Exploring the Role of Cannabidiol-Monoterpene Formulations in Modulating Anxiety Symptoms

Richard Leu, The University of Western Ontario

Supervisor: Laviolette, Steven, The University of Western Ontario
A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Neuroscience
© Richard Leu 2020

Follow this and additional works at: https://ir.lib.uwo.ca/etd

Part of the Alternative and Complementary Medicine Commons

Recommended Citation
Abstract

While cannabidiol (CBD) has been documented to elicit anxiolytic effects, only recently have studies demonstrated the anxiolytic efficacy of the aromatic monoterpenic linalool. Past research involving phytocannabinoid-phytocannabinoid formulations has shown that CBD may yield superior anxiolytic efficacy when administered alongside other cannabis compounds, with this phenomenon termed the “Entourage Effect” (EE); however, this EE has not been investigated in phytocannabinoid-terpene formulations. Furthermore, investigations involving intra-cranial CBD administration have shown the nucleus accumbens shell, known for affective processing, to be a therapeutic target of interest. Thus, using treatment interventions in behavioral and molecular assays, this thesis investigated the possibility of EE-potentiated anxiolysis within linalool-CBD formulations through olfactory and intra-NAcSh routes of concurrent acute administration, focusing on GABAAR and ERK signaling mechanisms previously shown to be mediated by linalool and CBD. Altogether, findings ultimately suggest that linalool-CBD formulations elicit EE-potentiated anxiolysis through GABAAR- and ERK-dependent mechanisms capable of reversing chronic stress-induced anxiety.

Keywords:
 Cannabidiol, CBD, THC, phytocannabinoid, nucleus accumbens shell, mesocorticolimbic pathway, entourage effect, monoterpenic, terpene, linalool, anxiety, anxiolysis, GABAAR, ERK, behavioral, molecular
Summary for Lay Audience

Commonly referred to as cannabis, the plant species *Cannabis sativa* contains two major substances, cannabidiol and tetrahydrocannabinol (THC), both of which modulate emotional processing. Although these substances are collectively termed “phytocannabinoids”, they yield contrasting psycho-modulatory effects. While THC has been shown to yield psychoactive symptoms, CBD has been demonstrated to reverse these symptoms when co- or post-administered, and also elicit anti-anxiety effects. Interestingly, developments in cannabis research have documented that when administered in conjunction, THC and CBD may yield greater anti-anxiety effects than those achieved alone with CBD plant extracts. This phenomenon of increased anti-anxiety effects following the combination of cannabis compounds has been termed the “Entourage Effect” (EE). Furthermore, studies demonstrated that administrations of these compounds into specific brain structures within a circuit known as the mesolimbocortical (MCL) pathway are especially effective in generating mood-altering effects. Within this MCL pathway, the nucleus accumbens brain structure—in particular its “shell” sub-region (NAcSh)—has shown to be a therapeutic target of interest, given that past studies employing intra-NAcSh administrations of these cannabis compounds reported significant mood-altering results. Importantly however, combinations involving terpenes (aromatic substances) found in cannabis have yet to be explored despite their anxiolytic potential, highlighting an area of interest in the investigation of the Entourage Effect.

Thus, this thesis sought to determine whether an EE existed in combined linalool (anxiolytic terpene) and CBD treatments, using olfactory and intra-NAcSh routes to respectively administer these compounds in rat models. Utilizing behavioral tests followed by molecular
analyses of biomarkers of interest, the findings of this thesis demonstrated that there exists EE-potentiated anti-anxiety effects following concurrent administration of odorous linalool and intra-NAcSh CBD. In addition, through incorporation of protein modulators and models of chronic stress, the observed EE was found to successfully reverse chronic stress-induced anxiety and elicit anti-anxiety effects through specific protein modulations. Ultimately, these findings highlight the therapeutic potential of cannabis as a natural alternative to conventional medications used to treat anxiety symptoms and disorders. With safe use profiles, CBD and linalool may serve as an adjunct—or even replacement—for these conventional medications riddled with unwanted and potentially dangerous, side-effects.
Acknowledgements

First and foremost, I would like to thank Dr. Steven Laviolette for his insightful guidance and supervision during my two years as a master’s student, as he has ultimately allowed me to develop the necessary skills required to be proficient in my research pursuits. Furthermore, the tireless support he has given me during the composition of this thesis has been absolutely invaluable, both in regard to thesis-specific developments and flexibility with accommodations during this COVID-19 crisis.

