CB$_1$ RECEPTOR ACTIVITY MODULATES RESPONSES IN THE FORCED SWIM TEST AND OPEN FIELD TEST PARADIGMS.

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Abstract

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CB_1 RECEPTOR ACTIVITY MODULATES RESPONSES IN THE FORCED SWIM TEST
AND OPEN FIELD TEST PARADIGMS.
(Under the direction of Linda Dykstra, PhD)

Cannabinoid type I (CB_1) receptor activation in the forced swim test (FST) and on locomotor activity (LMA) was examined by manipulating the endogenous agonist anandamide (AEA). AEA modifier URB597 prevents AEA metabolism and AM404 inhibits the reuptake of AEA in post synaptic neurons.

Antidepressant-like behavior was measured using time spent immobile (sec) in the FST, and distance traveled (cm), average velocity (cm/sec), entries into the center zone (#), and time spent in the center zone (min) in the open field test. LMA before (Pre-FST1), immediately after (Post-FST1), and a week after the FST (LMA-FST1) were measured.

Time spent immobile decreased with desipramine (DMI; 10 mg/kg), URB597 (1.0 and 3.2 mg/kg), and AM404 (1.0 mg/kg). Although 1.0 mg/kg of URB597 and AM404 reduced time spent immobile, distance traveled was not impaired. Overall, the decreases in time spent immobile were not because of LMA solely, and URB597 and AM404 have antidepressant properties.

DEDICATION

To the men and women around the world that have developed stress-induced depression.
The mechanism for the onset of this mental illness is very important and a potential therapy
may include pharmacological manipulation of the endocannabinoid system, specifically the
CB_1 receptor.

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

CHAPTER

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

2-arachidonoyl glycerol…………………………………………………………......2-AG

Adenylate cyclase………………………………………………………………….AC

Adenosine triphosphate…………………………………………………….…..ATP

Adrenocorticotropic hormone……………………………………………….…..ACTH

AEA metabolism inhibitor…………………………………………………....URB597

AEA reuptake inhibitor………………………………………………………...AM404

Anandamide…………………………………………………………………….…AEA

Calcium………………………………………………………………….………Ca^{2+}

Cannabinoid type 1 receptor……………………………………………….....CB_1

Cannabinoid type 2 receptor……………………………………………….. CB_2

Corticosterone……………………………………………………………….…..CORT

Corticotropin releasing hormone…………………………………………….....CRH

Cyclic adenosine monophosphate………………………………………….…cAMP

Desipramine…………………………………………………………………..…..DMI

Elevated plus maze……………………………………………………………….EPM

Fatty acid amide hydrolase………………………………………………….……FAAH

Forced swim test……………………………………………………………….…FST

Gamma-aminobutyric acid……………………………………………………..GABA

Hypothalamus Pituitary Adrenal…………………………………………………HPA

Intraperitoneal……………………………………………………………………..IP

Locomotor activity chamber…………………………………..…….…………LAC

Locomotor activity……………………………………………………………......LMA

Monoamine oxidase inhibitor………………………………………………….....MAO-I

CHAPTER 1

BACKGROUND AND SIGNIFICANCE

Etiology and illnesses associated with stress

Depression can be defined as a diminished interest or pleasure in most activities (Cryan, 2002). At least 18.8 million Americans over the age of 18 are affected by a depressive disorder in a given year (NIMH, 2009) and an estimated 2% of American children between the ages of 7-12 have major depression (Harvard Medical School, 2002). Prolonged exposure to stress has been linked to the development of depression and several pathological conditions (NIOSH, 1999; Stojanovich, 2008). Stress is a multifactorial disruption of an organism's physiological homeostasis that can be caused by a variety of external and internal stimuli that are predominately emotional, physical, psychological and environmental in nature.

Currently, treatment for depression and other anxiety-derived illnesses are managed by antidepressants. Antidepressants can block the reuptake of neurotransmitters (NTs) in the brain or inhibit NT metabolism (Highfield, 2001). The antidepressant therapies currently available include tricyclic antidepressants (TCA), selective serotonin reuptake inhibitors (SSRI) and monoamine oxidase inhibitors (MAO-I) (Long, 1998; Martinez, 2005). However, there are a growing number of human cases that report a resistance to the current antidepressant therapies (Fava, 2006). The endocannabinoid system has been proposed as an alternative therapy target for stress-induced depression.

Despair and helplessness are behavioral responses of human depression (Borsini,1988) that can be monitored in mouse models. Mouse models serve as a suitable

system to investigate the effectiveness of antidepressant therapies used to treat human depression (Lucki, 2001b; Petit-Demouliere, 2005). Mouse models have been used to assess the effects of the endocannabinoid system (cannabinoid type 1 (CB_1) receptor agonist, CB_1 receptor antagonist, AEA reuptake blocker, or AEA metabolism inhibitor), evaluate behavior response to a novel stressor in the absence of learned helplessness developed during FST pre-exposure, and to measure CORT and AEA release in response to FST exposure. In the FST a common behavioral response that is recorded and scored is time spent immobile. Time spent immobile is defined as a lack of locomotor activity and is a stress coping behavior mice commonly display.

In our study we determined whether pharmacological manipulation of CB_1 receptor activity effects time spent immobile, therefore suggesting antidepressant-like properties in response to acute forced swim (FST) exposure. In addition locomotor activity (LMA) was measured before (Pre-FST1), immediately after (Post-FST1), and a week after (LMA-FST1) the FST in open field tests, to determine if CB_1 receptor activity can reverse the effects of FST exposure on LMA.

Activation of the hypothalamic-pituitary-adrenal (HPA) axis

Hyperactivation of the HPA axis has been linked to stress. The basic pathway involved in HPA axis activation has been outlined (Figure 1). In addition, the HPA axis activity can be altered with cannabinoids and reduce stress related behavioral responses (Tasker, 2004). In response to stressful psychological and physical stimuli, corticotrophin releasing hormone (CRH) is synthesized and released from the hypothalamus (Barna, 2004;Tasker, 2004). CRH binds to receptors in the anterior pituitary and stimulates the synthesis and secretion of adrenocorticotropic hormone (ACTH). In turn, ACTH binds to receptors on the adrenal cortex, which activate adenylate cyclase (AC). Adenosine triphosphate (ATP) is converted into cyclic adenosine monophosphate (cAMP) in the

presence of active AC.

Increases in the intracellular levels of cAMP and several enzymes are involved in the biosynthesis and release of cortisol. The rodent equivalent to human cortisol is corticosterone (CORT). CORT release has been analyzed from rodents in response to FST exposure. In our laboratory we have observed a correlation between time spent immobile and CORT release.

Endocannabinoid Signaling

Endocannabinoids are endogenous neuromodulators involved in learning and memory, locomotor activity (LMA), depression, appetite, mood and behavior (Martin, 2002; Shearman, 2003; Barna, 2004; Hohmann, 2005; Hill, 2007; Benarroch, 2007; Griffith, 2009; LoVerme, 2008). The endocannabinoid system is composed of endocannabinoids that activate centrally and peripherally located cannabinoid receptors, cannabinoid type 1 (CB_1) and cannabinoid type 2 (CB_2) (Petit-Demouliere, 2005; Pertwee, 2006; Vaughan, 2006; LoVerme, 2008). Cannabinoid receptors are G-protein-coupled receptors (GPCR) responsible for inhibiting certain cellular functions and biological responses that include the release of neurotransmitters (NTs) from pre synaptic cells (Pertwee, 2006; Benarroch, 2007). The CB_1 receptor is the most abundant receptor in the central nervous system (CNS) and is located on pre synaptic neurons in the hippocampus (McLaughlin, 2007), hypothalamus (Arevalo, 2001), prefrontal cortex (Freund, 2003), periaqueductal gray (Vaughan, 2006), amygdale (Azad, 2003), and adipose tissue (Tzavara, 2003).

When specific stimuli (i.e. electrical impulse, neurotrabsmitters (NTs)) are received by pre synaptic neurons, a signaling cascade is initiated, NT are released and act on receptors on post synaptic neurons. The NTs released initiate an intracellular signaling cascade in post synaptic neurons that facilitate the synthesis and release of endocannabinoids (Fig. 2; Vaughan, 2006; Benarroch, 2007). Anandamide (AEA) and 2-arachidonoyl glycerol (2-AG)

are two of the most widely studied endogenous endocannabinoids (Hohmann, 2005; Vaughan, 2006; Benarroch, 2007; Hill, 2007).

Activation of the CB_1 receptor by endogenous and exogenous cannabinoid ligands inhibits AC, which decreases cAMP levels and inhibits glutamate release (Pertwee, 2006; Benarroch, 2007). When this reaction occurs in the hypothalamic paraventricular nucleus (PVN) and supraoptic nucleus, synaptic input of CRH neurons in the PVN is inhibited (Tasker, 2004). The lack of glutamate release in response to CB_1 receptor activation also effects postsynaptic cell desensitization (Di, 2003). The influx of calcium (Ca^{2+}) is blocked, whereas the efflux of potassium (K^+) is potentiated in pre synaptic cells in the presence of CB_1 receptor activation. The increase in K^+ efflux causes the pre synaptic cells to be hyperpolarized. Furthermore, activation of the CB_1 receptor reduces arachidonic acid and activates mitogen-activated protein kinases (MAPK) and phosphatidylinositol-3 kinase (PI3K) pathways. Antidepressant screening ssays such as forced swim test (FST; Porsolt, 1977; Porsolt, 1978; Borsini, 1988; Lucki, 2001a; Lucki, 2001b; Dalvi, 1999; Shearman, 2003; Tzavara, 2003; Steiner, 2008a; Steiner, 2008b), tail suspension test, (TST; Gobbi, 2005; Hill, 2005), and elevated plus maze (EPM; Haller, 2004; Griebel, 2005; Hill, 2007; Egashira, 2008) have been used to characterize the effects of endocannabinoids on immobility and locomotion.

