

**Cannabinoids & stress: The impact of endogenous and exogenous
cannabinoids on anxiety behaviors in an acute stress model**

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Abstract

Although the impact of cannabinoids (CBs) on anxiety has been thoroughly studied, current research paradigms fail to incorporate acute stressors. The present study investigated the synthetic CB HU-210's anxiolytic potential in an acute stress CD1 male mouse model, where the animals were subject to a 10-minute Forced Swimming (FS) test between treatment and behavioral tests. Surprisingly, HU-210 did not show anxiolytic action in the Open Field (OFT) and Elevated-Plus Maze (EPM) stressed mice as previously reported in the naïve model literature. The combination of acute stress and high HU-210 doses produced severe locomotor impairments in ambulatory movement that were not previously observed in unstressed mice. It is hypothesized that this anxiogenic phenotype results from the summation of exogenous CB treatment and stress-induced endocannabinoid (eCB) release.

Subsequently, the impact of the eCB signaling on anxiety behaviors was examined. Systemic administration of KML29, the selective inhibitor of 2-AG degradative enzyme, returned stress-induced anxiety-like behaviors to baseline levels, without significantly affecting locomotion. KML29's anxiolyticism was abolished when combined with the cannabinoid receptor antagonist AM281, implying this is a CB receptor-mediated process. A GABA_A receptor agonist muscimol was co-administered with KML29 in order to pharmacologically investigate the role of GABAergic neurotransmission in this anxiolytic phenomenon, but it did not alter KML29's effects.

Collectively, these findings suggest that exogenous CBs and acute stress act synergistically in an anxiogenic manner, but that enhanced 2-AG signaling in response to stress demonstrates anxiolytic potential.

Preface

Findings from the exogenous cannabinoid treatment presented in the current thesis were recently published:

1. Kinden, R. & Zhang, X. (2015). Cannabinoids & Stress: Impact of HU-210 on behavioral tests of anxiety in acutely stressed mice. *Behavioural brain research*, 284:225-230.

This thesis will present the data that I collected from the aforementioned study and additional findings from my subsequent experiments as part of my Master's project as a cohesive report.

Figures adapted from this paper include: Fig. 3.1, 3.2.1 & 3.2.2

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List of Abbreviations & Chemical Names

2-AG	2-Arachidonoylglycerol
ACTH	Adrenocorticotrophic Hormone
AEA	Anadamide or Arachidonylethanolamide; N-(2-Hydroxyethyl)-5Z,8Z,11Z,14Z-ei
AM281	1-(2,4-Dichlorophenyl)-5-(4-iodophenyl)-4-methyl-N-4-morpholinyl-1H-pyrazole-3-carboxamide
CB	Cannabinoid
CB ₁ R	Cannabinoid type-1 Receptor
CB ₂ R	Cannabinoid type-2 Receptor
CRH	Corticotropin-Releasing Hormone
eCB	Endogenous Cannabinoid or Endocannabinoid
DMSO	Dimethyl sulfoxide
EPM	Elevated-Plus Maze
FAAH	Fatty Acid Amine Hydrolase
FS	Forced Swimming Test
GABA	γ -Aminobutyric acid
Glu	Glutamate
GPCR	G-Protein Coupled Receptor
HPA-axis	Hypothalamic-Pituitary Adrenal axis
HU-210	(6aR)-trans-3-(1,1-Dimethylheptyl)-6a,7,10,10a-tetrahydro-1-hydroxy-6,6-dimethyl-6H-dibenzo[b,d]pyran-9-methanol
KML29	4-[Bis(1,3-benzodioxol-5-yl)hydroxymethyl]-1-piperidinecarboxylic acid 2,2,2-trifluoro-1-(trifluoromethyl)ethyl ester
MAGL	Monoacylglycerol lipase
Muscimol	5-Aminomethyl-3-hydroxyisoxazole
OFT	Open Field Test
THC	Tetrahydrocannabinol

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“Imagination is more important than knowledge. For knowledge is limited to all we now know and understand, while imagination embraces the entire world, and all there ever will be to know and understand.”

A. Einstein

Chapter 1: Introduction

1.1 – Defining Stress, Anxiety & Anxiety Disorders

1.1.1 – Stress

In recent years, the research community has heavily examined stress and its impacts on multiple facets of human behavior and health. However, the branching of stress-related research topics has led to a vague and ambiguous definition of the stress concept. For the purposes of this study, we adopted an operational definition of stress from Koolhaas et al. (2010), in which stress is a result of an environmental stressor that exceeds the natural regulatory capacity of an organism, especially in situations that are unpredictable and uncontrollable.

Exposure to a stressor leads to a cascade of psychophysiological responses in order to restore homeostasis. The psychological effects of stressors are evident in the manifestation of anxiety in response to disrupted homeostasis. The physiological manifestations of stress are characterized by the classic “Fight or Flight” response. The stress response is mainly mediated through the activation of the Hypothalamic-Pituitary-Adrenal (HPA) axis (Herman & Cullinan, 1997).

1.1.2 – HPA-axis

The HPA-axis constitutes of the hypothalamus, pituitary gland the adrenal cortex, which collectively result in a short-term “fight or flight” response and longer term adaptation to stress. The initial perception of a stressor stimulates the synthesis of corticotropin-releasing factor (CRF) in the periventricular nucleus (PVN) of the hypothalamus. Once released into the hypophysial portal vessels, CRF

binds to its receptors in the pituitary gland, which leads to the synthesis and release of adrenocorticotrophic hormone (ACTH) into systemic circulation. ACTH ultimately reaches the adrenal glands, above the kidneys, which in turn leads to glucocorticoid (GC) production and adrenaline release from the cortex. The activation GC receptors produce a variety of effects, such as increased heart and breathing rates, as well as mobilize energy stores (Rivier & Vale, 1983).

It's well established that glucocorticoid hormones elicit a wide variety of adaptive and advantageous physiological short-term changes in response to stress-induced HPA-axis activation. However, prolonged glucocorticoid effects can be detrimental on various biological systems, including immune, cardiovascular, neuroendocrine and metabolic functions (Chrousos, 2009). This is why that in the case of chronic HPA-axis activity, where the glucocorticoid system is hyperstimulated, a different set of physiological changes occurs that favors the habituation and desensitization of the stress response. Failure to adequately adapt to chronic stress can lead to the development of multiple different ailments, including depression and mood disorders (McEwen, 2005).

1.1.3 – Anxiety

Although it is often used synonymously with stress, anxiety is the psychological response to stress that is characterized by feelings of tension, apprehension and worry in response to an external stimulus. The National Institute of Mental Health (NIMH) defines anxiety as a normal, often adaptive reaction to stress. In general, anxiety is relatively mild and is often referred to as acute anxiety

as a result of its short and transient nature. However, when anxiety is persistent, it becomes distressing to an individual and disrupts their quality of life. Chronic anxiety lasting more than 6 months is a hallmark symptom of Anxiety Disorders (NIMH, 2009).

1.1.4 – Anxiety Disorders

Anxiety disorder is an umbrella term that encompasses many different forms of anxiety. It is characterized by irrational and excessive worry, apprehension and tension, which interfere with daily functioning (Rector et al., 2009). It is commonly accepted that excessive or inappropriate excitatory glutamate activity within anxiety-related brain circuits is an underlying factor in the pathology of anxiety disorders (Swanson et al., 2005). Anxiety disorders are divided into several subtypes in the Diagnostic and Statistical Manual of Mental Disorders (DSM-5; APA, 2013). The most common disorders are Post-Traumatic Stress Disorder (PTSD) and Generalized Anxiety Disorders (GAD) with a lifetime prevalence of 8% and 5%, respectively (Langlois et al., 2013). Panic disorder, social anxiety, phobias and other unspecified anxiety disorders are also defined in the DSM-5 (APA, 2013). Although these disorders' individual characteristics vary, they have two underlying commonalities: chronicity of anxiety-related symptoms and interference in daily quality of life.

According to the World Health Organization's mental health survey initiative, anxiety disorders are consistently the most prevalent class of mental disorder affecting the general population. Across the 17 countries participating in this survey initiative, anxiety disorders average an estimated lifetime prevalence of 16% in

comparison to the 12% lifetime prevalence of Mood Disorders. The high lifetime prevalence rates and the societal/economic costs, such as absenteeism and loss of productivity, of anxiety disorders emphasize the importance of efficacious treatment options (Kessler et al., 2009).

1.1.5 – Brain Circuitry Involved in Anxiety

The functional neuroanatomy of stress has been widely reviewed in the scientific literature. It is believed that the amygdala plays a critical role in mediating and interpreting stress responses due its complex interconnectivity. For instance, the amygdala receives extensive afferents from thalamic, cortical and subcortical projections and subsequently relays information through a series of efferent pathways, including the hypothalamus, hippocampus, periaqueductal gray mater, locus coeruleus and striatum. These proposed efferent pathways are believed to regulate stress and anxiety through the modulation of neuroendocrine, autonomic and skeletal-motor responses (Charney & Deutch, 1996).

One area that has gained interest in the modulation of anxiety-related behaviors is the amygdala, a collection of nuclei highly involved in emotional processing and responses. Alterations in amygdala activation have been associated with various anxiety disorders. For example, hyper-activation as measured by a functional MRI (fMRI) has been observed in patients with specific phobias and panic disorders, whereas hypo-activation was seen in PTSD (Etkin & Wager, 2007). There is also evidence to suggest that the communication between the basolateral amygdala (BLA) and the central amygdala (CeA) is critical to the bidirectional

modulation of anxiety-related behavior in an optogenetic study, where stimulation of the BLA terminals in the CeA lead to an anxiolytic effect and inhibition produced increases in anxiety behavior (Tye et al., 2011). In addition to the CeA, the BLA projects to the medial temporal memory system, striatum and nucleus accumbens, and possesses bidirectional connections with the pre-frontal cortex (Sah et al., 2003). Collectively, this firmly suggests a potential modulatory role for the BLA in the regulation of anxiety circuitry.

1.1.6 – Current Treatment Strategies for Anxiety Disorders

Despite the need for adequate anxiolytic treatment options, existing treatment options, including benzodiazepines (BZDs), monoamine oxidase (MAO) inhibitors and selective serotonin reuptake inhibitors (SSRIs), are restricted by their limited efficacy and problematic side effect profiles, which generally include sedation, memory disturbances, as well as issues with substance dependence, tolerance and abuse potential. The lack of knowledge surrounding the precise cellular and molecular mechanisms underpinning the psychopathologies characteristic of anxiety disorders is one of the contributing factors to the current constraints on the development of newer, more efficacious and less harmful pharmacological therapeutic interventions (Griebel & Holmes, 2013).

1.2 – Cannabinoids & The Cannabinoid System

1.2.1 – Overview

Traditionally used as a medicinal herb, cannabis was one of the most commonly used recreational drug by the general Canadian population in 2012, second only to alcohol (Canadian Center of Substance Abuse [CCSA], 2014). The activation of the cannabinoid system produces a desirable “high” effect, which is commonly reported by recreational marijuana users. The cannabinoid system is comprised of two primary receptor subtypes and endogenous ligands, in addition to synthetic and degrading enzymatic pathways. The ligands are lipophilic retrograde signaling molecules released from the post-synaptic axon terminal upon stimulation. The release of neuromodulators into the synaptic cleft activates pre-synaptic receptors, which ultimately reduces subsequent neurotransmitter release in the pre-synaptic neuron and produces the well-established euphoric effects of cannabinoids.

1.2.2 – Types of Cannabinoids

1.2.2.1 Exogenous Cannabinoids – There are two main types of exogenous cannabinoids: phytocannabinoids and synthetic cannabinoids. Phytocannabinoids are natural cannabinoid agents produced by the cannabis plant. In light of its growing recreational popularity in the 1950s, a group of researchers sought to identify the active ingredient in cannabis and successfully isolated Δ^9 -tetrahydrocannabinol (THC) via chromatography of hashish resin extract, which yielded the chemical structure of the molecule (Gaoni & Mechoulam, 1964).