I would also like to thank current and past members of my advisory committee, Dr. Walter Rushlow, Dr. Whitehead and Dr. Brian Corneil for their valuable supervision and professional wisdom provided over the past two years. In addition, thank you as well to my examiners, Tim Bussey, Susanne Schmid and Wataru Inoue for their flexibility in accommodating defense re-scheduling efforts and overall for their time they sacrificed to allow me the opportunity to defend my thesis—you have my sincere gratitude. Furthermore, I would like to extend a special thank you to my fellow laboratory members of the addiction research group, without whom I would not have been able to progress nearly as quickly and comfortably from a fledging researcher to the scientist I have become today. In particular, I would like to thank Dr. Maria Del Mar Rodriguez Ruiz, Dr. Hanna Szkudlarek, Dr. Marta de Felice and fellow research students Nathashi and Roger for their mentorship, support and overall camaraderie—you will never be forgotten. Importantly, I would like to thank MITAC, Canopy Health Innovations and NSERC for supporting my research efforts during the pursuance of my master’s degree. Lastly, thank you to my family and friends endlessly supported my endeavors; this journey has been quite a challenge, and I ultimately express my sincerest gratitude to everyone who played a part in it.
# Table of Contents

Abstract ............................................................................................................................................ i
Summary for Lay Audience .............................................................................................................. ii
Acknowledgements .............................................................................................................................. iv
Table of Contents ................................................................................................................................. v
List of Figures ........................................................................................................................................ viii
Abbreviations .......................................................................................................................................... x

1. Introduction ........................................................................................................................................ 1
   1.1 Overview of Anxiety and Anxiety Disorders .................................................................................. 3
      1.1.1 Defining Anxiety and Anxiety Disorders .................................................................................... 3
      1.1.2 Prevalence of Anxiety Disorders and Implications ...................................................................... 4
      1.1.3 Conventional Treatments for Anxiety Disorders ........................................................................... 5
   1.2 The Mesocorticolimbic Pathway ...................................................................................................... 7
      1.2.1 Nucleus Accumbens .................................................................................................................... 7
      1.2.2 Prefrontal Cortex ........................................................................................................................ 9
      1.2.3 Functional Connections between the PFC, NAc and Associated Brain Regions ....................... 10
      1.2.4 The Role of the Mesocortiolimbic Pathway and Receptor Targets in Anxiety Disorders ......... 12
      1.2.5 Overview of Notable Molecular Biomarkers in Anxiety Disorders .......................................... 16
   1.3 Cannabis: An Alternative Treatment for Anxiety Disorders ......................................................... 18
      1.3.1 Effects of Cannabis on Mental Health ......................................................................................... 18
      1.3.2 Properties and Mechanisms of Action of Cannabidiol ............................................................... 19
      1.3.3 Properties and Mechanisms of Action of the Mono-Terpene, Linalool .................................... 21
      1.3.4 The Entourage Effect ................................................................................................................. 22
   1.4 Research Aims and Hypothesis ....................................................................................................... 24