Summary

The endocannabinoid system, specifically the CB_1 receptor, is involved in stress behavioral responses such as time spent immobile and locomotion. Researchers report different findings about the influence of the endocannabinoid system, specifically the CB_1 receptor, on time spent immobile in the FST. The results may vary because of the different antidepressant screening tests, animal models, and the limited doses used. Furthermore, current anti-depressants are an effective form of treatment in less than half of those

diagnosed with depression, and reduces locomotion activity. The impact of the endocannbinoid system on modulating the time spent immobile, and it effects on locomotor activity are unclear. Therefore, expanding our understanding of the endocannabinoid system will support its use as an alternative therapy for the treatment of depression without impairing locomotion.

Significance

Alterations in the time spent immobile and HPA axis hormone release observed in acute stress conditions response to CB_1 receptor activity is similar to current antidepressant treatments. However, the effects of CB_1 receptor activity on stress behavioral responses under acute FST conditions remains unclear. In addition, many of the studies investigating the impact of the endocannabinoid system do not include the effects of CB_1 receptor activation on the locomotion concurrently. Our goal is to expand the range of AEA modifier doses, and extend our understanding how the endocannabinoid system modulates behavioral responses to the FST paradigm. The findings from this study will expand the understanding of the interaction between the endocannabinod system and behavioral responses to stress, and whether or not manipulation of the CB_1 receptor influences locomotion in the presence or absence of stress exposure.

CHAPTER 2

INTRODUCTION

The cannabinoid type 1 (CB_1) receptor is a G protein coupled receptor that is involved in modulating gamma-aminobutyric acid (GABA) and glutamate neurotransmitter release, calcium influx, and potassium eflux (Pertwee, 2006; Benarroch, 2007). Anandamide (AEA) is the endogenous agonist that binds to and activates the CB_1 receptor. In addition, the CB_1 receptor is involved in modulating a variety of behaviors such as learning and memory, locomotor activity, appetite, mood, anxiety, and depression (Martin, 2002; Shearman, 2003; Barna, 2004; Tasker, 2004; Hohmann, 2005; Benarroch, 2007; Hill, 2007; LoVerme, 2008; Griffith, 2009). Currently, treatment for depression and other anxiety-derived illnesses is managed with antidepressants, but because a growing number of human cases report a resistance to current antidepressant therapies (Fava, 2006), an alternative therapy is needed. Antidepressants can modulate neurotransmitters in the brain by altering reuptake and inhibiting metabolism of dopamine, serotonin, and norepinephrine (Highfield, 2001). Scientists are interested in exploring the modulatory effects of CB_1 receptor activation on responses to stress because CB_1 receptor activation has potential applicability as a possible therapeutic treatment for stress and depression to human.

The effect of CB_1 receptor activation on responses to stress is unclear because different behavioral assays have been used to determine the impact of the endocannabinoid system on behavioral responses to stress (Bowers, 2008). For example, Shearman (2003) demonstrated that CB_1 receptor agonism increased the time spent immobile in the tail suspension test (TST), a behavioral assay that has been used to examine stress as well as

the effects of antidepressants. Other research suggests that agonism (Jiang, 2005; Adamczyk, 2008; El-Alfy, 2010) and antagonism (Adamczyk, 2008; Takahashi, 2008) of the CB_1 receptor can reduce time spent immobile in the forced swim test (FST), another behavioral assay that has been used to examine the activity of potential antidepressants. However, Rutkowska (2006) reported that CB_1 receptor activation had no effect on time spent immobile in the FST. The culmination of all of this research suggests that further examination of CB_1 receptor activation and behavioral responses to stress are needed.

There is limited information regarding the effect of CB_1 receptor activation on locomotor activity (LMA). Data suggest that high concentrations of AEA, an endogenous CB_1 receptor ligand, result in sedation (Meybohm, 2008), which can, in turn, decrease LMA. Moreover, increases in AEA concentration can cause hypothermia, which can also decrease LMA and lead to immobility (Mogil, 1996; McLaughlin, 2003; Drugan, 2005). It is evident that understanding the effects of CB_1 receptor activation on LMA immediately after stress exposure is necessary to better understand the impact of CB_1 receptor activation on behavioral responses to stress.

One approach to studying the effects of CB_1 receptor activation on behavioral responses to stress and locomotion are to use an animal model that will examine the effects of various doses of endocannabinoid AEA modifiers. Endocannabinoid AEA modifiers indirectly activate CB_1 receptors by increasing the concentration of AEA (Beltramo, 1997; Kathuria, 2003). As a result, AEA modifiers increase the probability of AEA binding to CB_1 receptors and activating CB_1 receptors. The mechanisms known to increase AEA concentrations include 1) inhibition of AEA reuptake into neuronal cells and 2) inhibition of fatty acid amide hydrolase (FAAH) metabolism. AM404, which block the reuptake of AEA into neuronal cells, and URB597, which inhibits the enzymatic activity of FAAH, are endocannabinoid modifiers that are known to increase AEA concentrations (Beltramo, 1997;

Kathuria, 2003), and thereby increase CB_1 receptor activity. Some reports show that manipulating of AEA with URB597 and AM404 either decreases the behavioral response to FST stress (Gobbi, 2005; Hill, 2005a; Adamczyk, 2008), or has no effect (Naidu, 2008). Activating CB_1 receptors indirectly with endocannabinoid AEA modifier, rather than direct ligands, may provide a better assessment of CB_1 receptor activation in response to stress.

The effects of CB_1 receptor activity on behavioral responses to stress under acute conditions remains unclear. In addition, many of the studies investigating the impact of the endocannabinoid system do not include the effects of CB_1 receptor activation on LMA concurrently. We hypothesize that the endocannabinoid system, specifically the CB_1 receptor, regulates behavioral responses to stress in C57Bl/6 mice as observed in the FST and open field test. To test our hypothesis we 1) validated the FST paradigm assay as a screen for potential antidepressants; 2) examined the effects of a range of doses of URB597 and AM404 by scoring the time spent immobile in response to FST exposure; and 3) assessed the effects of URB597 and AM404 on LMA by measuring a variety of locomotor assessments in the open field test. Understanding the effect of CB_1 receptor activation on behavioral responses to stress will aid in understanding the CB_1 receptor in the onset and modulation of responses to stress and locomotion.

CHAPTER 3

MATERIALS AND METHODS

Animals

Adult male C57BL/6 mice were purchased from Jackson Labs (Raleigh, NC; 10 weeks of age upon delivery), for these studies. The age of the mice during these studies was between 3 and 6 months. All mice were group housed (four per cage) in standard Plexiglass cages in a colony room maintained on a reversed 12-hr light/dark cycle (lights on at 7:00 p.m., lights off at 7:00 a.m.). Mice had continuous access to food and water throughout the study and were habituated to the intraperitoneal (i.p.) injection procedure (handling) and the colony room environment for 2 weeks prior to any experimental manipulation. Mice were exposed to the testing environment for at least two days prior to the initiation of an experiment. All testing procedures were conducted during the dark cycle between 10:00 a.m. and 3:00 p.m. The "Guide for the Care and Use of Laboratory Animals" (National Research Council, National Academy of Sciences, Washington, D. C., 1996) was adhered to during all test sessions.

Forced Swim Test (FST) Procedure

The FST assay was used to measure and analyze behavioral responses to stress. The FST parameters included a glass cylinder filled with 3000 mL of water at $25 + 1°C$. The water was changed between each FST session. Each FST session was recorded for 6 min (JVC camcorder) and time spent immobile was measured in the FST assay. Time spent immobile in the FST was defined as a lack of forearm or hind leg movement, or minimal body movement to keep their head and body afloat. A stopwatch was used to score to the nearest second.

On the day of testing, mice received an intraperitoneal (i.p.) injection desipramine (DMI) (10 mg/kg and 20 mg/kg), AM404 (0.32-3.2 mg/kg), or URB597 (0.03-3.2 mg/kg). Drugs were administered 30 min prior to FST exposure. The full 6 min of each FST session was scored and analyzed. The time spent immobile in the FST session was expressed as the mean $\pm$ standard error of the mean (SEM).

Open Field Test Procedure

LMA was assessed in an LAC (28.6x28.6x20.7cm; Med-Associates, St. Albans, VT). Infrared photobeams in the LAC were spaced 5/8 of an inch apart. LMA was defined as a disruption of 3 consecutive photobeams.   Interruptions of the photobeams indicated horizontal activity as measured in distance traveled and were expressed in centimeters (cm). The parameters assessed for the present study were total distance traveled (cm), the average velocity (cm/s), number of entries into the center zone (#), and time spent in the center zone (min). Immediately after the LMA session, the mice were removed from the LAC and returned to their homecage. Between each LMA session, the LAC's were cleaned with 70% ethanol and dried. The Activity Monitor software program was used to analyze the data collected (Med-Associates, St. Albans, VT).