This discovery prompted the development of many high-affinity synthetic forms of cannabinoids based on THC's chemical structure. For instance, Mechoulam and colleagues (1988) synthesized an enantiomeric pair of pharmacologically distinct compounds, one of which demonstrated potent cannabimimetic activity and would be later named HU-210. Structural analysis of HU-210 revealed a tricyclic benzopyran structure that gives rise to the compound's lipophilic properties, which enables it to cross the blood-brain barrier. Previously synthesized enantiomers failed to show such pharmacological selectivity, but HU-210 bound to cannabinoid receptors with a 1500x greater affinity than its enantiomeric counterpart (Howlett et al., 1990) and 70-800x greater than THC (Mechoulam et al., 1988).

1.2.2.2 Endogenous Cannabinoids – It was another 30 years before the discovery and isolation of endogenous cannabinoids, otherwise known as endocannabinoids (eCB). The first brain cannabinometric agent isolated was Anandamide (AEA), which bound to the same cannabinoid receptors as THC and produced similar psychoactive and behavioral side effects (Devane et al., 1992; Mechoulam & Fride, 1995). AEA acts as a partial agonist at cannabinoid receptors (Mackie, Devane & Billie, 1993) and as a full agonist at TRVP1 vanilloid receptors (Smart et al., 2000). The discovery and characterization of 2-arachidonoylglycerol (2-AG) shortly followed. This particular endocannabinoid exhibits full agonism at central cannabinoid receptors and is reportedly found at 200x higher concentrations than AEA within brain tissue (Stella, Schweitez & Piomelli, 1997).

AEA and 2-AG have distinct synthetic and degradation enzymatic pathways, which are believed to be triggered by post-synaptic increases in internal calcium concentrations. Briefly, 2-AG is synthesized from the catalytic action of phospholipase C (PLC) and diacylglycerol lipase (DAGL) on inositol phospholipids, and mainly degraded by monoacylglycerol lipase (MAGL), which are located on the pre-synaptic membrane. AEA is synthesized from membrane lipids via a transacylation-phosphodiesterase pathway, and degraded by fatty acid amine hydrolase (FAAH), which is found on intracellular membranes of post-synaptic cells (Ohno-Shosaku et al., 2012). A variety of pharmacological agents have been developed to target the synthetic and degrading enzymes in order to experimentally manipulate eCB concentrations, such as KML29 (highly selective MAGL inhibitor), PF3845 (FAAH inhibitor) and RHC80267 (DAGL inhibitor).

Although known to act as retrograde signaling molecules, it wasn't until years after its initial discovery that a physiological role for eCB signaling was reported. A clever sequential experimental design revealed that eCBs mediate the transient suppression of GABA neurotransmission observed at hippocampal synapses after the depolarization of glutamatergic pyramidal neurons (Wilson & Nicoll, 2001). A more recent study pinpoints the involvement of 2-AG, not AEA, in retrograde synaptic suppression within the CNS (Tanimura et al., 2010). These findings highlight the distinct roles of different endogenous cannabinoids in the retrograde modulation of neurotransmission.

1.2.3 – Cannabinoid Receptors

There are two main classes of cannabinoid receptors: CB₁R and CB₂R. A third class of cannabinoid receptors has been proposed, but is widely and hotly debated in the research community. For the scope of the current research project, we will only focus on the centrally located CB₁Rs, seeing as CB₂Rs mainly execute anti-inflammatory effects within the periphery.

1.2.3.1 Receptor subtype – CB₁Rs belong to the Class A of the G-protein coupled receptors (GPCRs) superfamily, which contains 1000-2000 different subtypes of receptors. Structurally speaking, these receptors are generally characterized by 7 trans-membrane domains and contain a toggle switch that detects structural changes that result from agonist binding to the receptor and causes rotamerization of the 6th trans-membrane domain. This conformational change promotes the binding of G-proteins to the receptor. The specific intracellular signaling cascades activated by CB₁Rs are dependent on the G-protein coupled to the receptor (Turu & Hunyady, 2010).

CB₁Rs are primarily coupled to the G_{i/o} family of G-proteins, which mediate the majority of the psychoactive effects of cannabis, including euphoria, appetite stimulation and lethargy. These effects are a result of the inhibitory nature of these G-proteins and their suppressing effects on adenylyl cyclase, as well as the suppression of voltage-gated calcium channels and stimulation of inward-rectifying potassium channels (GIRKs). Although preferential coupling of the CB₁ to G_{i/o} proteins has been established in the literature, these receptors can also couple with G_q proteins. The activation of the CB₁ G_q protein coupled receptors stimulates the

phospholipase C- β (PLC- β) intracellular signaling cascade, which ultimately reduced intracellular calcium concentrations (Review by Boisier et al., 2010).

1.2.3.2 Anatomical localization of CB₁Rs – Stimulation of the cannabinoid system produces a variety of physiological effects, which reflects its diffuse distribution throughout the central nervous system. A group of researchers developed a radioactive synthetic cannabinoid, [3H]CP55,940, which was specifically developed as a CB₁R agonist. Autoradiography revealed a unique and conserved distribution pattern amongst humans and several mammalian species, including dogs, mice, rats and monkeys. The greatest density of CB₁Rs were found in the basal ganglia, hippocampus and the cerebellum, which correlate with the main side effects of cannabinoid consumption: impaired working memory and altered locomotor abilities. Notable densities of CB₁Rs were also found in the amygdala, cingulate cortex and hypothalamus (Herkenham et al., 1990).

1.2.3.3 Subpopulation localization of CB₁Rs – CB₁Rs are believed to be the most highly expressed GPCR in the central nervous system, but its expression is cell population specific. Marsicano and Lutz (1999) devised an *in situ* hybridization experiment that sought to detect CB₁R mRNA while simultaneously measuring mRNA targeting glutamatergic and GABAergic cells. Two very distinct CB₁R-expressing cell populations emerged, where one highly expressed CB₁Rs and the other exhibited low, but significant, levels. The majority of the cells expressing high levels of CB₁Rs were GABAergic interneurons. Interestingly, low CB₁R-expressing cells in the hippocampus, amygdala and entorhinal cortex were non-GABAergic in

nature, and believed to be projecting glutamatergic neurons (Marsicano & Lutz, 1999).

1.2.3.4 Effects of CB₁Rs on Neurotransmission – The activation of CB₁Rs induces a cascade of intra-cellular events that ultimately inhibits future neurotransmitter release in the pre-synaptic terminal. Briefly, CB₁R signal transduction via $g_{i/o}$ -proteins generally inhibits cyclic AMP (cAMP) production by suppressing adenylyl cyclase, as well as regulates calcium influx and inward-rectifying potassium (GIRK) ion channels. Stimulation of CB₁Rs suppress internal calcium and increase potassium concentrations, which repolarize the membrane and reduces the probability of subsequent neurotransmitter release. It is important to note that signal transduction via CB₁Rs is ligand-dependent, where full and partial agonist/antagonist/inverse agonist actions depend on the specific $g_{i/o}$ -protein associated with a receptor (Turu & Hunyady, 2010).

1.3 – Cannabinoids & Anxiety

1.3.1 – Historical Overview of Medicinal Cannabis

The use of cannabis for its mood- and perception-altering abilities, and medicinal properties has been reported for centuries, across various cultures. The first documentation of medicinal cannabis use for the treatment of anxiety was reported in Sanskrit and Hindi literature, dating over 2,600 years (Grierson, 1894). Despite its status as a mainstream medicine during the 19th century, contradictory political standpoints, severe quality control issues on exported goods from India to

the UK and subsequent prohibitions saw a rapid decline in Western medicinal cannabis use in the early 20th century (Dixon, 1923).

Today, cannabis remains listed under restricted substances, but became available for medicinal purposes in Canada as of 2001. Generally, cannabis can be prescribed for patients suffering from severe pain associated with multiple sclerosis, spinal injuries, arthritis, cancer and AIDS, as well as epileptic seizures. However, cannabis can be prescribed for disorders other than those stated above with the support of a medical practitioner (Controlled Drugs & Substance Act [CDSA], 2014).

1.3.2 – Role of cannabinoids in modulation of anxiety

Although the central mechanisms underlying stress habituation remain unknown, a large body of research over the past 20 years implies an emerging role of the cannabinoid system as an important regulator of the stress response and a prime candidate in mediating stress adaptation. First and foremost, many brain regions involved in anxiety circuitry contain high densities of CB₁Rs, including the amygdala, hippocampus, hypothalamus and prefrontal cortex (Herkenham et al., 1990). This implies that the cannabinoid system has the appropriate anatomical localization in order to target disrupted brain circuits involved in anxiety-related disorders.

Secondly, CB₁Rs are largely expressed on GABAergic and Glutamatergic pre-synaptic terminals, where they suppress calcium influx and subsequent neurotransmission upon stimulation (Freund et al., 2003). This provides

cannabinoids with a biphasic mechanism to indirectly enhance or directly inhibit neurotransmission in anxiety circuitry. That is to say, GABAergic CB₁Rs could indirectly stimulate circuits via its suppression of tonic GABA inhibition on glutamatergic neurons or directly inhibit its hyperactivity via Glutamate CB₁Rs.

In addition to the optimal localization of CB₁Rs in the CNS, stress-induced HPA-axis activation has been shown to modulate the endogenous cannabinoid system. Repeated restraint stress in Sprague Dawley rats consistently produced a decrease in AEA content in corticolimbic structures, such as the frontal cortex, hypothalamus, amygdala and hippocampus, whereas it exclusively enhanced 2-AG concentrations in the amygdala. Furthermore, this divergent modulation of eCBs contributed to their distinct roles in terminating HPA-axis activity. The authors demonstrated that the inhibition of AEA hydrolysis via URB597 treatment suppressed stress-induced corticosterone hyper-secretion, implying that AEA is critical to this adaptive process. Furthermore, systemic and intra-basolateral amygdala (BLA) pharmacological blockade of CB₁Rs via AM404 treatment after repeated stress prevented stress-induced corticosterone suppression. Collectively, these results support the notion that AEA is a tonic modulator of neurotransmission, where HPA-axis activity suppresses corticolimbic concentrations of AEA to enhance corticosterone activity, and that 2-AG and CB₁Rs attenuate HPA-axis activity through its inhibitory actions on neurotransmission in the BLA (Hill et al., 2010a).

Consequently, a variety of synthetic ligands have been developed to target CB₁Rs and the behavioral consequences of its modulation have been thoroughly investigated in the literature. Research has shown that acute pharmacological

antagonism of CB₁Rs induces an anxiogenic behavioral phenotype in unstressed rats (Navarro et al., 1997), whereas agonism produced an anxiolytic effect similar to that of Diazepam, a commonly prescribed anxiolytic (Patel & Hillard, 2006). Initial investigations focused on cannabinoid modulation of anxiety yielded contradictory findings. The issue laid in the inconsistency of doses used to examine the anxiolytic potential of cannabinoids. Further empirical research revealed that cannabinoids modulated anxiety-related behavior in a biphasic manner. Specifically, cannabinoids elicited an anxiogenic behavioral phenotype at high doses, whereas low doses produced anxiolytic effects (Hill & Gorzalka, 2004).