2. Methods .............................................................................................................................................. 25
   2.1 Animals and Housing ..................................................................................................................... 25
   2.2 Surgical Procedures ....................................................................................................................... 25
   2.3 Drug Preparation and Administration ............................................................................................ 27
   2.4 Chronic Unpredictable Stress Protocol ......................................................................................... 29
   2.5 Experimental Cohorts and Treatment Group Designations ......................................................... 30
   2.5 Behavioral Testing ......................................................................................................................... 31
2.5.1 Open Field Test ................................................................. 31
2.5.2 Light Dark Box Test .......................................................... 31
2.5.3 Social Motivation and Recognition ................................... 32
2.5.4 Elevated Plus Maze .......................................................... 32
2.5.5 Context-Dependent Fear Conditioning .............................. 33
2.5.6. Spontaneous Alternation .................................................. 34
2.5.7 Novel Object Recognition .................................................. 34
2.5.8 Testing Timelines .............................................................. 35
2.6 Molecular Analyses ............................................................. 36
2.7 Histology ............................................................................ 37
2.8 Statistical Analyses .............................................................. 37
3. Results: Behavioral Assays .................................................... 39
  3.1 Acute Treatment Behavioral Results .................................... 39
    3.1.1 Open Field Test ............................................................. 39
    3.1.2 Light Dark Box Test ....................................................... 41
    3.1.4 Social Motivation and Recognition Test .......................... 44
    3.1.3 Elevated Plus Maze Test ............................................... 46
    3.1.5 Context-Dependent Fear Conditioning ......................... 48
  3.2 Flumazenil Challenge Behavioral Results ............................. 50
    3.2.1 Open Field Test ............................................................. 50
    3.2.2 Light Dark Box Test ....................................................... 52
    3.2.3 Context-Dependent Fear Conditioning (Recall) .............. 54
  3.3 EPA Challenge Behavioral Results ...................................... 55
    3.3.1 Open Field Test ............................................................. 55
    3.3.2 Light Dark Box Test ....................................................... 57
    3.3.3 Context-Dependent Fear Conditioning (Acquisition) ....... 59
  3.4 Chronic Stress Behavioral Results ...................................... 61
    3.4.1 Open Field Test ............................................................. 61
    3.4.2 Spontaneous Alternation ............................................... 63
    3.4.3 Novel Object Recognition .............................................. 65
    3.4.4 Light Dark Box Test ....................................................... 67
    3.4.5 Social Motivation and Recognition Test .......................... 69
    3.4.6 Elevated Plus Maze ....................................................... 71
4. Results: Molecular Assays (Acute Treatment) ................................................................. 73
  4.1 GSK-3β Protein Analysis ....................................................................................... 74
  4.2 Akt Protein Analysis ............................................................................................. 78
  4.3 ERK Protein Analysis .......................................................................................... 82
  4.4 JNK Protein Analysis .......................................................................................... 86
Discussion .................................................................................................................. 90
Conclusions and Future Directions ............................................................................ 121
References .................................................................................................................. 124
Curriculum Vitae ......................................................................................................... 147
List of Figures

Figure 1 Schematic of the Mesocorticolimbic Circuit and Associated Anatomical Connections .......................................................... 15
Figure 2 Visualization of intra-NAcSh surgical guide cannulations .............................................................. 26
Figure 3 Visualization of odor exposure apparatus ......................................................................................... 28
Figure 4 Experimental Cohorts and Associated Treatment Group Designations .................................. 30
Figure 5 Timeline of Various Test Cohorts ................................................................................................. 35
Figure 6 Open Field Test, total locomotion: baseline treatment cohort ..................................................... 40
Figure 7 Light Dark Box Test, time spent in the center compartment and total compartmental transitions: baseline treatment cohort ........................................................................... 43
Figure 8 Social Motivation and Recognition Test, SMI and SRI: baseline treatment cohort .... 45
Figure 9 Elevated Plus Maze Test, open arm exploration time and total transitions between open and closed arms: baseline treatment cohort ........................................................................... 47
Figure 10 Context-Dependent Fear Conditioning—Acquisition and Recall: baseline treatment cohort ...................................................................................................................... 49
Figure 11 Open Field Test, total locomotion and center/total locomotion (%): flumazenil challenge cohort .......................................................................................................................... 51
Figure 12 Light Dark Box Test, total time in light compartment and total compartmental transitions: flumazenil challenge cohort .......................................................................................... 53
Figure 13 Context-Dependent Fear Conditioning—Recall: flumazenil challenge cohort ..................... 54
Figure 14 Open Field Test, total locomotion and center/total locomotion (%): EPA challenge cohort ........................................................................................................................................ 56
Figure 15 Light Dark Box Test, total time in light compartment and total compartmental transitions: EPA challenge cohort ...................................................................................................................... 58
Figure 16 Context-Dependent Fear Conditioning—Acquisition: EPA challenge cohort........60