Prior to the day of testing, each mouse was conditioned to the open field test for 1 hr. The mice were placed in the center of the LAC and allowed free movement. After conditioning, the mice were returned to their home cage. On the day of testing, each mouse was exposed to the open field test prior to the FST session (Pre-FST1) in order to determine baseline activity levels. For Pre-FST1, each mouse was placed in a LAC for 1 hr before the FST session, then returned to its home cage. The initial 30 min of the open field test was analyzed. The mice then received an i.p. injection 30 min before the FST session (see FST Procedure), and immediately after the FST session, the mice were towel dried and placed in

the LAC for 30 min (Post-FST1). Following the open field test, the mice were returned to their homecage. A week after the FST session, each mouse received an i.p. injection 30 min prior to the open field test, and their LMA was recorded for 30 min (LMA-FST1). Following the open field test, the mice were returned to their homecage. The mice received either DMI (10 mg/kg and 20 mg/kg), AM404 (0.32-3.2 mg/kg), or URB597 (0.03-3.2 mg/kg) prior to testing. The influence of CB_1 receptor activity on the behavioral response to FST exposure on LMA was recorded and analyzed using change difference analysis.

Drugs and Treatment

URB597 was provided by National Institutes on Drug Abuse (NIDA; Bethesda, MD). DMI, an anti-depressant, was purchased from Sigma-Aldrich (St. Louis, MO) and AM404 from Tocris (Ellisville, MO). URB597 and AM404 were dissolved in an ethanol, alkamuls, and 0.9% saline solution (1:1:18) combination. DMI was dissolved in 0.9% saline solution. Drugs were administered through i.p. injections at a volume of 10 mg/ml.

Data analysis

The data for each test group included the mean $\pm$ SEM for 1) time spent immobile; 2) total distance traveled; 3) average velocity; 4) number of entries into the center zone; and 5) time spent in the center zone. The statistical significance for time spent immobile was analyzed using SAS and SPSS 18.0 software and *one-way analysis of variance* (*ANOVA*). Student t-tests were used when appropriate to compare the effect of treatment on time spent immobile. Significance was set at a p value $\leq$ 0.05. The effect of each compound on the distance traveled, the average velocity, the number of entries into the center zone, and time spent in the center zone were analyzed using the *one-way ANOVA*. The *one-way ANOVA* calculated the overall statistical difference between the doses tested and vehicle for each LMA session (Pre-FST1, Post-FST1, and LMA-FST1). Significance was set at a p value $\leq$ 0.05. *Post-hoc* analysis calculated the statistical difference between the individual

11

doses tested and vehicle for each LMA session. Significance was set at a p value $\leq$ 0.05.

CHAPTER 4

RESULTS

A. EFFECT OF THE FORCED SWIM TEST ON BEHAVIORAL RESPONSES

Validation of the FST paradigm with DMI

Figure 3 shows the effects of 10 mg/kg and 20 mg/kg of desipramine (DMI), on time spent immobile in the FST paradigm. One-way ANOVA revealed a significant decrease for time spent immobile at 10 mg/kg of DMI for the full 6 min FST session ($F_{(2,21)}=4.867$, p=0.013). In contrast, 20 mg/kg of DMI did not decrease time spent immobile during the full 6 min ($F_{(2,21)}=4.867$, p>0.05). These results validate the FST procedure with 10 mg/kg of DMI. Therefore, this procedure will be used to screen potential antidepressants in future pharmacological studies.

Effects of URB597 on time spent immobile

Figure 4 shows the effect of URB597 on time spent immobile in the FST paradigm. One-way ANOVA analysis, followed by an unpaired *t-test* analysis revealed a significant increase in time spent immobile at 0.32 mg/kg of URB597 (p=0.016) and a significant decrease at 1.0 mg/kg (p=0.001) and 3.2 mg/kg of URB597 (p=0.001). These findings suggest that manipulating AEA concentrations with URB597 produced biphasic effects for time spent immobile.

Effects of AM404 on time spent immobile

Figure 5 shows the effects of AM404 on time spent immobile in the FST paradigm. One-way ANOVA, followed by an unpaired *t-test* analysis revealed a significant decrease for time spent immobile at 1.0 mg/kg of AM404 (p=0.039), but no effects at 0.32 mg/kg or 3.2

mg/kg of AM404.

B. EFFECT OF THE FORCED SWIM TEST ON LOCOMOTOR ACTIVITY

Effect of DMI and FST1 exposure on locomotor activity

In a previous experiment we validated the FST paradigm with 10 mg/kg of DMI and showed that DMI decreased time spent immobile (Figure 3). The effect of 10 mg/kg of DMI on time spent immobile in the FST paradigm was repeated, and one-way ANOVA revealed a significant reduction for time spent immobile in response to 10 mg/kg of DMI ($F_{(1,15)}$ =6.106, p=0.026; Appendix A). These data confirm the reduction in time spent immobile in the FST paradigm in response to DMI.

Figure 6 shows the effect of DMI on distance traveled immediately after FST exposure (Post-FST1) and a week after FST exposure (LMA-FST1). *One-way ANOVA* analysis revealed that FST exposure sharply reduced distance traveled in Post-FST1, and 10 mg/kg of DMI did not reverse the decrease in distance traveled ($F_{(1,17)}$=0.663, p=0.427; Table 1). Importantly, 10 mg/kg of DMI significantly decreased distance traveled in LMA-FST1 ($F_{(1,17)}$=8.791, p=0.009; Table 1). These results indicate that although 10 mg/kg of DMI decreased time spent immobile in the FST, it is likely that these effects were not due to an overall increase in locomotor activity (LMA).

Effect of URB597 and FST1 exposure on locomotor activity

Figure 7 shows the effects of URB597 on distance traveled for Post-FST1 and LMA-FST1 using the open field test. *One-way ANOVA* analysis revealed that FST exposure sharply reduced distance traveled in Post-FST1, but the URB597 doses used did not reverse the these effects (Table 1). In addition, a URB597 dose dependent increase for distance traveled during LMA-FST1 was observed ($F_{(5,48)}$=2.722, p=0.040; Table 1). Specifically, *Post hoc* analysis revealed a significant increase in distance traveled was observed at 0.32 mg/kg (p= 0.027), 1.0 mg/kg (p=0.003), and 3.2 mg/kg (p=0.010) when

14

compared to vehicle LMA (Table 1). These findings suggest that FST exposure affected distance traveled in the open field test, and URB597 was unable to reverse the effect (Post-FST1). Furthermore, URB597 enhanced LMA in a dose dependent manner in the absence of stress (LMA-FST1). Therefore, it is likely that the increase in time spent immobile at 0.32 mg/kg of URB597 is not due to overall changes in LMA. However, the decrease in time spent immobile at 1.0 mg/kg and 3.2 mg/kg of URB597 may be explained by the changes in LMA.

Effect of AM404 and FST1 exposure on locomotor activity

Figure 8 shows the effect of AM404 and FST exposure on distance traveled in Post-FST1 and LMA-FST1. *One-way ANOVA* analysis revealed that FST exposure sharply reduced distance traveled in Post-FST1, but AM404 did not reverse these effects (Table 1). However, 3.2 mg/kg of AM404 significantly decreased the distance traveled in LMA-FST1 ($F_{(1,28)}$ =12.764, p=0.001; Table 1). Therefore, the dose of AM404 (1.0 mg/kg) that decreased time spent immobile in the FST paradigm, did not alter locomotion during LMA-FST1.

CHAPTER 5

DISCUSSION

The primary goals of these experiments were to employ the forced swim test (FST) to screen for the antidepressant effects of the endocannabinoid system, specifically CB_1 receptor activity, and to assess CB_1 receptor activity on locomotor activity (LMA). The main findings of the present study were that 1) pharmacological manipulation of cannabinoid type 1 (CB_1) receptors with AEA modifiers and with DMI reduced time spent immobile in a FST at 25°C; 2) selected doses of AM404 and DMI reduced distance traveled in the open field test in LMA-FST1 (a week after FST exposure); 3) neither URB597 nor AM404 reversed the immediate effects of FST exposure on LMA; 4) the doses of DMI and AM404 that decreased time spent immobile did not produce parallel increases in LMA; and 5) the doses of URB597 and AM404 that were examined did not alter the average velocity, time in the center zone, nor the number of center zone entries compared to vehicle.

DMI reduced time spent immobile at the doses that did not increase LMA

It is well established that FST exposure is sufficient to screen for the potential effects of drugs as a treatment for depression (Porsolt, 1977; Porsolt, 1978). Here we report that 10 mg/kg of DMI reduced time spent immobile in response to FST exposure in two independent experiments. Our results are consistent with published data in which the greatest decrease in time spent immobile was observed in C57Bl/6 mice in response to 10 mg/kg DMI and FST exposure at 25°C (Figure 3; Lucki, 2001; Hill, 2005a). Therefore, we established an FST paradigm to screen potential antidepressants.