Until recently, the molecular and cellular mechanisms underlying this biphasic modulation of anxiety behavior remained unclear. A study has shed some light on the molecular underpinnings of this phenomenon through its investigation of cannabinoid agonism (CP-55,940) in two lines of conditional CB₁R knockout mice, where either cortical glutamatergic or forebrain GABAergic CB₁Rs were genetically knocked out in female and male mutant mice via a flox-Cre system. It turns out that CB₁Rs differentially modulate anxiety behavior through distinct neuronal populations. As previously mentioned, high doses of cannabinoid agonism elicits an anxiogenic behavioral phenotype in the EPM and holeboard tests, but this effect was abolished in GABA-CB₁R KO mice. Additionally, where low doses of CB₁R agonists typically produced anxiolytic effects in wild-type controls, Glutamate-CB₁R KO mice exhibited an increase in anxiety behavior in the aforementioned tests. Collectively, these results imply that CB₁R activation is critical to maintaining the balance of

GABAergic and Glutamatergic neurotransmission, which is shown to be disrupted anxiety disorders (Rey et al., 2012).

Collectively, these findings imply a strong role for the cannabinoid system in the modulation of anxiety brain circuitry and behavior through the regulation of GABA and Glutamatergic neurotransmission. Cannabinoids seem to play a critical role in the termination of HPA-axis activation through its negative regulation of neurotransmission in the BLA and corticolimbic structures.

1.3.3 – Classic experimental design in anxiety-cannabinoid literature

Generally, an unstressed behavioral paradigm is employed to investigate the anxiolytic potential of various cannabinometric agents. Specifically, subjects receive a cannabinoid pre-treatment prior to testing in one or more anxiety-sensitive behavioral apparatuses, such as the OFT, EPM, dark-light box, etc. The performance of the cannabinoid-treated subjects is then compared to that of vehicle-treated controls, and statistically analyzed for significant effects.

1.5 – Rationale for the Current Study

A major limitation with the aforementioned protocol is that the subjects themselves are not exposed to stress, which compromises the design's external validity, as the ultimate goal of these experiments is to eventually provide evidence that supports the use of cannabinoids as a novel therapeutic intervention for anxiety disorders. One group of researchers have examined the effects of cannabinoids on stress-related behaviors in a chronic restraint stress model in Sprague Dawley rats,

which revealed that the effects of high and low doses of cannabinoids have different effects under stressed and unstressed conditions (Hill & Gorzalka, 2004). There are no reports on whether acute stress is able to alter cannabinoid-induced behaviors, which is the gap in the literature the present study seeks to address.

In addition to investigating a novel paradigm, we also sought to examine the therapeutic potential of exogenous cannabinoids as a clinical tool to alleviate symptoms of anxiety-related disorders, as they are currently used clinically to treat other ailments, such as nausea in cancer patients and multiple symptoms of multiple sclerosis (MS). However, the combination of cannabinoid treatment and acute stress exposure did not produce the hypothesized anxiolytic behavioral effects, so we next sought to investigate eCB modulation of anxiety-related behaviors. As previously stated, 2-AG is more highly expressed in the central nervous system in comparison to AEA (Piomelli, 1997), and it is 2-AG that is generally accepted to be the key eCB in retrograde synaptic suppression (Tanimura et al., 2010). Intuitively, the impact of enhanced 2-AG signaling, specifically, on anxiety-related behaviors was examined in the second part of this study.

1.6 - Aims

First and foremost, the primary aim of the current study was to investigate the effects of exogenous cannabinoids on anxiety-related behaviors in an acutely stressed model, which had never been examined. It was hypothesized that measures of anxiety in an acute stressed paradigm would be similarly modulated by cannabinoid pre-treatment, as previously observed in the unstressed models. That

is to say that high doses of exogenous cannabinoids would increase anxiety behaviors, whereas low doses would produce an anxiolytic effect.

Due to unexpected effects of acute stress exposure on the impact of exogenous cannabinoid on anxiety-related behaviors, we further sought to investigate the impact of enhanced eCB signaling on measures of anxiety. It was hypothesized that enhanced 2-AG signaling via the pharmacological blockade of its degrading enzyme MAGL would produce anxiolytic effects. If it did indeed decrease anxiety behaviors, then it was hypothesized that this anxiolytic behavioral phenotype was CB₁R-dependent and likely involved receptors found at Glutamatergic pre-synaptic terminals.

Chapter 2: Materials & Methods

2.1 – Experimental Subjects

Experiments were conducted on 7-week-old male CD1 mice (30-40 grams). The mice were housed in cages of 4 under standard laboratory conditions (*ad libitum* food and water; 12 hour light-dark cycle [7AM-7PM]). All experimental subjects were handled in accordance with the guidelines of the Canadian Council on Animal Care and the protocols approved by the Animal Care Committee of the Institute of Mental Health Research (Ottawa, Ontario).

2.2 – Drugs & Drug Administration

Multiple drugs were used to investigate the cellular and molecular mechanisms underlying cannabinoid modulation of anxiety-related behaviors, including:

- AM281 (Tocris – Burlington, ON), a potent and selective CB₁R antagonist. AM281 was administered at 3 mg/kg (Zhang et al., 2012) 4 hours prior to acute stress exposure.
- HU-210 (Sigma – St. Louis, USA), a common CB₁R agonist, was administered at four doses (50, 25, 10 and 5 µg/kg) 30 minutes before acute stress. The 50 & 10 µg/kg doses were chosen based on the study by Hill & Gorzalka (2004), and the 25 & 5 µg/kg were added as complimentary and intermediate doses due to unforeseen results.

- KML29 (Cayman – Ann Arbor, USA), a highly selective MAGL inhibitor, was chosen for this study because of its ability to selectively enhance 2-AG content without affecting AEA concentrations and FAAH activity in the brain (Chang et al., 2012). KML29 was administered 4 hours prior to acute stress exposure at doses of 5, 10 and 20 mg/kg.
- Muscimol (Tocris), a potent and selective GABA_A agonist, was selected for this study due to its ability to bind directly to the endogenous GABA binding site on these receptors, as opposed to the majority of prescribed anxiolytics (Johnston et al., 1968). Muscimol was administered at 0.05 and 0.1 mg/kg 30 minutes prior to acute stress exposure.

AM281, HU-210 and KML29 were dissolved in a vehicle of DMSO: Tween 80: 0.9% NaCl (1:1:18), whereas Muscimol was dissolved in a vehicle of distilled water (DW). Both the DMSO:Tween80:Saline and DW vehicles were also tested. All treatments were received via intra-peritoneal (IP) injections with a 26-gage ½-inch needle in a volume of 0.05 ml/kg.

2.3 – Experimental Approach

In order to measure changes in anxiety-related behaviors across treatment conditions, the present study used three behavioral tests: the Open Field Test (OFT) and Elevated-Plus Maze (EPM), and used a forced swimming test (FS) as an acute stressor, as previously described (Kinden & Zhang, 2015). Briefly, mice were randomly assigned to a stressed or non-stressed paradigm (Fig. 2.1). In the stressed condition, mice received a drug or its vehicle and habituated for 30 minutes before

being exposed to the acute stressor (10-min FS). After an hour resting period, the animals were placed in the OFT (bottom right corner; facing the center) and left to explore for 5 minutes, during which the time spent in the center region was manually scored and the ambulatory movement was recorded. Immediately after, the mice were transferred to the central square of the EPM, facing the open arm, for 5 minutes. The time spent in the open arms, central square and closed arms were manually scored.

In the non-stressed condition, mice were pre-treated with a drug or its vehicle and habituated for 30 minutes prior to behavioral testing. Non-treated mice also underwent stressed and unstressed experimental paradigms as controls. All testing apparatuses were cleaned with 70% ethanol between animals.

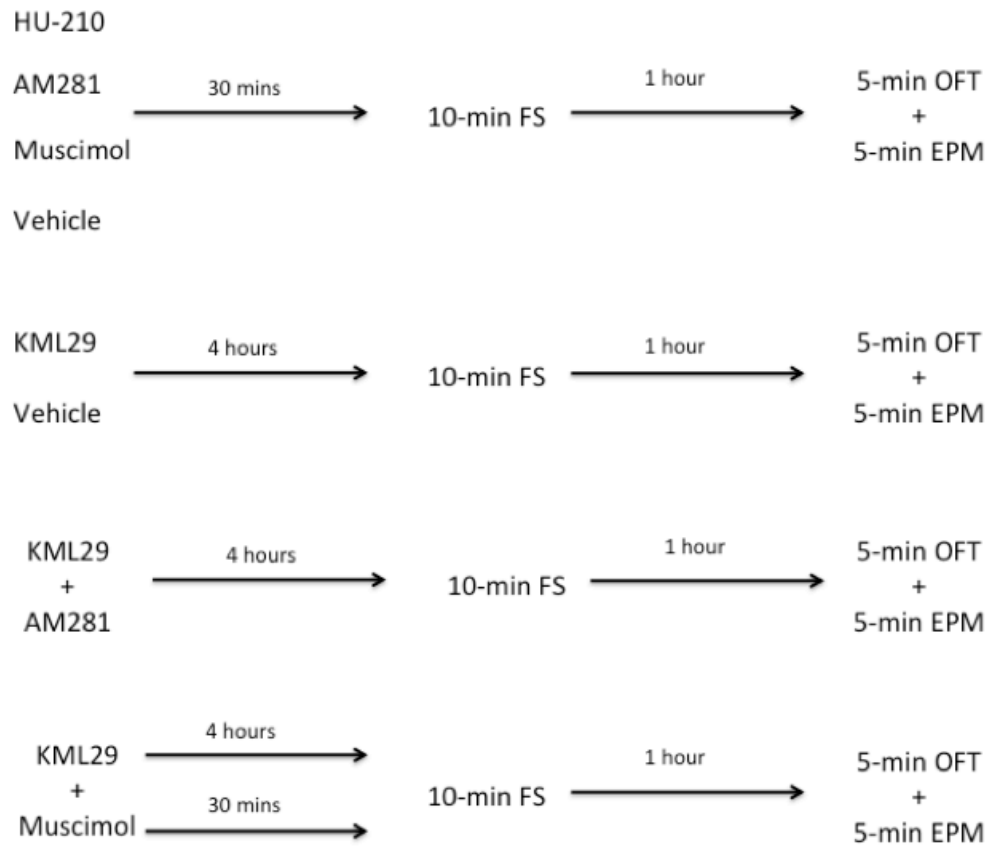


Figure 2.1: Graphical representation of the experimental approach and cannabinoid administration time. Mice received a given treatment prior to a 10-minute FS test (acute stressor) and were subsequently tested in behavioral tests (OFT & EPM, 5 minutes each) 1-hour after acute stress exposure. Administration time points vary for different pharmacological treatments.

2.4 – Exclusion Criteria

Previous literature has established that cannabinoids alter locomotor activity (Sañudo-Peña et al., 2000). The measures of anxiety used in the present study rely on the animal's ability to move from one section to another within the apparatuses. In order to rule out impaired locomotion as a confounding variable, an exclusion criterion was implemented. If the ambulatory movement of a mouse was ± 2 standard deviations from the mean of its group, the data was omitted from future statistical analyses.

2.5 – Statistical Analyses

Statistics were completed on Graphpad Prism (version 6.0) and SPSS (version 22). Statistical significance was set at $p < 0.05$. Two-way t-tests were done with Welch's Corrections, whereas multivariate ANOVA and One-way ANOVAs with Fisher's LSD tests.

Chapter 3: Results

3.1 – Efficacy of Acute Stress Paradigm

Prior to investigating the anxiolytic potential of exogenous cannabinoid treatment in an acute stress model, the paradigm had to be established to ensure that the acute stressor of choice was sufficient to elicit an anxiogenic behavioral phenotype. Two pairs of mouse groups were examined: unstressed vs. stressed paradigms and naïve (i.e. untreated) vs. vehicle treatment. A one-way ANOVA revealed that there was no significant difference in ambulatory movement amongst these four groups (Fig. 3.1A, $F_{(3,24)} = 0.8591$, $p=0.4757$). In accordance to our hypotheses, a series of two-way T-test showed that naïve stressed mice spent significantly less time in the center region of the OFT arena (Fig. 3.1B, $*p=0.0058$) and the open arm of the EPM (Fig. 3.1C, $*p=0.0099$) in comparison to unstressed controls. The same increase in anxiety-like behavior in the OFT (Fig. 3.1D, $*p=0.0026$;) and EPM (Fig. 3.1E, $*p=0.0049$) of stressed versus unstressed mice was also observed in vehicle-treated mice. In summary, these data imply that the acute stress paradigm design successfully induces an anxiety phenotype in untreated and vehicle-treated mice, without impairing locomotor activity.