Figure 17 Open Field Test, total locomotion and center/total locomotion (%): Chronic stress cohort.................................................................62

Figure 18 Spontaneous Alternation, normalized alternation counts (%)........................64

Figure 19 Novel Object Recognition, novelty preference score (%)..................................66

Figure 20 Light Dark Box Test, total time in light compartment and total compartmental transitions: chronic stress cohort.................................................................68

Figure 21 Social Motivation and Recognition Test, SMI and SRI: chronic stress cohort.........70

Figure 22 Elevated Plus Maze Test, open arm exploration time and total transitions: chronic stress cohort.................................................................72

Figure 23 Effects of Treatment Conditions on p-GSK3β, T-GSK3β, p/T-GSK3β levels......76-77

Figure 24 Effects of Treatment Conditions on p-Akt, T-Akt, p/T-Akt levels.....................80-81

Figure 25 Effects of Treatment Conditions on p-ERK, T-ERK, p/T-ERK levels...............84-85

Figure 26 Effects of Treatment Conditions on p-JNK, T-JNK, p/T-JNK levels...............88-89

Figure 27 Summary of Baseline Treatment Cohort Results from Behavioral Assays............90

Figure 28 Summary of Flumazenil Challenge Cohort Results from Behavioral Assays.........95

Figure 29 Summary of EPA Challenge Cohort Results from Behavioral Assays...............98

Figure 30 Summary of Chronic Stress Cohort Results from Behavioral Assays...............101

Figure 31 Summary of Phosphorylated/Total Protein Ratio Results from Western Blot Assays
...............................................................................................................................106