The relationship between time spent immobile and locomotion is unclear; however,

studies show that decreases in time spent immobile lead to increases in time spent swimming suggesting a complementary relationship (Gobbi, 2005). The assessment of LMA after stress and in response to drug administration remains to be elucidated. We observed a significant decrease in distance traveled following 10 mg/kg of DMI in LMA-FST1, consistent with the current literature (Figure 6; Pähkla, 2000). Moreover, 10 mg/kg of DMI did not reverse the large decreases in LMA observed following FST exposure; however, 10 mg/kg of DMI decreased the number of center zone entries in Post-FST1 ($F_{(1, 17)}$=9.502, p=0.007; Appendix C) and LMA-FST1 ($F_{(1, 17)}$=9.938, p=0.007; Appendix C), and the time in the center zone in Post-FST1 ($F_{(1, 17)}$=9.139, p=0.008; Appendix D). DMI did not alter the average velocity compared to VEH (Appendix B). These data indicate that although DMI increased mobility in the FST paradigm, these increases in mobility were not explained by increases in distance traveled. Furthermore, the antidepressant effects of DMI are seen in the presence of stress.

URB597 reduced time spent immobile at the doses that did alter LMA

To examine the role of the endocannabinoid system on responses to stress, increasing doses of URB597 (0.03-3.2 mg/kg), a fatty acid amid hydrolase (FAAH) metabolism inhibitor, were used. URB597 increases AEA concentration in post synaptic neurons by inhibiting AEA metabolism by FAAH (Kathuria, 2003). We show 0.32 mg/kg of URB597 significantly increased time spent immobile, whereas 1.0 mg/kg and 3.2 mg/kg of URB597 significantly reduced time spent immobile in the 25°C FST. The increase in time spent immobile with 0.03-0.32 mg/kg of URB597 is in contrast to reports indicating that URB597 decreases time spent immobile in response to FST exposure (Gobbi, 2005; Hill, 2007; Adamczyk, 2008). The increase in time spent immobile in response to 0.03-0.32 mg/kg of URB597 may be the result of an increase in AEA concentration and CB_1 receptor activity, therefore leading to sedation (Meybohm, 2008).

Additional reasons for this discrepancy includes 1) different species and strains used (Borsini, 1988; Hall, 2001); 2) different stressor paradigm used (Campos, 2010); 3) the effect of handling on behavioral responses prior to testing (Pryce, 2005; Burn, 2008); and 4) changes in CB_1 receptor availability. It is also plausible that the increase in time spent immobile at 0.03-0.32 mg/kg URB597 was caused by changes in CB_1 receptor availability. Glucocorticoids are synthesized and released in response to stress. Reports show that glucocorticoids negatively regulate CB_1 receptor transcription (Maileux, 1993) and density (Hill, 2008), therefore reducing receptor availability and increasing AEA binding to non-CB_1 receptors. Therefore, it is possible that stress exposure and activation of the CB_1 receptor may influence glucocoticoids and response to stress. On the other hand, the increase in CB_1 receptor activity may have resulted in desensitization and down regulation of CB_1 receptors on pre synaptic neurons, which has been reported in response to chronic unpredictable mild stress (Hill, 2005b). The biphasic effect of URB597 on time spent immobile is not uncommon. El-Alfy (2010) reports a biphasic effect of delta-9 tetrahydrocannabinol (THC), an exogenous CB_1 receptor agonist, on time spent immobile in an acute FST and the tail suspension test (TST). The influence of acute stress exposure on CB_1 receptor expression is not well characterized, and may explain the behavioral responses we discovered in these studies. These data suggest that further investigation of the concentration of AEA in response to stress exposure and URB597 are needed.

Furthermore, the decrease in time spent immobile from 0.32-3.2 mg/kg of URB597 may be the results of another enzyme compensating for the loss of FAAH to metabolize AEA, AEA binding to and activating cannabinoid type 2 (CB_2) receptor or a non-cannabinoid receptor to decrease time spent immobile. Stress exposure may alter lipid metabolism (Poleszak, 2008). Altering the metabolism of AEA with increasing doses of URB597, in addition to stress exposure, may have contributed to the difference in time spent immobile in

our studies.

We show that 1.0 mg/kg and 3.2 mg/kg of URB597 decreased time spent immobile in the FST (Figure 4), however, its effects on LMA is unknown. Studies show significant reductions in locomotion and sedation with high concentrations of AEA and CB_1 receptor activity in the absence of stress (Meybohm, 2008). Reductions in locomotion and sedation would be a confounding response in evaluating AEA modifiers in the FST to measure predictive antidepressant behavioral responses. We report a significant dose dependent increase for distance traveled in response to 0.32-3.2 mg/kg of URB597 was observed in LMA-FST1 (Figure 7). These data are inconsistent with the findings by Adamczyk (2008), which show no effect of URB597 on distance traveled in the absence of stress exposure. URB597 did not alter the average velocity compared to VEH (Appendix B). This finding is unique because mice show an effect of URB597 in the absence of stress (LMA-FST1), therefore, supporting the involvement of the CB_1 receptor in LMA as measured in the open field test. Also, *one-way ANOVA*, followed by *Post hoc* analysis revealed a significant increase in the number of center zone entries in LMA-FST1 at 0.32 mg/kg (p=0.032), 1.0 mg/kg (p= 0.013), 3.2 mg/kg (p=0.16) compared to vehicle (Appendix C). Furthermore, *one-way ANOVA*, followed by *Post hoc* analysis revealed a significant increase for the time spent in the center zone in at 0.03 mg/kg (p=0.000) in Post-FST1 (Appendix D) and at 0.32 mg/kg (p=0.009) in LMA-FST1 (Appendix D) compared to vehicle. It remains unclear whether the behavioral responses observed in the FST are a result of AEA metabolism inhibition, increasing AEA concentrations, or activation of CB_1 receptor; thus further investigation is needed.

AM404 reduced time spent immobile at the doses that did not increase LMA

An alternative approach to examine the effects of the endocannabinoid system on the response to stress is to study the effects of AM404 (0.32-3.2 mg/kg), an AEA reuptake

inhibitor. AM404 increases AEA concentrations by preventing the reuptake of AEA into post synaptic neurons and preventing its metabolism, therefore increasing the activation of CB_1 receptors (Beltramo, 1997; Giuffrida, 2000). We showed that 0.32-3.2 mg/kg of AM404 produced a trend towards a decreased in time spent immobile in response to FST exposure at 25°C, and that 1.0 mg/kg of AM404 significantly decreased time spent immobile under these conditions (Figure 5). These findings are consistent with those that report that AM404 decreases time spent immobile in response to FST exposure (Hill, 2005a; McLauglin, 2007; Adamczyk, 2008). These data indicate that indirect activation of the CB_1 receptor has antidepressant properties in the FST paradigm.

It has been established that the CB_1 receptor activity modulates locomotion. The effect of AM404 on AEA and CB_1 receptor activity and the relationship between time spent immobile and LMA are unknown. We report that 3.2 mg/kg of AM404 significantly decreased distance traveled in LMA-FST1 (p=0.001; Figure 8). These results that are consistent with the findings that AM404 (Gonzalez, 1999) and AEA (Fride, 1993) decrease motion and increase time spent inactive in the open field test. However, our results are in contrast with the data of Adamczyk (2008), which shows no effect of AM404 on distance traveled. These data suggest that 1.0 mg/kg of AM404 not only decreased time spent immobile in the FST, this dose of AM404 did not alter LMA in LMA-FST1. AM404 did not alter the average velocity (Appendix B), number of entries into the center zone (Appendix C), time spent in the center zone (Appendix D) compared to VEH. Therefore, 1.0 mg/kg of AM404 has the potential to be a therapeutic drug for depression, based on our screening assay, and did not alter LMA. The mechanism of action of AM404 to affect CB_1 receptor activity differs in its effects on LMA in the presence or absence of FST exposure; therefore, further investigation into how AM404 modulates time spent immobile in the FST is needed.

CHAPTER 6

CONCLUSION

Taken together, these studies demonstrate for the first time that AEA modifiers URB597, AM404, and the antidepressant DMI are effective at reducing time spent immobile in response to acute FST exposure. Moreover, employing different mechanisms to increase the concentration of AEA using AEA modifiers (URB597 and AM404) may modulate time spent immobile and LMA differently, and these differences may be the result of physiological changes caused by FST exposure. As such, activation of the CB_1 receptor indirectly with AEA modifiers may have some beneficial properties to reduce behavioral responses to stress. Further investigation is needed to determine if the compounds used are effective as potential antidepressant and whether or not they have side effects in human studies.

