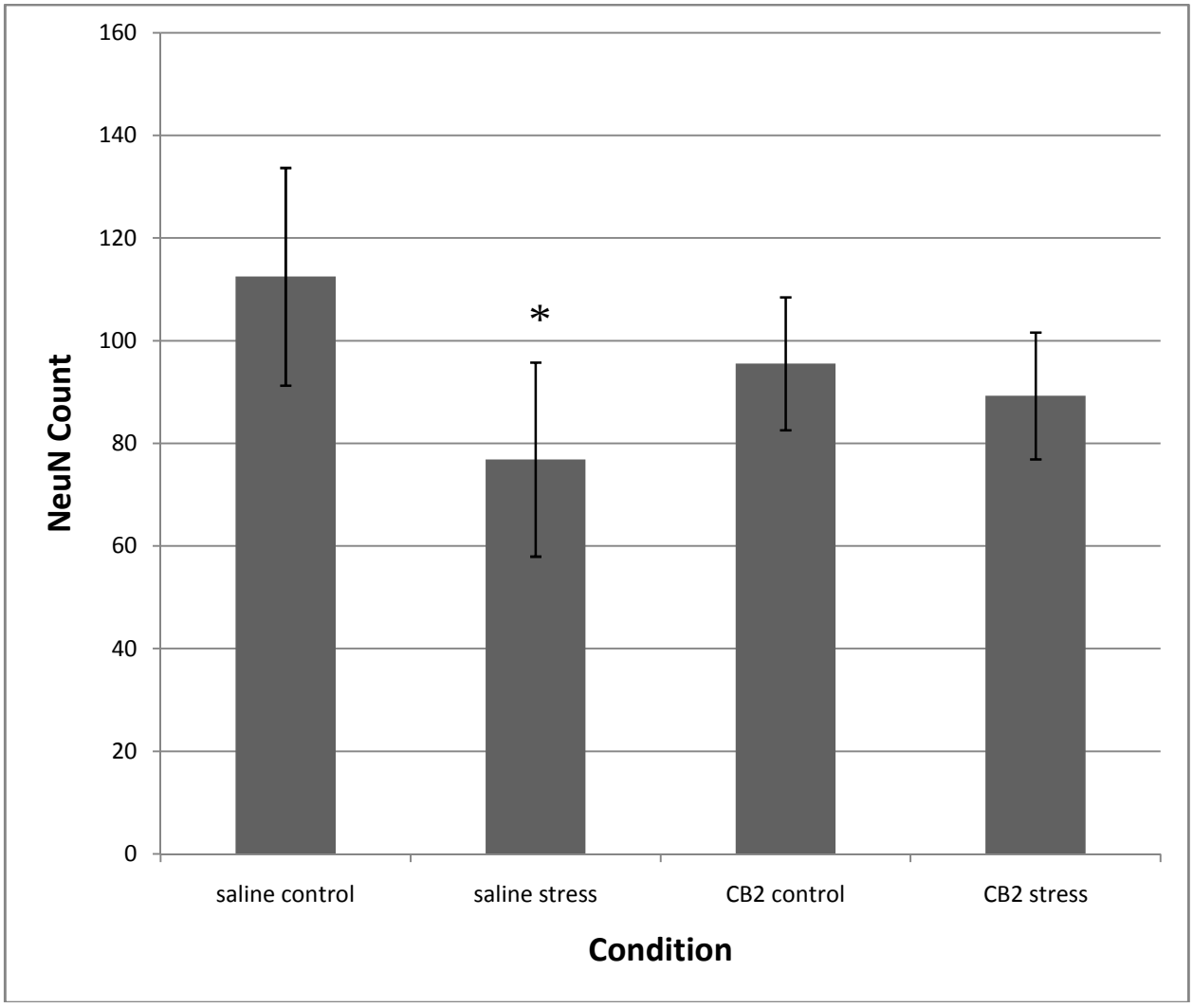


Figure 11.
The effects of drug and stress condition on neuronal nuclei cell counts in the CA3 region of the hippocampus. The saline stress rats were seen to have significantly lower counts than all three other groups.



*$p < 0.05$