Figure 32 Summary of Linalool, CBD Cellular Targets and Molecular Pathways Associated with GABAA R-mediated anxiolysis.................................................................118
**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-AG</td>
<td>2-Arachidonoglycerol</td>
</tr>
<tr>
<td>5-HT</td>
<td>5-hydroxytryptamine, serotonin</td>
</tr>
<tr>
<td>5-HT&lt;sub&gt;1A/2A/3A&lt;/sub&gt;R</td>
<td>5-hydroxytryptamine or serotonin 1A/2A/3A receptor</td>
</tr>
<tr>
<td>ADCY1</td>
<td>Adenylate Cyclase 1</td>
</tr>
<tr>
<td>AEA</td>
<td>Anandamide</td>
</tr>
<tr>
<td>Akt</td>
<td>Protein Kinase B</td>
</tr>
<tr>
<td>AktSer473</td>
<td>Protein Kinase B Serine-473 residue</td>
</tr>
<tr>
<td>AktThr308</td>
<td>Protein Kinase B Threonine-308 residue</td>
</tr>
<tr>
<td>AMPH</td>
<td>Amphetamine</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>CB</td>
<td>Cannabinoid</td>
</tr>
<tr>
<td>CBD</td>
<td>Cannabidiol</td>
</tr>
<tr>
<td>CB&lt;sub&gt;1/2&lt;/sub&gt;R</td>
<td>Cannabinoid receptor type 1 and 2</td>
</tr>
<tr>
<td>CBT</td>
<td>Cognitive Behavioral Therapy</td>
</tr>
<tr>
<td>CFC</td>
<td>Context-dependent Fear Conditioning</td>
</tr>
<tr>
<td>CUS</td>
<td>Chronic Unpredictable Stress</td>
</tr>
<tr>
<td>CUS-AirV&lt;sub&gt;PBS&lt;/sub&gt;</td>
<td>Odorous vehicle (air) &amp; intracranial PBS, CUS-exposed</td>
</tr>
<tr>
<td>CUS-L2000-CBD</td>
<td>Odorous 2000 μL linalool &amp; intracranial 5 ng/0.5μL cannabidiol, CUS-exposed</td>
</tr>
<tr>
<td>DA</td>
<td>3,4-dihydroxyphenethylamine or dopamine</td>
</tr>
<tr>
<td>D1/D2/D3</td>
<td>Dopamine receptor 1/2/3</td>
</tr>
<tr>
<td>EC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Electrical Current (half maximal effective drug concentration)</td>
</tr>
<tr>
<td>EE</td>
<td>Entourage Effect</td>
</tr>
<tr>
<td>EPA</td>
<td>Eicosapentanoic Acid</td>
</tr>
<tr>
<td>EPM</td>
<td>Elevated Plus Maze</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular Signal-Regulated Kinase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>ERK1/2</td>
<td>Extracellular Signal-Regulated Kinases 1 and 2</td>
</tr>
<tr>
<td>ES</td>
<td>Effect Size</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma-Aminobutyric acid, γ-aminobutyric acid</td>
</tr>
<tr>
<td>GABA&lt;sub&gt;A&lt;/sub&gt;R</td>
<td>Gamma-Aminobutyric acid receptor A</td>
</tr>
<tr>
<td>GSK3</td>
<td>Glycogen Synthase Kinase 3</td>
</tr>
<tr>
<td>GSK3β</td>
<td>Glycogen Synthase Kinase 3 Beta</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinases</td>
</tr>
<tr>
<td>JNK1/2</td>
<td>c-Jun N-terminal kinase isoforms 1 and 2</td>
</tr>
<tr>
<td>L200-CBD</td>
<td>Odorous 200 μL linalool &amp; intracranial 5 ng/0.5μL cannabidiol</td>
</tr>
<tr>
<td>L2000-CBD</td>
<td>Odorous 2000 μL linalool &amp; intracranial 5 ng/0.5μL cannabidiol</td>
</tr>
<tr>
<td>L200-V&lt;sub&gt;PBS&lt;/sub&gt;</td>
<td>Odorous 200 μL linalool &amp; intracranial phosphate-buffered saline</td>
</tr>
<tr>
<td>L2000-V&lt;sub&gt;PBS&lt;/sub&gt;</td>
<td>Odorous 2000 μL linalool &amp; intracranial phosphate-buffered saline</td>
</tr>
<tr>
<td>LA</td>
<td>Linalool Acetate</td>
</tr>
<tr>
<td>LDB</td>
<td>Light Dark Box</td>
</tr>
<tr>
<td>LO</td>
<td>Linalool</td>
</tr>
<tr>
<td>LTD</td>
<td>Long-term depression (in the context of neuronal synapses)</td>
</tr>
<tr>
<td>LTP</td>
<td>Long-term potentiation (in the context of neuronal synapses)</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>NAc</td>
<td>Nucleus Accumbens</td>
</tr>
<tr>
<td>NAcc</td>
<td>Nucleus Accumbens core region</td>
</tr>
<tr>
<td>NAcSh</td>
<td>Nucleus Accumbens shell region</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
</tr>
<tr>
<td>NMDAR</td>
<td>N-methyl-D-aspartate receptor</td>
</tr>
<tr>
<td>NOR</td>
<td>Novel Object Recognition</td>
</tr>
<tr>
<td>OF</td>
<td>Open Field</td>
</tr>
<tr>
<td>OR</td>
<td>Opioid Receptor</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein Kinase A</td>
</tr>
<tr>
<td>S1/S2/S3</td>
<td>Stage 1, Stage 2, Stage 3 (in the context of a behavioral assay)</td>
</tr>
<tr>
<td>----------</td>
<td>---------------------------------------------------------------</td>
</tr>
<tr>
<td>SA</td>
<td>Spontaneous Alternation</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SMI</td>
<td>Social Motivation Index</td>
</tr>
<tr>
<td>SRI</td>
<td>Social Recognition Index</td>
</tr>
<tr>
<td>SSRI</td>
<td>Selective serotonin reuptake inhibitor</td>
</tr>
<tr>
<td>THC</td>
<td>$\Delta^9$-tetrahydrocannabinol</td>
</tr>
<tr>
<td>$V_{\text{AirCBD}}$</td>
<td>Odorous vehicle (air) and intracranial 5 ng/0.5μL cannabidiol treatment</td>
</tr>
<tr>
<td>$V_{\text{Air}V_{\text{PBS}}}$</td>
<td>Odorous vehicle (air) and intracranial phosphate-buffered saline treatment</td>
</tr>
<tr>
<td>VTA</td>
<td>Ventral Tegmental Area</td>
</tr>
</tbody>
</table>
1. Introduction