Figure 6. Effects of DMI on distance traveled in the open field test. C57Bl/6 mice received vehicle (VEH) and 10 mg/kg of desipramine (DMI). A) Baseline distance traveled recorded in the absence of drug administration and prior to FST1 exposure (Pre-FST1). B) Distance traveled recorded immediately following drug administration and FST1 exposure (Post-FST1). C) Distance traveled recorded a week after FST1 exposure and in the presence of drug administration (LMA-FST1). Abscissae, Groups of mice (1-2; A), VEH and DMI (B, C). Ordinates, distance traveled measured in centimeters (cm). The group numbers correspond to the drug administered to each group during the Post-FST1 and LMA-FST1 experiments (1=VEH, 2=10 mg/kg of DMI mg/kg). Data were calculated using the mean $\pm$ SEM of two independent experiments. An asterisk indicates statistical significance between the control and the treatment used, a p value $\leq$ 0.05 (*). n=9-10.
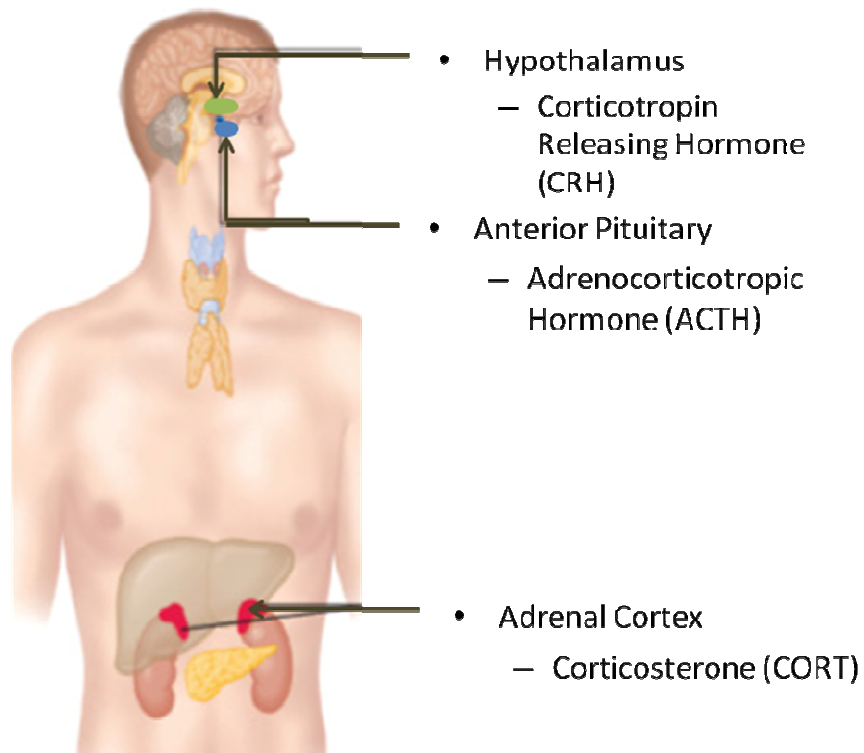
Figure 7. Effects of URB597 on distance traveled in the open field test. C57Bl/6 mice received vehicle (VEH), 0.03 mg/kg, 0.1 mg/kg, 0.32 mg/kg, 1.0 mg/kg, or 3.2 mg/kg of URB597. A) Baseline distance traveled recorded in the absence of drug administration and prior to FST1 exposure (Pre-FST1). B) Distance traveled recorded immediately following drug administration and FST1 exposure (Post-FST1). C) Distance traveled recorded a week after FST1 exposure and in the presence of drug administration (LMA-FST1). Abscissae, Groups of mice (1-6; A), VEH and URB597 (B, C). Ordinates, distance traveled measured in centimeters (cm). The group numbers correspond to the drug administered to each group during the Post-FST1 and LMA-FST1 experiments (1=VEH, 2=0.03 mg/kg URB597, 3=0.1 mg/kg URB597, 4=0.32 mg/kg URB597, 5=1.0 mg/kg URB597, 6=3.2 mg/kg URB597). Data were calculated using the mean $\pm$ SEM of three independent experiments. An asterisk indicates statistical significance between the control and the treatment used, a p value $\leq$ 0.0125 (*). n=8-10.

Figure 8. Effects of AM404 on distance traveled in the open field test. C57Bl/6 mice received vehicle (VEH), or 0.32 mg/kg, 1.0 mg/kg, or 3.2 mg/kg of AM404. A) Baseline distance traveled recorded in the absence of drug administration and prior to FST1 exposure (Pre-FST1). B) Distance traveled recorded immediately following drug administration and FST1 exposure (Post-FST1). C) Distance traveled recorded a week after FST1 exposure and in the presence of drug administration (LMA-FST1). Abscissae, Groups of mice (1-4; A), VEH and doses of AM404 (B, C). Ordinates, distance traveled measured in centimeters (cm). The group numbers correspond to the drug administered to each group during the Post-FST1 and LMA-FST1 experiments (1=VEH, 2=0.32 mg/kg of AM404, 3=1.0 mg/kg of AM404, 4=3.2 mg/kg of AM404). Data were calculated using the mean $\pm$ SEM of two independent experiments. An asterisk indicates statistical significance between the control and the treatment used, a p value $\leq$ 0.0125 (*). n=8.

Figure 1

- Hypothalamus
  - Corticotropin Releasing Hormone (CRH)
- Anterior Pituitary
  - Adrenocorticotropic Hormone (ACTH)
- Adrenal Cortex
  - Corticosterone (CORT)

http://www.vitalifenetworks.com/images/Cortisol_image.jpg

Figure 2
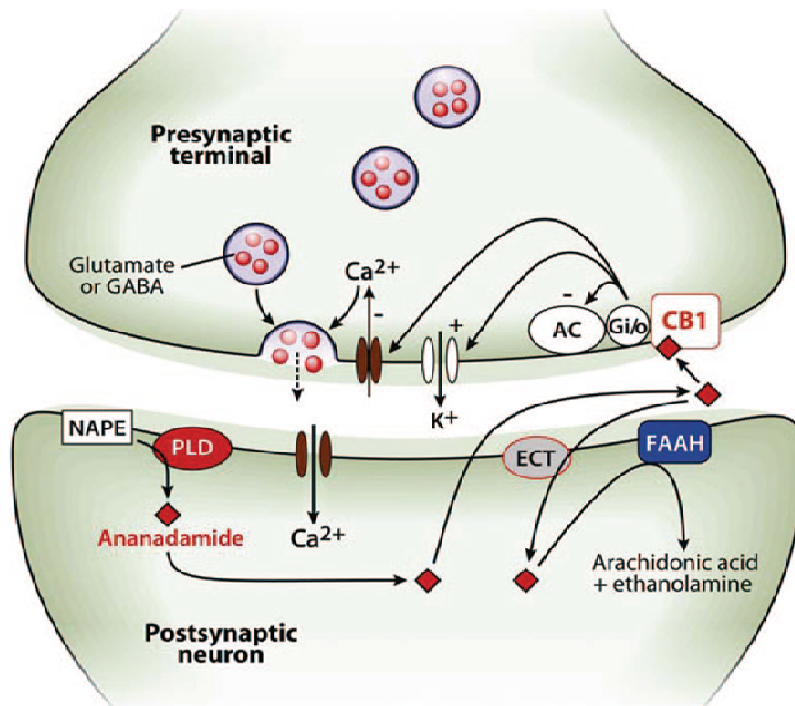
Figure 3
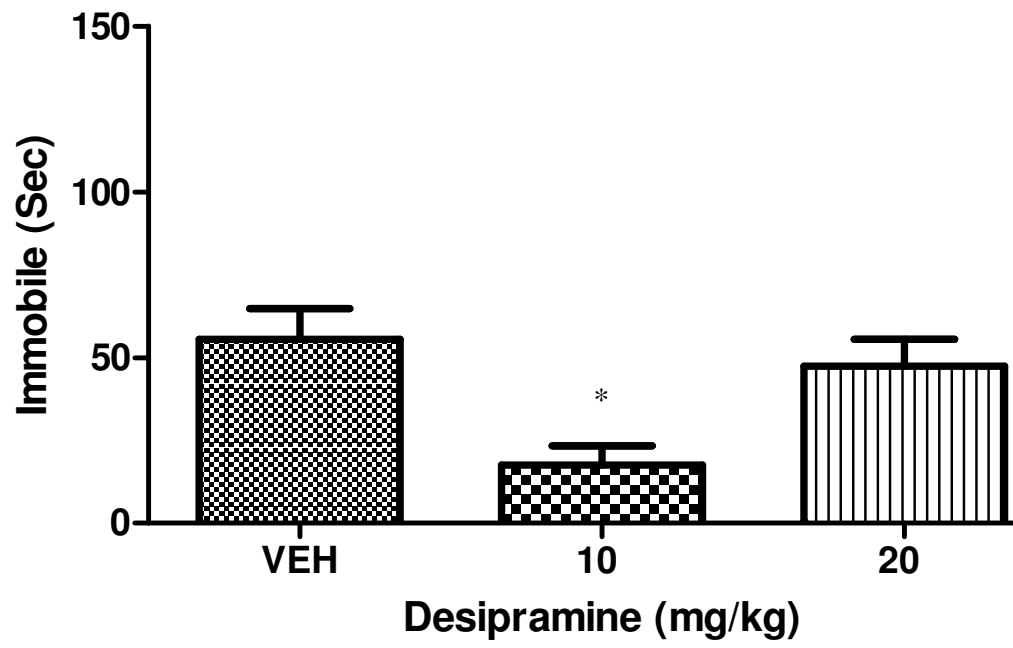
Figure 4

Figure 5

Figure 6

A

Distance Traveled (Pre-FST1)

B   **Distance Traveled (Post-FST1)**

C   **Distance Traveled (LMA-FST1)**

Figure 7

A

Distance Traveled (Pre-FST1)

B

Distance Traveled (Post-FST1)

C

Distance Traveled (LMA-FST1)