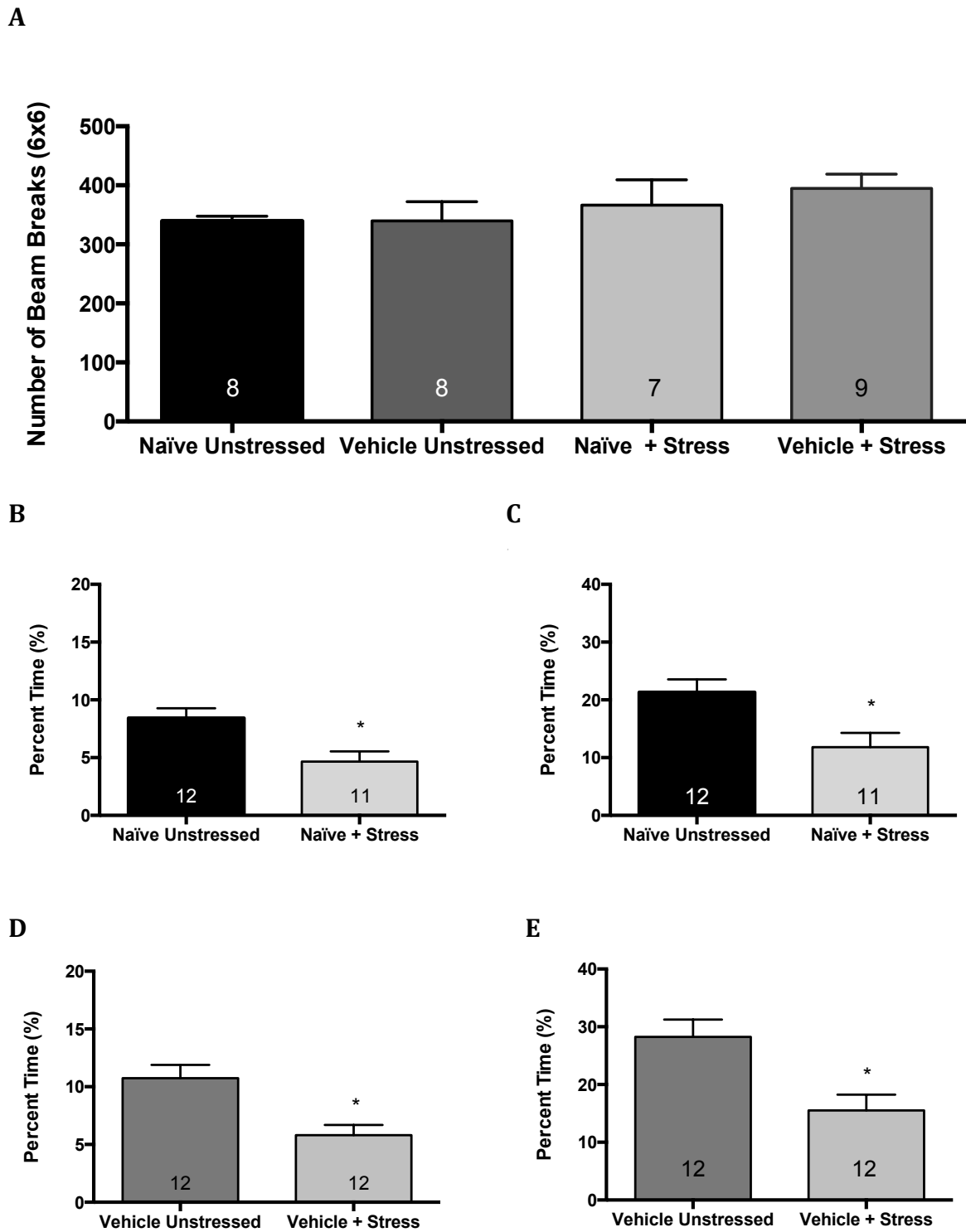


Fig. 3. 1: Acute stress induced anxiogenic behaviour in the OFT and EPM, without locomotor impairments (A; $p=0.4757$). Naive mice exposed to acute stress spent significantly less time in the center of the OFT (B; $*p=0.0058$) and open arms of the EPM (C; $*p=0.0026$) in comparison to unstressed controls. The same phenomenon was observed in vehicle treated mice (D - $*p=0.0049$; E - $*p=0.0049$). Error bars - Standard Error (SEM).

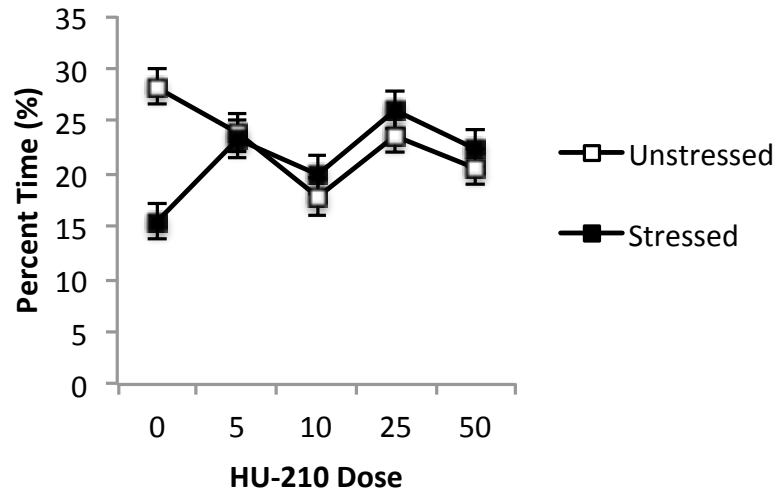
3.2 – Exogenous Cannabinoid Treatment

In order to investigate whether exogenous cannabinoid treatment had the same effects in stressed and unstressed models, the impact of HU-210 pre-treatment on anxiety-related behaviors were examined in the acute stress paradigm. Ten groups (n=12) of mice each received a vehicle or 5, 10, 25 or 50 µg/kg of HU-210 treatment with or without acute stress (FS - 10 min), followed a 5-minute sessions in the OFT and EPM.

3.2.1 – Multivariate analysis of measures of anxiety in the EPM

A multivariate analysis of variance (MANOVA) revealed no significant effects of stress ($F_{2,92}=0.789$, $p=0.457$) or HU-210 treatment ($F_{8,186}=0.894$, $p=0.523$), or interactions ($F_{8,186}=1.73$, $p=0.093$) between the two independent variables on measures of anxiety in the EPM (Fig. 3.2.1). Due to the lack of significant effects observed in the multivariate analysis, univariate main effects and interactions, as well as pairwise comparisons could not be investigated.

A



B

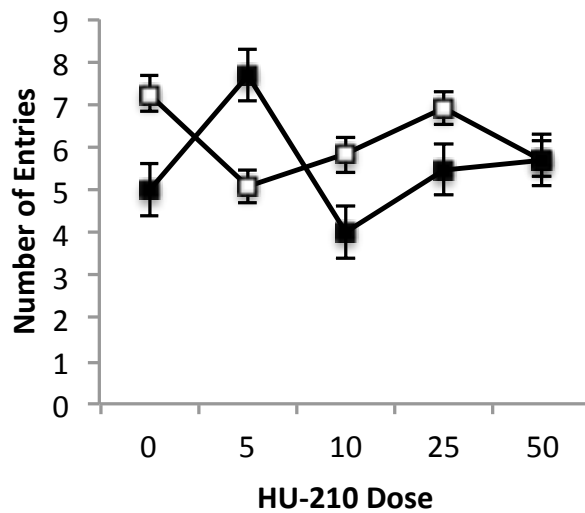


Figure 3.2.1: Multivariate ANOVA of the main effects and interactions across exogenous cannabinoid treatment groups in the EPM. There were no main effects of stress ($p=0.457$) or treatment ($p=0.523$), nor was there a significant interaction between the two ($p=0.093$). *Unstressed* - $n=8$; *Stressed* - $n=8$. Error bars - Standard Error (SEM).

3.2.2 –Multivariate analysis of measures of anxiety & locomotion in the OFT

In the OFT, a MANOVA revealed that there was a main effect of treatment ($F_{12,294}=4.056$, $p<0.001$) and stress ($F_{3,96}=6.759$, $p<0.001$), as well as a significant interaction between stress and treatment (treatment*stress; $F_{12,294}=4.769$, $p<0.001$) (Fig. 3.2.2). There were also significant between-subject effects of treatment*stress on the percent time spent in the center (Fig. 3.2.2A – $F_{4,98}=3.828$, $p=0.006$), the number of center entries (Fig. 3.2.2B – $F_{4,98}=2.501$, $p=0.047$) and ambulatory movement (Fig. 3.2.2C – $F_{4,98}=6.565$, $p<0.001$).

A clear behavioral trend emerged in unstressed, but not stressed, mice, where an increase in HU-210 dosage systematically reduced the percent time the mice spent in the center region of the OFT arena. Specifically, the analysis of the confidence intervals from the MANOVA showed a significant difference in behavior when mice received a vehicle or treatment of 5 μ g/kg of HU-210, where mice spent significantly more time in the center of the arena when compared to stressed mice receiving the same pre-treatment dose ($p<0.05$), but were not significantly different from each other in both stressed and unstressed subjects; therefore, no anxiolytic effect of lower dose HU-210 administration was observed. HU-210 treatments of 25 and 50 μ g/kg in unstressed mice spent significantly less time in the center region in comparison to unstressed controls ($p<0.05$). This is indicative of an anxiogenic effect at high HU-210 doses in the absence of acute stress exposure.

In stressed mice, almost all treatment levels failed to show any statistical difference in performance in the OFT, except at 25 μ g/kg HU-210, where the mice spent significantly more time in the center in comparison to vehicle controls and

unstressed mice receiving the same ($p < 0.05$). This anomalous anxiolytic effect at 25 $\mu\text{g}/\text{kg}$ was addressed when measures of locomotion were investigated.

Two tests of locomotor activity were administered in the present study: center entries and ambulatory movement. Center entries were unchanged in unstressed animals across treatment levels, but stressed animals exhibited a dose-dependent reduction (treatment*stress – $F_{4,98}=2.501$, $p=0.047$). Mice receiving 25 and 50 $\mu\text{g}/\text{kg}$ doses of HU-210 made significantly fewer entries in the center region of the OFT in comparison to unstressed controls receiving the same dose. The administration of 25 and 50 $\mu\text{g}/\text{kg}$ doses of HU-210 also produced statistically significant decreases in ambulatory movement in stressed animals when compared to unstressed mice pre-treated with the same doses, as well as vehicle-treated stressed controls ($p < 0.05$). Similar to the effects of HU-210 on center entries, ambulatory movement remained unaffected by dose across all treatment levels in unstressed mice, whereas stressed mice showed a significant and steady decreasing effect with increasing dosage. Collectively, these results are indicative of locomotor impairments following the administration of 25 and 50 $\mu\text{g}/\text{kg}$ doses of HU-210, but only when these doses are combined with acute stress exposure. As our behavioral tests are rooted in the rodent's ability to move from one region of the testing apparatuses to the other, conclusions on anxiety behavior in mice receiving 25 or 50 $\mu\text{g}/\text{kg}$ doses of HU-210 in conjunction with acute stress exposure cannot be drawn due to locomotor impairments observed in these animals; therefore, the conclusions that an "anxiolytic" effect was observed at 25 $\mu\text{g}/\text{kg}$ is not valid.