Figure 8

A **Distance Traveled (Pre-FST1)**

B **Distance Traveled (Post-FST1)**
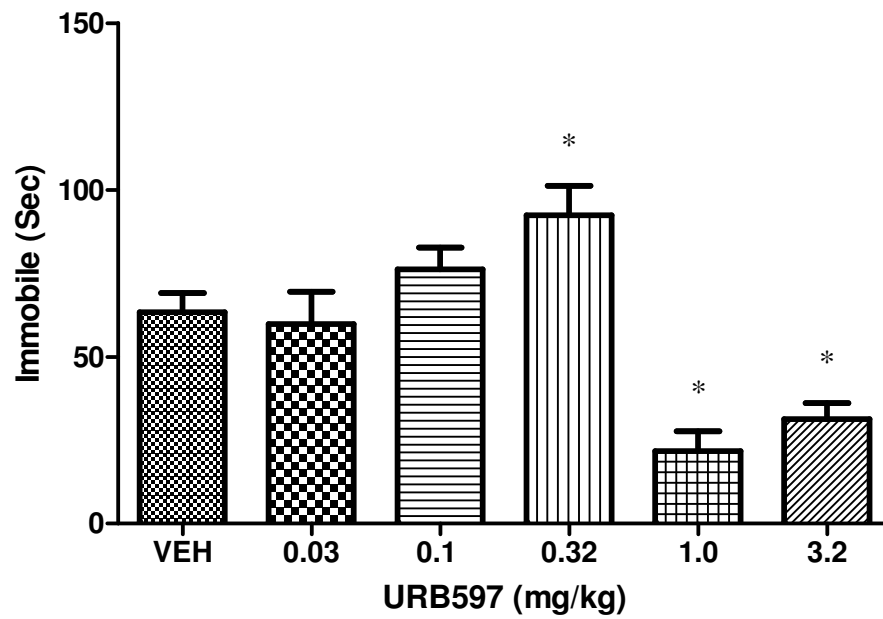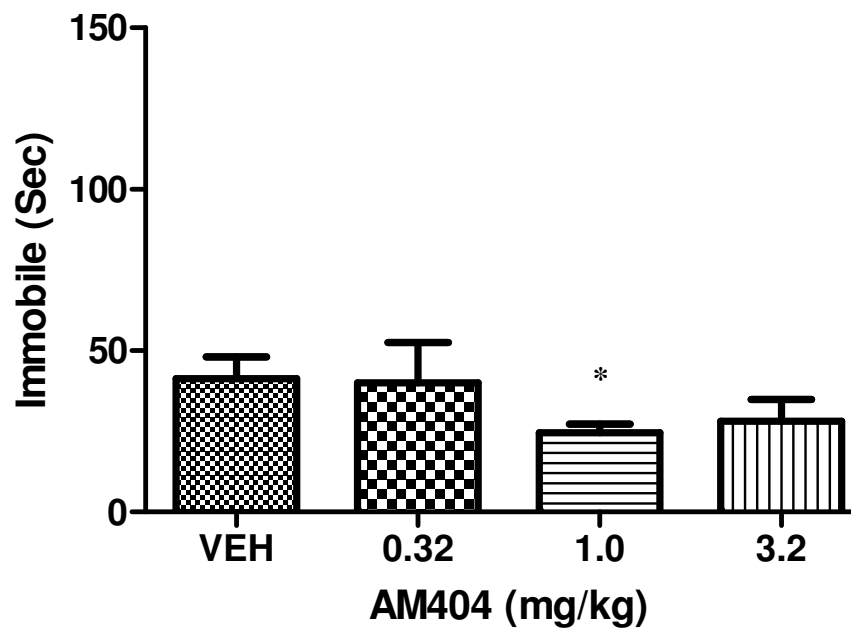
C **Distance Traveled (LMA-FST1)**

Table 1. Locomotor activity assessment of distance traveled (cm) mean values (± SEM) for treatment with vehicle, 10 mg/kg of DMI, 0.03 mg/kg-3.2 mg/kg of URB597, and 0.1 mg/kg-3.2 mg/kg of AM404 measured in the open field test (30 min session).

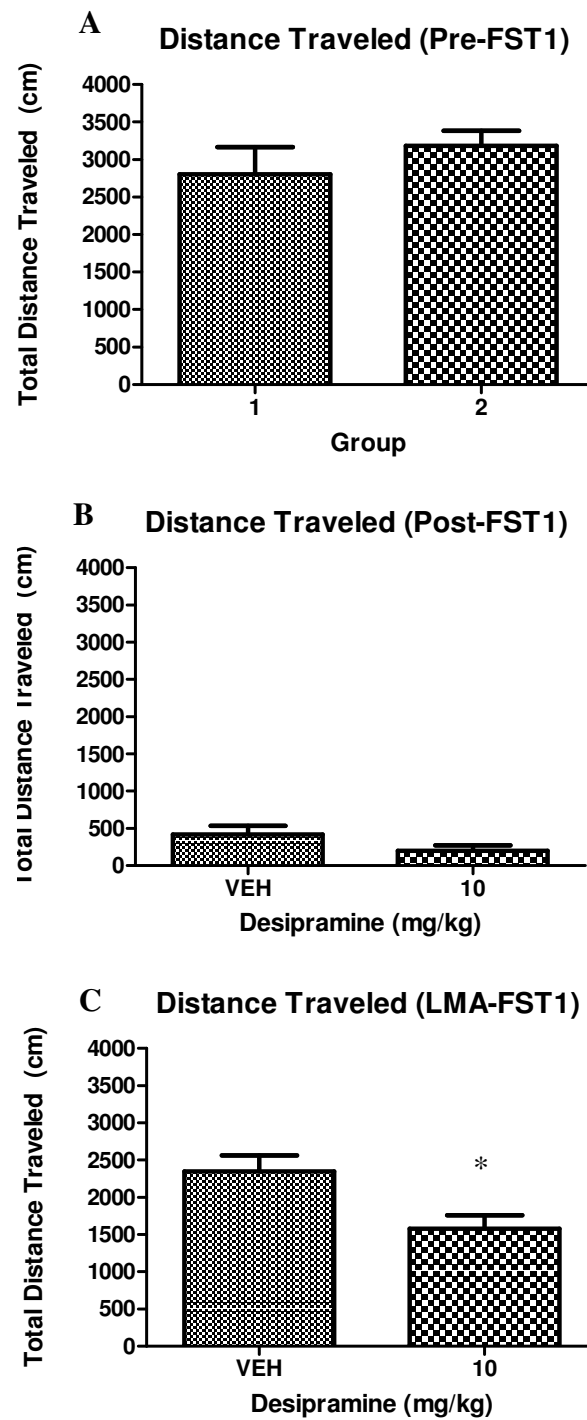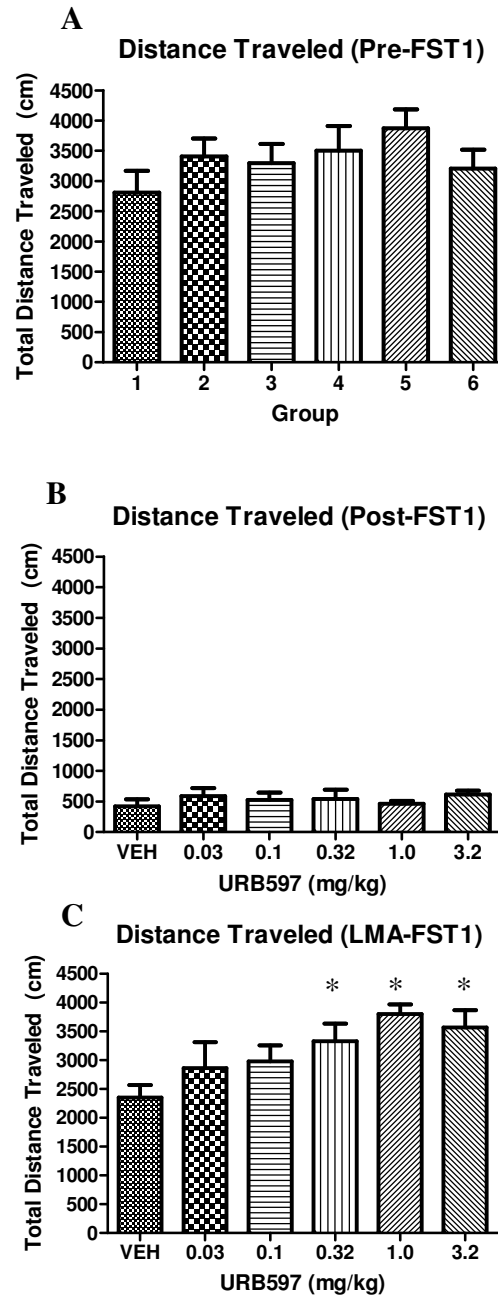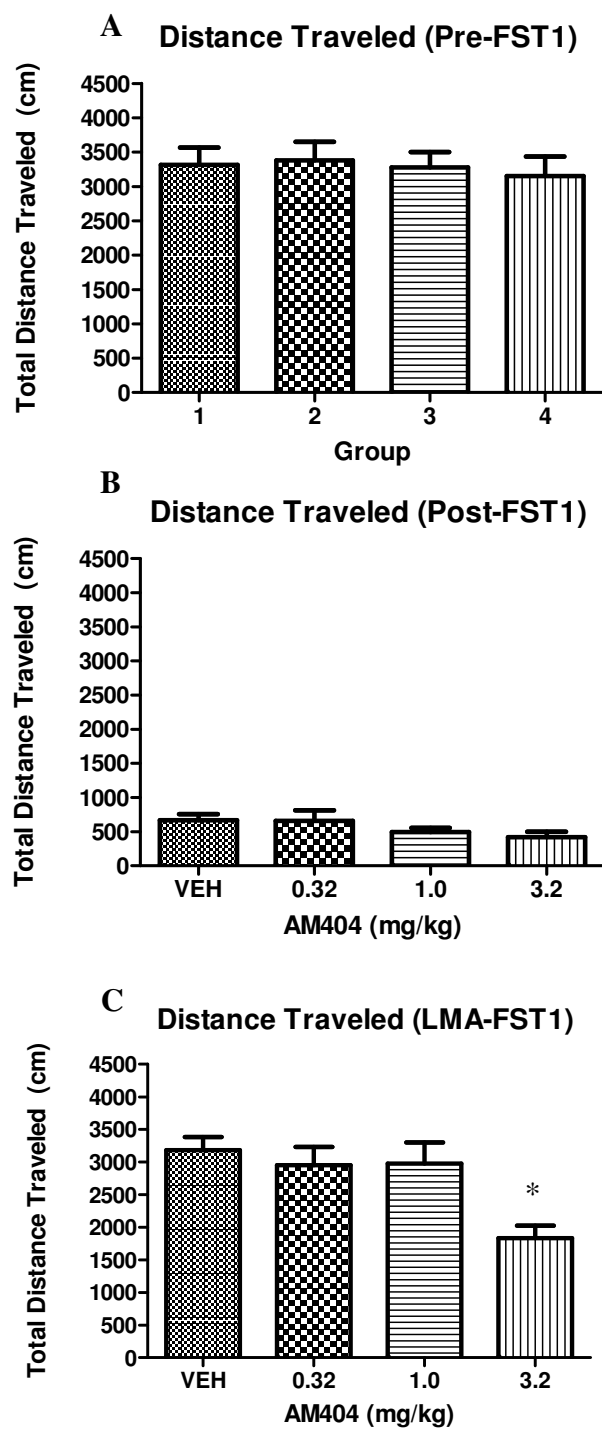| Treatment	Pre-FST1	Post-FST1	LMA-FST1
Vehicle	2805.0 (± 359.5)	419.6 (± 114.8)	2348.0 (± 218.1)
10 DMI	3181.0 (± 202.9)	197.3 (± 73.80)	*1579.0 (± 181.1)
Vehicle	2805.0 (± 359.5)	419.6 (± 114.8)	2348.0 (± 218.1)
0.03 URB597	3405.0 (± 295.3)	588.3 (± 127.8)	2859.0 (± 448.8)
0.1 URB597	3295.0 (± 321.3)	524.9 (± 120.5)	2977.0 (± 279.0)
0.32 URB597	3504.0 (± 407.1)	540.3 (± 151.2)	*3329.0 (± 303.1)
1.0 URB597	3875.0 (± 306.7)	460.1 (± 46.74)	*3800.0 (± 166.3)
3.2 URB597	3205.0 (± 311.5)	613.7 (± 63.51)	*3564.0 (± 303.5)
Vehicle	3319.0 (± 248.8)	666.3 (± 87.86)	3182.0 (± 202.6)
0.32 AM404	3381.0 (± 272.3)	659.2 (± 153.60)	2953.0 (± 279.5)
1.0 AM404	3277.0 (± 266.6)	491.7 (± 63.26)	2979.0 (± 322.3)
3.2 AM404	3150.0 (± 286.3)	418.3 (± 78.52)	*1832.0 (± 189.1)

Statistical significance determined by *one-way ANOVA* analysis of the change difference: * p value $\leq$ 0.05

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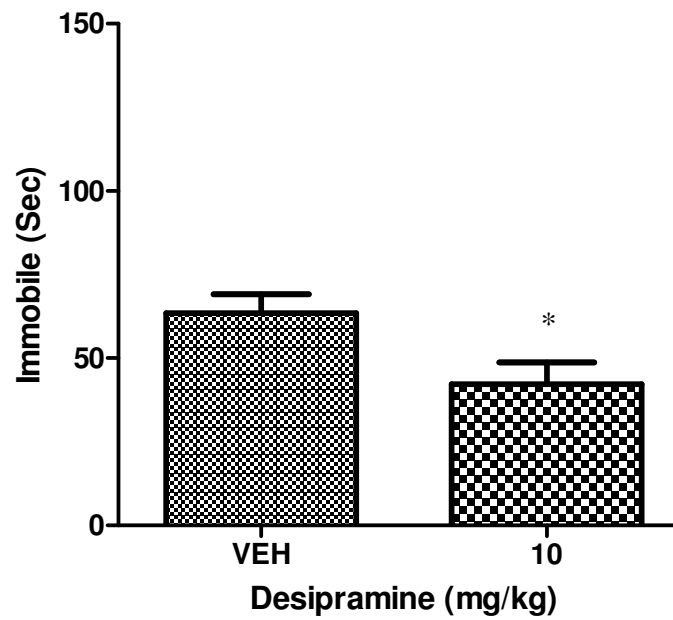
APPENDIX

Appendix A. **Effects of DMI on time spent immobile in FST1 (25° C).** C57Bl/6 mice received vehicle or 10 mg/kg of DMI prior to FST exposure. Time spent immobile for the full 6 min of the FST session. Abscissae, VEH and DMI. Ordinates, time spent immobile measured in seconds. Data were calculated using the mean $\pm$ SEM. An asterisk indicates statistical significance between the control and the treatment used, a p value $\leq$ 0.05 (*). n=8-9.

Appendix B. **Effects of DMI, URB597, and AM404 on average velocity in the open field test.** C57Bl/6 mice received vehicle (VEH), 10 mg/kg of DMI, 0.03-3.2 mg/kg of URB597, or 0.3203.2 mg/kg of AM404. A) Baseline center zone entries recorded in the absence of drug administration and prior FST1 exposure (Pre-FST1). B) Center zone entries recorded immediately following drug administration and FST1 exposure (Post-FST1). C) Center zone entries recorded a week after FST1 exposure and following drug administration (LMA-FST1). Abscissae, Groups of mice (1-6; A), VEH, DMI, URB597, AM404 (B, C). Ordinates, average velocity measured in centimeters per second (cm/sec). The group numbers correspond to the drug administered to each group during the Post-FST1 and LMA-FST1 experiments (DMI:1=VEH, 2=10 mg/kg of DMI; URB597: 1=VEH, 2=0.03 mg/kg of URB597, 3=0.1 mg/kg of URB597, 4=0.32 mg/kg of URB597, 5=1.0 mg/kg of URB597; 6=3.2 mg/kg of URB597; AM404: 1=VEH, 2=0.32 mg/kg of AM404, 3=1.0 mg/kg of AM404, 4=3.2 mg/kg of AM404. Data were calculated using the mean $\pm$ SEM. An asterisk indicates statistical significance between VEH and DMI, URB597, AM404, p value $\leq$ 0.05 (*). n=8-10.