Finally, there were no statistically significant differences in ambulatory movement when comparing stressed and unstressed groups receiving either 5 or 10 $\mu\text{g}/\text{kg}$ of HU-210.

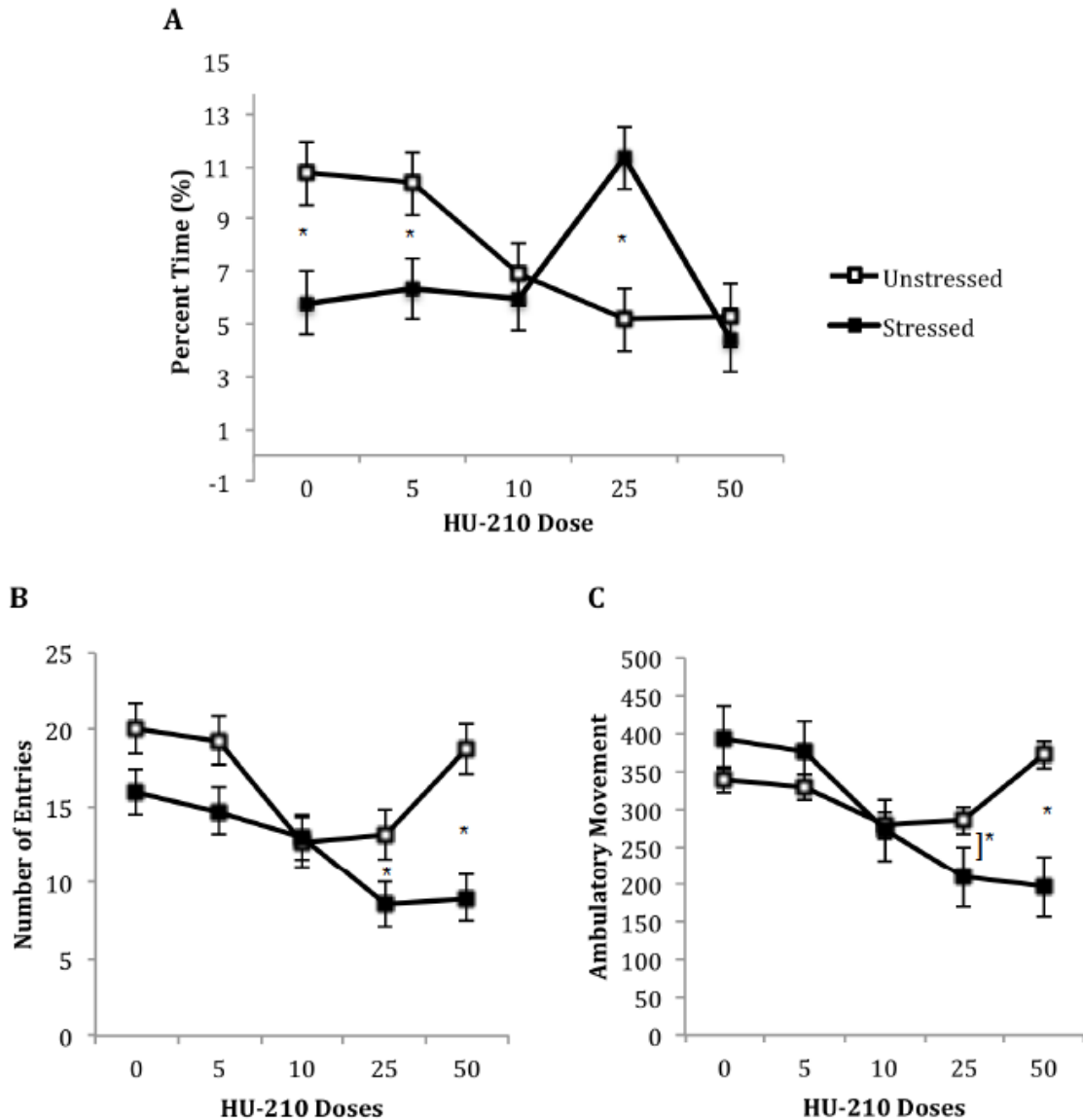


Figure 3.2.2: A MANOVA revealed main effects of stress ($p < 0.001$) and treatment ($p < 0.001$), and a significant interaction between the two ($p < 0.001$), were observed in the percent time spent in the center (A), number of entries (B) and ambulatory movement (C) in the OFT. * - $p < 0.05$ between stressed and unstressed performance at a given dose. Unstressed - $n = 8$; Stressed - $n = 8$. Error bars - Standard Error (SEM).

3.3 – Endogenous Cannabinoid Treatment

Due to our observation that the exogenous cannabinoid HU-210 did not produce clear-cut effects on anxiety-related behavior in stressed and unstressed mice, a second series of experiments sought to measure the impact of enhanced eCB signaling via KML29-mediated inhibition of MAGL activity on anxiety-related behaviors.

3.3.1 – Optimal KML29 dose

To examine the dose effects of KML29, 5 groups (n=8) of mice were tested: vehicle (with and without stress) and 3 doses of KML29 (5, 10 & 20 mg/kg) in acutely stressed mice. Mice were treated 4-hours prior to acute stress exposure in accordance with administration protocols by Chang et al. (2012). According to a one-way ANOVA (Fig. 3.3.1), significant group effects were observed in the OFT (A – $F_{4,43}= 3.519$, $p=0.0143$) and EPM (B – $F_{4,43}=2.637$, $p=0.0468$). Specifically, post-hoc analysis showed that only the highest dose of KML29 tested (20 mg/kg) was able to significantly reduce anxiety-related behavior to unstressed baseline levels in both the OFT ($p=0.0011$) and EPM ($p=0.0207$). The lowest dose (5 mg/kg) of KML29 tested didn't have an effect on anxiety-related behaviors in the OFT ($p=0.1369$) and EPM ($p=0.3014$). The intermediate dose (10 mg/kg) was able to significantly increase the percent time the mice spent in the center of the OFT ($p=0.0084$), but did not significantly alter EPM behavior ($p=0.3414$) from vehicle-treated, stressed controls. Consequently, all subsequent experiments used the 20 mg/kg KML29 dose, as it alleviated anxiety symptoms in both tests behavioral tests.

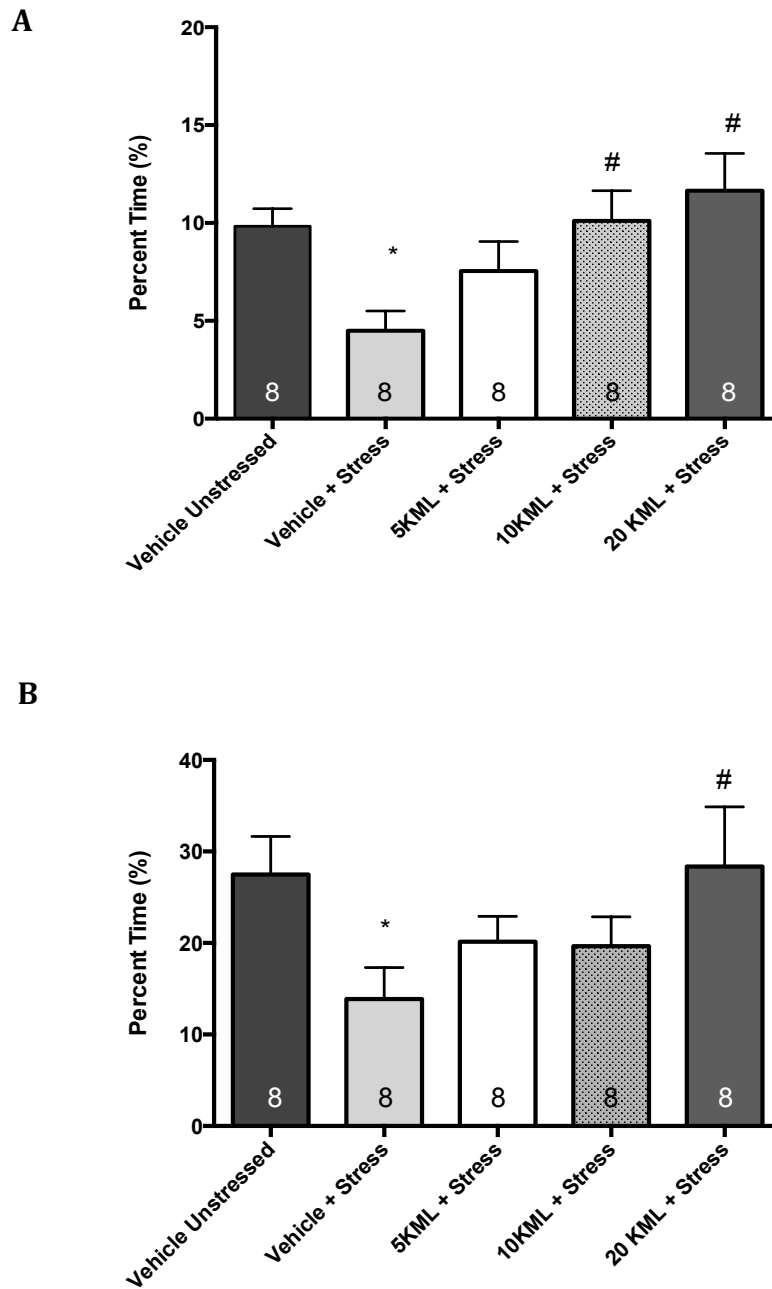
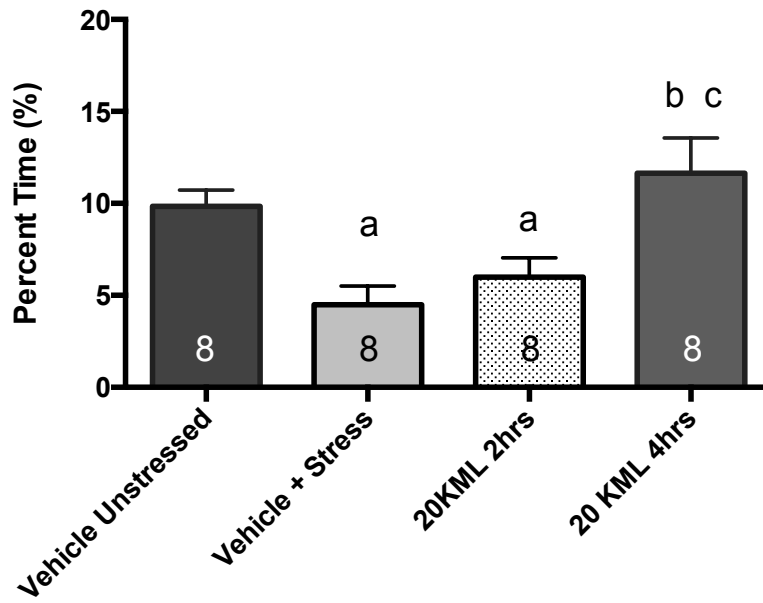


Figure 3.3.1: Effects of KML29 pre-treatment and acute stress exposure on anxiety-related behaviors. Both 10 and 20 mg/kg of KML29 treatments restored the percent time spent in the OFT center to that of vehicle-treated, unstressed controls (A - $p=0.0143$). Only 20mg/kg KML29 had an anxiolytic effect in the EPM (B - $p=0.0468$). * - $p<0.05$ vs. *Vehicle Unstressed*; # - $p<0.05$ vs. *Vehicle + Stress*. Error bars - Standard Error (SEM).