Appendix C. **Effects of DMI, URB597, AM404 on center zone entries in the open field test.** C57Bl/6 mice received vehicle (VEH), 10 mg/kg of DMI, 0.03-3.2 mg/kg of URB597, or 0.32-3.2 mg/kg AM404. A) Baseline center zone entries recorded in the absence of drug administration and prior FST1 exposure (Pre-FST1). B) Center zone entries recorded immediately following drug administration and FST1 exposure (Post-FST1). C) Center zone entries recorded a week after FST1 exposure and following drug administration (LMA-FST1). Abscissae, Groups of mice (1-6; A), VEH, DMI, URB597, AM404 (B, C). Ordinates, number of center zone entries measured in a cumulative number (#). The group numbers correspond to the drug administered to each group during the Post-FST1 and LMA-FST1 experiments (DMI:1=VEH, 2=10 mg/kg of DMI; URB597: 1=VEH, 2=0.03 mg/kg of URB597, 3=0.1 mg/kg of URB597, 4=0.32 mg/kg of URB597, 5=1.0 mg/kg of URB597; 6=3.2 mg/kg of URB597; AM404: 1=VEH, 2=0.32 mg/kg of AM404, 3=1.0 mg/kg of AM404, 4=3.2 mg/kg of AM404. Data were calculated using the mean $\pm$ SEM. An asterisk indicates statistical significance between VEH and DMI, URB597, AM404, p value $\leq$ 0.05 (*). n=8-10.
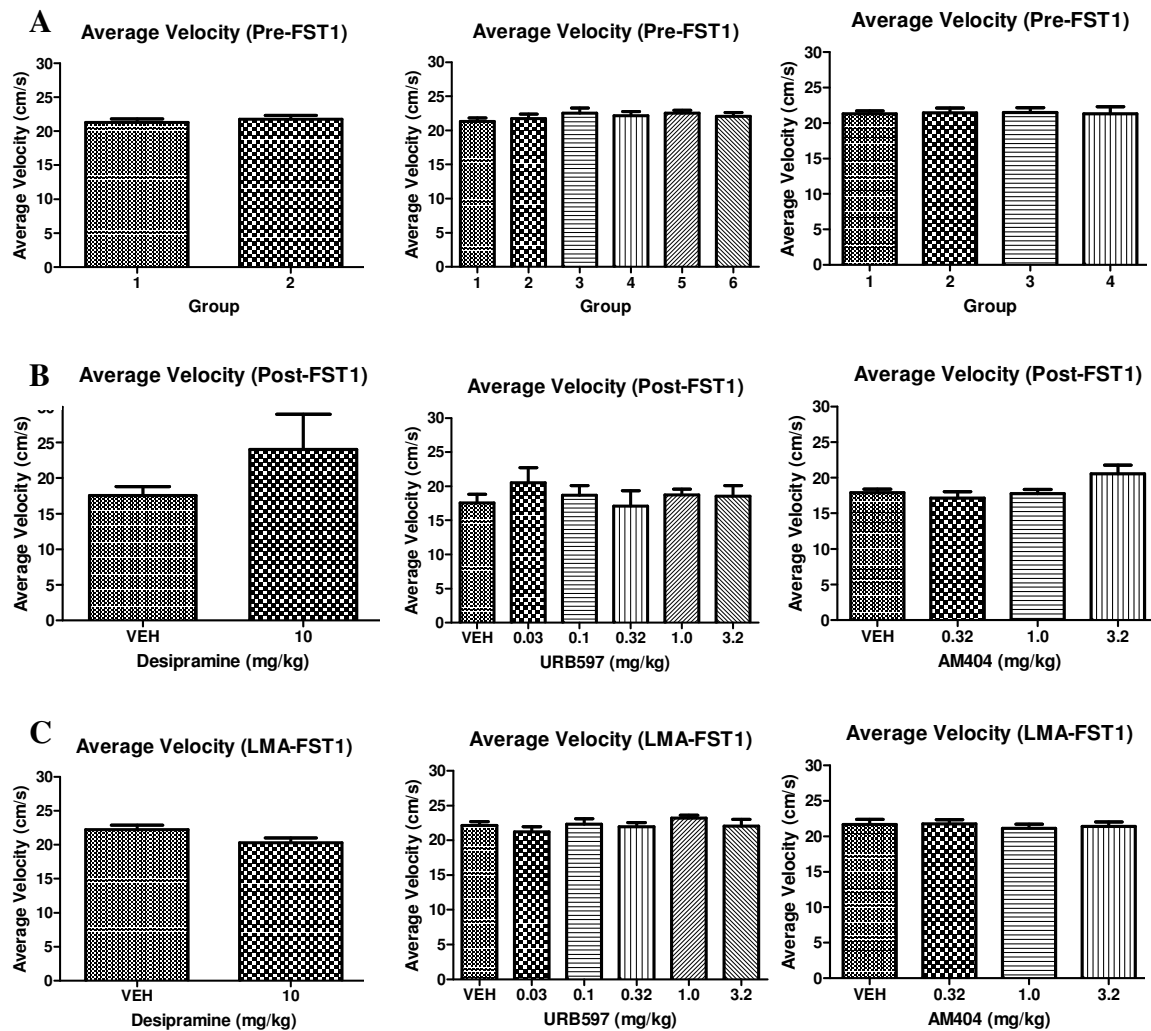
Appendix D. Effects of DMI, URB597, AM404 on time in the center zone in the open field test. C57Bl/6 mice received vehicle (VEH), 10 mg/kg of DMI, 0.03-3.2 mg/kg of URB597, 0.32-3.2 mg/kg of AM404. A) Baseline time in the center zone recorded in the absence of drug administration and prior FST1 exposure (Pre-FST1). B) Time in the center zone recorded immediately following drug administration and FST1 exposure (Post-FST1). C) Time in the center zone recorded a week after FST1 exposure and following drug administration (LMA-FST1). Abscissae, Groups of mice (1-6; A), VEH, DMI, URB597, AM404 (B, C). Ordinates, time in the center zone measured in minutes (min). The group numbers correspond to the drug administered to each group during the Post-FST1 and LMA-FST1 experiments (DMI:1=VEH, 2=10 mg/kg of DMI; URB597: 1=VEH, 2=0.03 mg/kg of URB597, 3=0.1 mg/kg of URB597, 4=0.32 mg/kg of URB597, 5=1.0 mg/kg URB597; 6=3.2 mg/kg of URB597; AM404: 1=VEH, 2=0.32 mg/kg of AM404, 3=1.0 mg/kg of AM404, 4=3.2 mg/kg of AM404. Data were calculated using the mean $\pm$ SEM. An asterisk indicates statistical significance between VEH and DMI, or URB597, p value $\leq$ 0.05 (*). n=8-10.
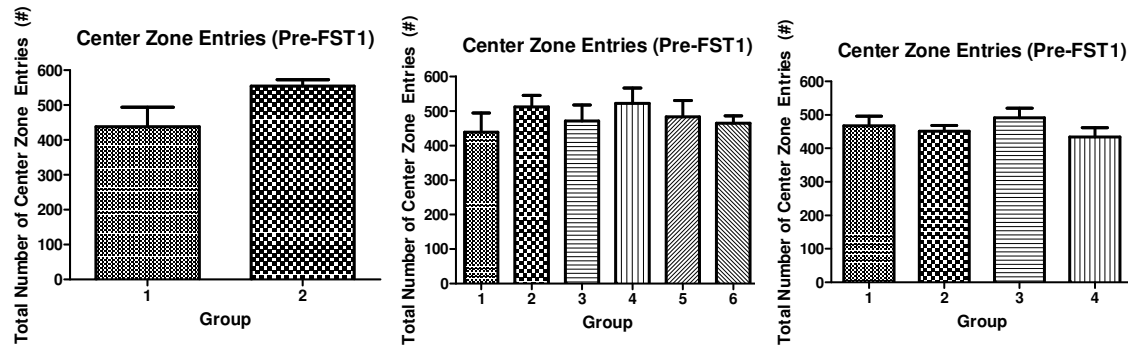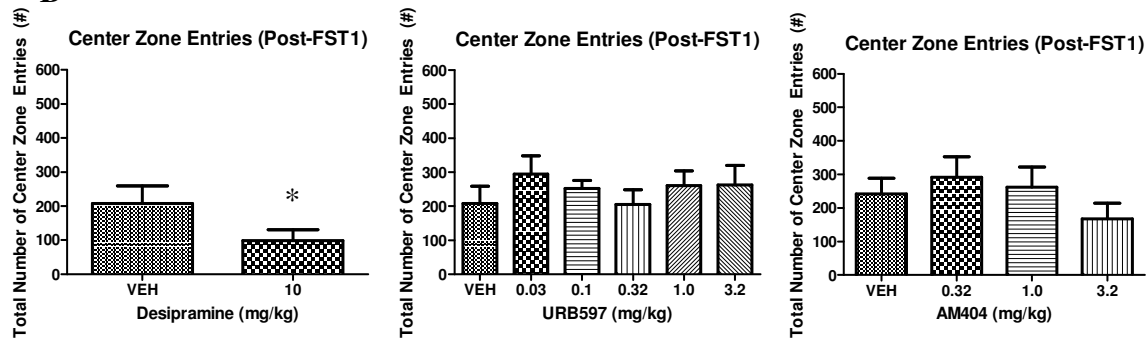
Appendix A

* p value < 0.05

Appendix B

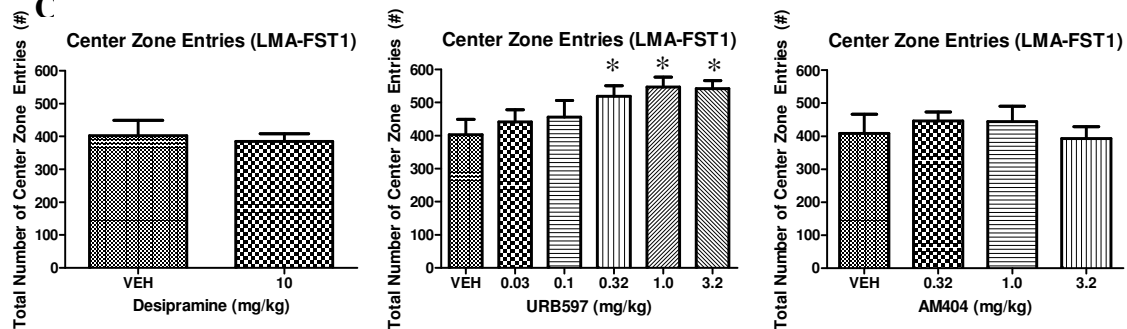**A** Average Velocity (Pre-FST1) — Group

B Average Velocity (Post-FST1) — Desipramine (mg/kg), URB597 (mg/kg), AM404 (mg/kg)

C Average Velocity (LMA-FST1) — Desipramine (mg/kg), URB597 (mg/kg), AM404 (mg/kg)

* p value ≤ 0.05

Appendix C

A

Center Zone Entries (Pre-FST1)

Center Zone Entries (Pre-FST1)

Center Zone Entries (Pre-FST1)

B

Center Zone Entries (Post-FST1)

Center Zone Entries (Post-FST1)

Center Zone Entries (Post-FST1)

C

Center Zone Entries (LMA-FST1)

Center Zone Entries (LMA-FST1)

Center Zone Entries (LMA-FST1)

* p value < 0.05

Appendix D

A

B

C

* p value ≤ 0.05