3.3.2 – Optimal KML29 administration time

The previous set of experiments examined KML29 doses when administered 4 hours prior to acute stress exposure, the recommended time course for this drug (Chang et al., 2012). A pilot study in our lab had previously used a 2-hour pre-treatment regime (unpublished data), so an additional set of experiments investigated whether there was any difference in the anxiolytic effects of KML29 when administered 2 or 4 hours prior to acute stress (Fig. 3.3.2). A one-way ANOVA revealed significant group effects in the OFT ($F_{3,29}=6.781$, $p=0.0013$) and EPM ($F_{3,29}=1.960$, $p=0.1421$). Analysis of the confidence intervals revealed that the 2-hour pre-treatment condition was insufficient to induce significant effects on measures of anxiety in comparison to vehicle-treated, stressed controls in both behavioral tests (OFT: $p=0.9080$; EPM: $p=0.5463$). Consequently, all subsequent doses of KML29 will be administered 4 hours prior to acute stress exposure at its optimal dose of 20mg/kg.

A



B

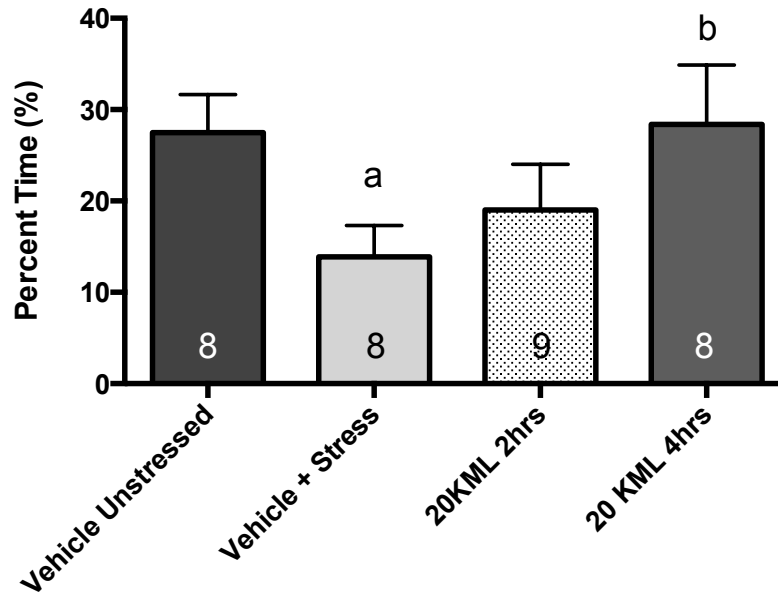


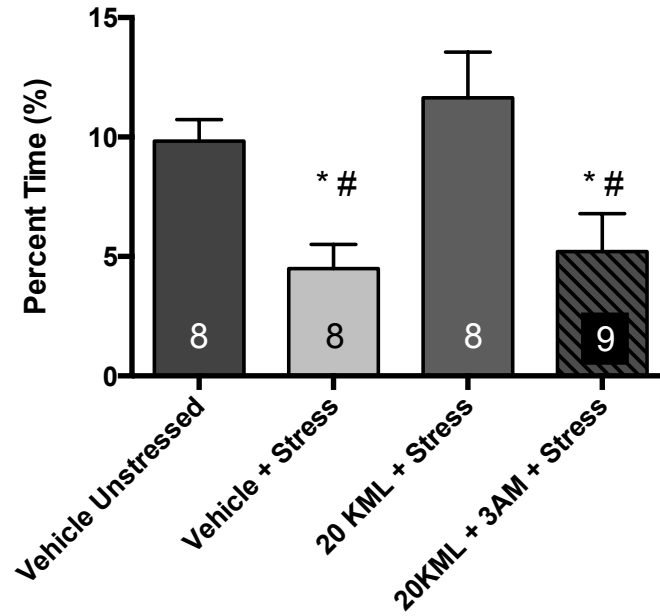
Figure 3.3.2: Effect of time point administration on the anxiolytic effects of KML29. Only 20 mg/kg KML29 4 hours pre-treatment produced an increase the percent time spent in OFT center (A - $p=0.0013$) and in the EPM open arm (B - $p=0.1421$) vs. vehicle-treated, stressed controls. a - $p<0.05$ vs. *Vehicle Unstressed*; b - $p<0.05$ vs. *Vehicle + Stress*; c - $p<0.05$ vs *20KML 2hrs + Stress*. Error bars - Standard Error (SEM).

3.3.3 – Impact of KML29 and AM281 co-administration of measures of anxiety

As previously reported, acute stress exposure significantly reduced time spent in the center of the OFT and in the open arm of the EPM (Fig. 3.1), which was reversed with the administration of 20 mg/kg KML29 4-hours before stress (Fig. 3.3.1). Next, the role of CB1Rs in KML29's effects was investigated by pharmacologically blocking the receptor via AM281 treatment (Fig. 3.3.3).

According to the one-way ANOVA performed, there were group specific effects of combined 20 mg/kg KML29 and 3 mg/kg AM281 treatment on anxiety-related behavior in both the OFT (Fig. 3.3.3A – $F_{3,29}=5.955$, $p=0.0027$) and the EPM (Fig. 3.3.3B – $F_{3,28}=2.621$, $p=0.0704$). The Fisher's LSD post-hoc test revealed that mice receiving the combined treatment did not perform significantly different from stressed controls in the percent time spent in the OFT center (Fig. 3.3.3A; $p=0.7211$) and the EPM open arms (Fig. 3.3.3B; $p=0.7492$). These results demonstrate that the blockade of CB1Rs via AM281 antagonism blocks the previously established anxiolytic effects of KML29 in both behavioral tests. This implies that CB1R activity is essential to KML29's mediation of anxiety behavior.

A



B

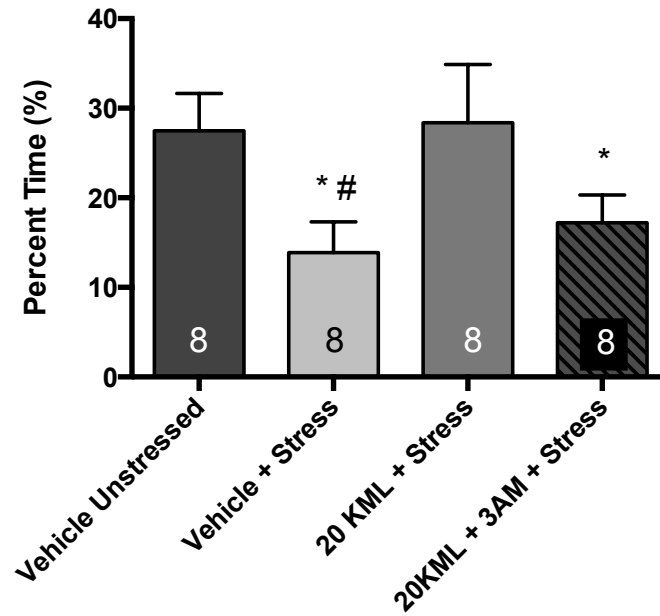


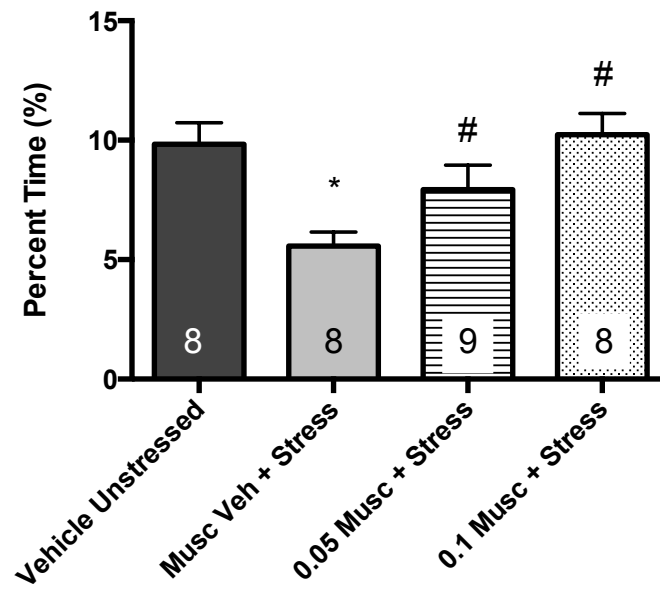
Figure 3.3.3: Impact of AM281 and KML29 co-administration on effects of KML29. Mice receiving both KML29 and AM281 spent significantly less time in the OFT center vs. KML29 treatment alone (A - $p=0.0027$). In the EPM, combination treatment didn't affect behavior vs. unstressed controls or KML29 alone (B - $p=0.0704$). * - $p<0.05$ vs. *Vehicle Unstressed*; # - $p<0.05$ vs. *20KML + Stress*. Error bars - Standard Error (SEM).

3.3.4 – Impact of Muscimol treatment alone on anxiety behaviors

In the last 50 years, benzodiazepines have been the prototypic anxiolytic and are specifically designed to target the GABA_A receptor through positive allosteric modulation, which enhances GABAergic neurotransmission (Griebel & Holmes, 2013). We reasoned that if KML29 produced anxiolytic effects through reduced release of pre-synaptic GABA, then the pre-treatment of stressed mice with a post-synaptic GABA_A receptor agonist would abolish the anxiolytic effects of KML29 by restoring GABAergic neurotransmission. In other words, if KML29 mediates its anxiolytic effect via GABA CB1Rs, then the impact of Muscimol, an orthosteric agonist of GABA_A, should block this phenomenon. First, the impact of Muscimol treatment alone on our measures of anxiety must be investigated (Fig. 3.3.4).

A one-way ANOVA revealed significant group effects in the OFT (Fig. 3.3.4A – $F_{3,28}=5.279$, $p=0.0052$) and EPM (Fig. 3.3.4B – $F_{3,27}=2.423$, $p=0.0876$). According to the Fisher's LSD test, 0.05 mg/kg Muscimol significantly increased the percent time spent in the center of the OFT ($p=0.0157$), but did not affect anxiety behavior in the EPM ($p=0.0814$). At a dose of 0.1mg/kg, Muscimol significantly increased the percent time spent in the center region of the OFT ($p=0.0052$) and in the open arm of the EPM ($p=0.0158$) vs. vehicle-treated, stressed controls. Consequently, a dose of 0.1mg/kg was used in subsequent experiments investigating the impact of Muscimol and KML29 co-administration on anxiety behaviors.

A



B

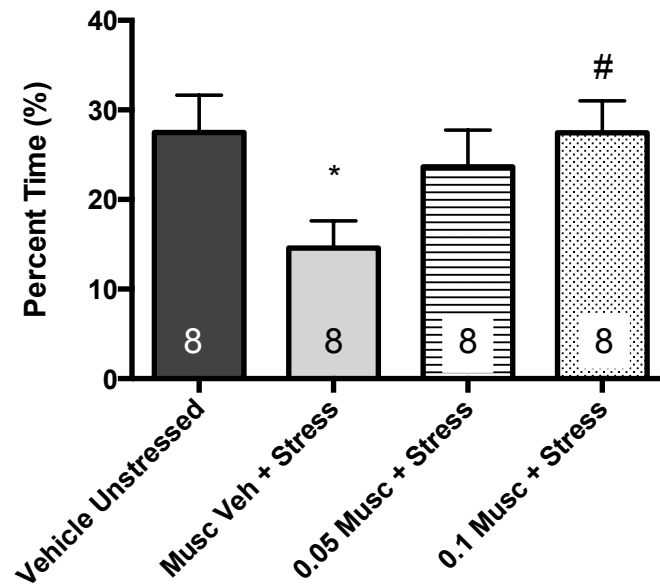


Figure 3.3.4: Impact of Muscimol treatment on anxiety behavior. Muscimol 0.1 mg/kg had an anxiolytic effect on the percent time spent in the center of the OFT (A - $p=0.0052$) and in the open arm of the EPM (B - $p=0.0876$). * - $p<0.05$ vs. *Vehicle Unstressed*; # - $p<0.05$ vs. *Vehicle + Stress*. Error bars - Standard Error (SEM).

3.3.5 – Impact of combined Muscimol and KML29 treatment on anxiety

Next, we sought to characterize the impact of muscimol treatment on the anxiolytic effects of KML29, which was examined by co-administering both drugs prior to acute stress exposure (Fig. 3.3.5). According to the one-way ANOVA, there were no significant group effects of combined treatment on measures of anxiety in the OFT (Fig. 3.3.5A – $F_{5,42}=2.050$, $p=0.0911$) and EPM (Fig. 3.3.5B – $F_{5,41}=1.757$, $p=0.1434$). Post hoc analysis revealed that combined treatment significantly increased the percent time spent in the center of the OFT vs. vehicle-treated stressed mice ($p=0.0183$), but did not significantly alter anxiety-related behavior when compared to mice receiving KML29 with Muscimol's vehicle ($p>0.05$) or Muscimol alone ($p>0.05$). Their performance was also not significantly different from vehicle-treated, unstressed controls ($p>0.05$).

In the EPM, a similar behavioral pattern was observed, where mice receiving combined treatment did not perform significantly different from unstressed vehicle-treated controls ($p=0.1885$), or those treated with KML29 and Muscimol vehicle ($p=0.2703$) or Muscimol alone ($p=0.1884$).

Collectively, these results suggest that KML29 does not mediate its anxiolytic effects by interfering with GABA neurotransmission, as the combined treatment did not significantly alter the mice's performance in comparison to KML29 or Muscimol treatments alone.

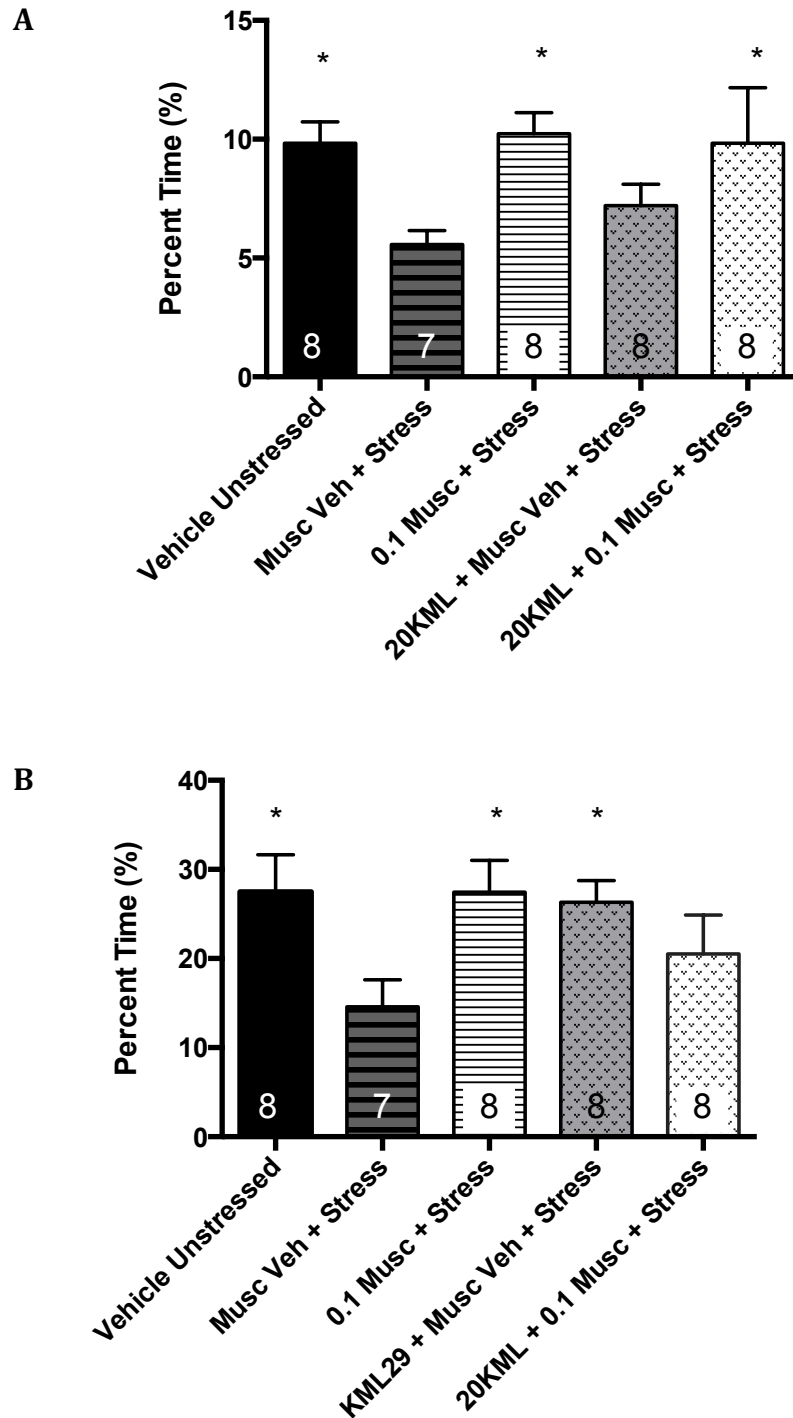


Figure 3.3.5: Impact of KML29 and Muscimol co-administration on the effects of KML29. In the OFT (A - 0.0911), receiving combined treatment spent significantly more time in the center than vehicle stressed mice ($p=0.0304$), but not differently from KML29 with Muscimol vehicle ($p=0.1591$) or Muscimol alone ($p=0.8315$). Similarly in the EPM (B - $p=0.1434$), combined treatment didn't alter performance vs. vehicle unstressed ($p=0.1755$), KML29 with Muscimol ($p=0.2590$), and Muscimol alone ($p=0.1783$). * - $p<0.05$ vs. *Musc Veh + Stress*. Error bars - Standard Error (SEM).

3.3.6 – Effects of endogenous cannabinoid modulation on locomotion

Due to the apparition of locomotor side effects associated with elevated exogenous cannabinoid administration in the first series of experiments, the ambulatory movement of mice treated with any drug targeting the cannabinoid system was screened. A one-way ANOVA revealed that there were no significant effects to report between all conditions receiving a cannabinoid treatment in comparison to vehicle-treated controls (Fig. 8 - $F_{7,57}=0.9797$, $p=0.4550$). This implies that any differences in behavior observed between experimental groups were not related to altered locomotor abilities.

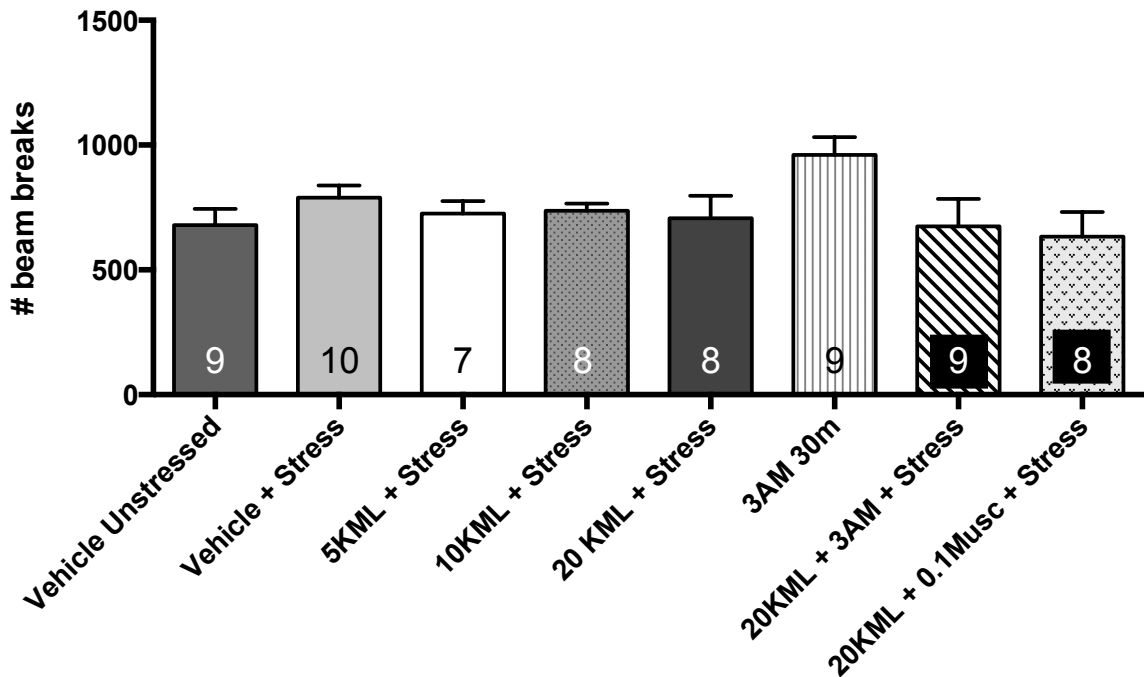


Figure 3.3.6: Examination of the impact of eCB modulation on locomotor activity in a 5-minute OFT session ($F_{7,57}=0.9797$, $p=0.4550$). There was no significant effect on ambulatory movement between all groups receiving any type of cannabinoid treatment administered. Error bars – Standard Error (SEM).

Chapter 4: Discussion

4.1 – Exogenous cannabinoids modulate stress differently under stressed and unstressed conditions

The anxiety-cannabinoid paradigms used to investigate the anxiolytic potential of novel cannabinoid agents lack the inclusion of acute stressors in their experimental design. The present study primarily sought to address this gap in the literature. It was hypothesized that animals receiving cannabinoid pre-treatment prior to acute stress exposure would impact behavior in a biphasic manner, as previously observed in unstressed models. However, a distinct behavioral profile emerged when the exogenous cannabinoid HU-210 was combined with acute stress exposure.

In the absence of acute stress, HU-210 administration produced a clear trend: increased dosage produced enhanced anxiogenic behaviors. The two highest doses of cannabinoids (25 & 50 $\mu\text{g}/\text{kg}$) significantly decreased the time spent in the center region of the OFT in comparison to vehicle treatment controls in unstressed animals. Furthermore, the performance of these unstressed, cannabinoid-treated mice was not significantly different that of vehicle-treated, stressed controls. These findings are consistent with the current cannabinoid-anxiety framework, where high doses of cannabinoids elicit an anxiogenic phenotype, as observed in unstressed models (Hill & Gorzalka, 2004). Although an anxiolytic effect was not yielded in unstressed mice receiving low doses of HU-210, the 5 $\mu\text{g}/\text{kg}$ dose was not significantly different from vehicle controls in unstressed mice, which was not

observed in unstressed subjects. Specifically, animals receiving 5 & 10 $\mu\text{g}/\text{kg}$ doses of HU-210 did not perform significantly different from stressed, vehicle treated-controls. Ultimately, the performance of mice receiving 5 $\mu\text{g}/\text{kg}$ without stress exposure was significantly higher than that observed in unstressed mice receiving the same dose of HU-210. These findings reveal that the same dose elicits two distinct behavioral phenotypes when paired with or without acute stress.

Interestingly, the combination of 25 $\mu\text{g}/\text{kg}$ pre-treatment of HU-210 and acute stress exposure produced a significant increase in the percent time spent in the center of the OFT, in comparison to vehicle-treated, stressed controls and unstressed mice treated with the same dose. However, further analysis revealed that the combination of acute stress and higher doses of CB treatment significantly decreased ambulatory movement and the number of center entries in comparison to unstressed controls. Specifically, 50 and 25 $\mu\text{g}/\text{kg}$ doses of HU-210 produced severe locomotor impairments in stressed mice that were not observed in unstressed controls receiving the same dose. In other words, these locomotor impairments seem to be specific to the combination of high CB doses and acute stress exposure. The behavioral tests used in the present study are rooted in the mice's ability to move from one region of the maze to other (i.e. from the periphery to the center; from open to closed arms); therefore, conclusions of CB modulation of anxiety cannot be made from the data of these groups.

The emergence of an anxiogenic behavioral phenotype observed in the present study is consistent with a study that investigated the impacts of CBs on anxiety in a chronic restraint stress model, which reported a significant increase in

anxiety-related behaviors in the EPM and heightened responsiveness to HU-210 in rats at low doses. As reported by Hill & Gorzalka (2004), doses of HU-210 that were previously anxiolytic (i.e. 10 ug/kg) did not alter anxiety behavior in rats receiving the same dose in chronically stressed rats in comparison to stressed, vehicle-treated controls. The authors suggested that this behavioral switch in their chronic stress model was due to a stress-induced increase in CB1R activity or sensitization of a downstream mediator (Hill & Gorzalka, 2004).

4.2 – Summation hypothesis

The sensitization of CB1R agonist effects following stress exposure has been previously documented in the literature. For instance, a study sought to investigate whether cannabinoids and stress interacted in the modulation of amygdala activation, as measured by Fos expression. Fos is a marker for cell activation, therefore greater expression can be measured in order to investigate the activation levels in given brain areas subsequent to stimulation. The study showed that environmental stress induced a reduction in amygdalar Fos expression. Furthermore, the authors demonstrated that stress and CB agonism synergistically increased Fos expression in the central amygdala, more so than either condition on its own. In other words, the combination of CBs and stress produced an inversion Fos expression patterns in the amygdala as compared to that elicited by stress exposure alone (Patel et al., 2005).

The results of the current study fall in line with the results from Patel and colleagues (2005), as the effects of a given dose of cannabinoid produced distinct

behavioral phenotypes with or without acute stress exposure. Specifically, the 5 $\mu\text{g}/\text{kg}$ dose of HU-210 administered to stressed mice resulted in a significant reduction in the percent time spent in the center of the OFT in comparison to unstressed mice receiving the same dose. Additionally, the cannabinoid-stress combination at 25 and 50 $\mu\text{g}/\text{kg}$ doses of HU-210 synergistically increased the exhibition of the locomotor side effects, more so than either experimental manipulation on its own. In other words, only the combination of stress and higher cannabinoid doses – neither stress (i.e. vehicle-treated stressed controls) or 25 and 50 $\mu\text{g}/\text{kg}$ HU-210 alone (i.e. treatment in unstressed mice) – produced significant alterations in locomotor abilities.

This “switch” phenomenon, where the combination of cannabinoids and stress produces a differential response profile than that obtained from stress exposure or treatment alone as observed in the present study, has also been noted in other recent studies. CB1R agonism prior to stress reportedly induced long-term potentiation (LTP) in the anterior bed nucleus of the stria terminalis (aBNST) of the amygdala, where stress exposure by itself triggered LTD (Glangetas et al., 2013). This shift in synaptic plasticity towards LTP in response to stress and cannabinoid pre-treatment has been shown to occur at CB1Rs in GABAergic synapses, where decreased GABA activity lead to elevated glutamatergic neurotransmission through its release from GABA’s tonic inhibition (Reich et al., 2013). However, there is no evidence that these shifts in synaptic plasticity have any behavioral effects. Although the present study provides evidence of a behavioral switch phenomenon, further

experiments could explore whether these shifts in synaptic plasticity underly the changes in behavioral phenotype.

Traditionally, this switch phenomenon has been attributed to the sensitization of CB1R agonism and downstream mediators. However, an additional theoretical framework can also be proposed in light of a more recent study, which confirmed that eCBs are synthesized and released in response to HPA-axis activity in order to restore baseline activity levels following acute stress exposure, in both male and female experimental subjects (Roberts et al., 2014). Intuitively, it is plausible that the emergence of an anxiogenic behavioral profile following the administration of a typical anxiolytic dose of HU-210 could result from the summation of both the exogenous cannabinoid treatment and the subsequent stress-induced eCB release.

Similarly, the summation of endogenous and exogenous CBs could also explain the apparition of significant locomotor deficits in stressed, but not unstressed mice treated with the same intermediate doses of HU-210. Activation of the CB signalling system in the basal ganglia produces mainly inhibitory effects on movement, where very high doses (i.e. 100 $\mu\text{g}/\text{kg}$ HU-210) result in the development of cataleptic behaviors (Sañudo-Peña et al., 2000). The cataleptic behavioural phenotype observed in the present study was only exhibited in mice that received intermediately high doses of HU-210 (25 & 50 $\mu\text{g}/\text{kg}$) before acute stress exposure. In other words, the dose of the drug was not high enough to induce these locomotor side effects alone.

Future experiments that seek to test this summation hypothesis should focus on inhibiting the biosynthetic pathways of eCBs during exogenous CB treatment in order to investigate the contributing roles of these neurotransmitters in the exhibition of the behavioural phenotypes described in the present study. For instance, RHC-80267 mainly inhibits the synthesizing activity of diacylglycerol lipase (DAGL), the primary synthetic enzyme for 2-AG. The co-administration of RHC-80267 with HU-210, or another CB₁R agonist, could provide powerful insight on the mechanisms that underlie the sudden onset of severe locomotor deficits in stressed, but not in unstressed, mice receiving high doses of CB pre-treatment prior to acute stress exposure. Alternatively, ineffective CB₁R antagonist doses (i.e. that have no effect on anxiety) could be administered in order to investigate its impact on anxiety-related behaviors.

4.3 - Enhanced eCB signaling elicits anxiolyticism without impairing locomotion

The increase in anxiety behaviors resulting from the combination of exogenous cannabinoid pre-treatment and acute stress exposure do not support the potential clinical use of this drug class for anxiety-related disorders. In actuality, the findings of the present study suggest that these two elements work synergistically, at least acutely, in an anxiogenic fashion. However, as previously described, this behavioral switch may be a result of a summation of the exogenous CB treatment and stress-induced eCB release. Intuitively, this study shifted its focus towards characterizing the impact of enhanced eCB signaling on anxiety-related behaviors, to see if its experimental manipulation might have potential clinical relevance as an effective therapeutic intervention.

Indeed, indirectly enhancing 2-AG signaling via the pharmacological blockade of its main degrading enzyme, MAGL, produced significant anxiolytic effects in acutely stressed mice in a dose-dependent manner. In contrast to mice treated with exogenous cannabinoids, mice treated with high and low doses of KML29 did not exhibit altered ambulatory movement. In other words, KML29 treatment produced desirable anti-anxiety effects without inducing locomotor deficits. This finding is especially important in terms of clinical application, as novel therapeutic interventions continuously seek to minimize side-effect profiles.

In light of these results, CB₁R was pharmacologically blocked by AM281 in order to better characterize the mechanisms through which KML29 exerted its anxiolytic effects. Where KML29 treatment alone was sufficient to produce an

anxiolytic effect on behavior similar to that observed in vehicle-treated unstressed mice, those receiving the combined treatment of KML29 and AM281 did not perform significantly different from stressed controls across all behavioral measures of anxiety. These results imply that KML29-induced enhancement of 2-AG requires CB₁R activity to mediate its anxiolytic effect.

As previously mentioned, cannabinoid agonism at the CB₁R suppresses neurotransmission in a pre-synaptic fashion. The majority of anxiolytics on the market stimulate the binding sites of post-synaptic GABA_A receptors; therefore, it is unlikely that pre-synaptic, CB₁R-mediated inhibition of GABAergic neurotransmission is responsible for modulating the anxiolytic effects of KML29. However, in order to empirically rule out GABA CB₁R in the mediation of KML29's anxiolyticism, a complex experiment was designed. If GABA CB₁R mediated the anxiolytic effects, then the stimulation of post-synaptic GABA_A receptors via Muscimol administration should interfere with KML29's anxiolytic effects by blocking its inhibitory mechanism of action on neurotransmission. In theory, if GABA CB₁R mediate the effects of KML29, then the stimulation of post-synaptic GABA_A receptors should cancel out the KML29-induced anxiolyticism. However, the co-administration of Muscimol and KML29 did not alter behavior from that of vehicle-treated controls, or from Muscimol and KML29 treatments, alone. This implies that GABA CB₁R do not likely mediate the anxiolytic effects produced by KML29 pre-treatment. However, it is important to note that Muscimol itself has an anxiolytic effect, and therefore a ceiling effect on anxiety behavior could have been reached; therefore, very limited conclusions can be drawn from these experiments.

Due to methodological limitations, a similar paradigm involving the pharmacological stimulation of Glutamatergic neurotransmission to investigate its effects on KML29 anxiolyticism is intangible. However, future research could investigate the role of Glutamatergic CB₁Rs in modulating anxiety via cell type-specific CB₁R knockout models. Several different KO mouse lines exist, however the use of improved-Cre (iCre) is favorable due to its optimized codon usage for mammal models, which enhances Cre expression levels, and decreases undesired splicing and epigenetic silencing throughout development in comparison to traditional Cre codons (Shimshek et al., 2002). Our lab has previously used 3 strains of mutant mice, which were created by breeding floxed CB₁R mice with transgenic mice specifically expressing an inducible version of iCreERT2 in GABA, Glutamate and Glial cells (Han et al., 2012). This cell type-specific KO model would prove to be a powerful tool to investigate the individual roles of GABA, Glutamatergic and Glial CB₁Rs in cannabinoid modulation of anxiety.

Furthermore, investigating the impact of localized CB injections would also provide powerful insight on the specific brain regions involved in this phenomenon. The basolateral amygdala (BLA), composed of the basal and lateral nuclei, is involved in processing emotional arousal and responses, as well as fear learning. As previously mentioned, the amygdala possesses a high density of CB₁Rs (Herkenham et al., 1990), which are expressed at low levels by glutamatergic projection neurons and at high concentrations in GABAergic cells in this region (Marsicano & Lutz, 1999). Moreover, stress-induced activation of the HPA-axis stimulates 2-AG synthesis and release exclusively in the amygdala, which is required for the

corticosterone-mediated termination of the stress response (Hill et al., 2010a). In addition to the findings of the present study, these results collectively support a neuromodulator role for cannabinoid in the regulation of anxiety-related behavior, specifically via BLA activity modulation. Intra-cannular injections of eCB modulators into the BLA would provide a unique opportunity to investigate whether the systemic injection effects of KML29 treatment prior to acute stress exposure can be replicated by a contained, local injection in a specific nucleus.

Although an acute stress paradigm sheds light on the fundamental mechanisms of cannabinoid modulation of anxiety behaviors, future experiments should adopt a chronic stress model in order to better investigate the molecular and cellular mechanisms underlying anxiety-related disorders. Acute and chronic stress create very different biochemical environments, which could result in the exhibition of differential behavioral phenotypes in response to cannabinoid treatment, as reported in the present study between unstressed and stressed mice. Anxiety disorders are characterized by persistent forms of anxiety and, therefore, should be studied in a chronic stress paradigm, which would more accurately and validly model its symptoms in animal research. For example, a witness defeat or unpredictable mild chronic stress models would provide an optimal experimental framework in order to investigate the impact of chronic stress on cannabinoid modulation of anxiety.

4.4 – Conclusion

The current study has presented some novel effects on the exogenous and endogenous CB system's impact on behavioral measures of anxiety that have not yet been reported in the literature. Firstly, the combination of exogenous cannabinoid administration and acute stress exposure seem to produce an anxiogenic additive effect, thought to result from the summation of stress-induced eCB release with the exogenous treatment administered. Further investigations are required to confirm the summation hypothesis, specifically examining the impact of pharmacological blockade of eCB synthesis and exogenous CB administration on locomotor impairments in an acutely stressed model. Secondly, the present study provides evidence that enhanced 2-AG signaling through the pharmacological blockade of degrading enzyme MAGL via KML29 treatment has anxiolytic effects, without altering locomotor activity. Furthermore, this effect was shown to be CB₁R-dependent, and is not likely mediated by GABAergic CB₁Rs.

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