*Cannabis sativa*, commonly referred to as marijuana or simply cannabis, is one of the most cultivated and consumed psychoactive substances in the world. According to the World Health Organization (WHO), there exists an estimated 147 million annual users of cannabis, which equates to roughly 2.5% of the world population, overshadowing the annual prevalence usage of other drugs such as cocaine (0.2%) and opiates (0.2%). Yielding a rich history consisting of both positive and negative affective experiences following its ingestion or inhalation, cannabis has begun to garner serious interest in the scientific field due to its medicinal properties and potential for pharmacotherapeutic applications (Callaway, 2004; Izzo et al, 2009; Pertwee, 2008).

Currently, over 500 chemical substances and 100 phytocannabinoids have been successfully isolated and identified from *Cannabis sativa*, including cannabidiol (CBD) and Δ9-tetrahydrocannabinol (THC). THC is the primary psychoactive compound in cannabis and is believed to be responsible for the negative side-effects of cannabis use, including episodes of anxiety, psychosis and cognitive deficits, especially following chronic adolescent exposure. In contrast, research into the psychotropic effects of CBD has shown quite the opposite (D’Souza et al, 2005). Indeed, as the predominant non-psychotropic phytocannabinoid found within *Cannabis sativa*, clinical and pre-clinical evidence has shown that CBD may possess many therapeutic effects potentially applicable in the clinical setting (Crippa et al., 2018; Millar et al., 2019). For example, considerable evidence has suggested that it may not only be effective in attenuating the psychoactive effects elicited by THC, but also effective alone in reducing both depression and anxiety symptoms in animal models (Boggs et al, 2017; Fogaça et al, 2014; Murkar et al, 2019). While the exact pharmacological mechanisms through which CBD produces
its purported therapeutic effects remain unclear, the potential for pharmacotherapeutic effects of CBD for a range of neuropsychiatric symptoms, including anxiety, depression and schizophrenia, is receiving considerable pre-clinical and clinical research focus (Campos et al., 2012; Pertwee, 2008; Petrocellis and Marzo, 2009).

Psychoactive effects, however, have also been discovered in a lesser studied group of compounds within cannabis known as the ‘terpenes’. Often referred to as essential oil components, terpenes are the substances found within cannabis which provide the plant with its distinctive aromatic profile, with at least 20,000 characterized and 200 reported to be contained in the plant (Russo, 2011). Commonly found terpenoids in both cannabis and other plants include limonene, α-pinene, β-myrcene, linalool, β-caryophyllene, nerolidol and phytol amongst others. Aside from their distinct aroma, these terpenes have also been documented in animal models to elicit a variety of physiological effects upon administration (via inhalation or ingestion), including anti-anxiety, analgesic and anti-convulsant effects (Elisabetsky et al., 1995; Peana et al., 2006; Russo, 2001). While terpene yields are often less than 1% in cannabis assays, individual terpenoid concentrations as small as 0.05% have been deemed to be of pharmacological significance in altering brain activity levels (Adams and Taylor, 2010).

Given that the scientific field regarding cannabis is still relatively young and many mechanisms of action remain unclear, there exists a need to pursue further research in order to both examine the properties of CBD and terpenes as well as elucidate their underlying mechanisms. More importantly, recent research has highlighted the potential for phytocannabinoid-terpenoid therapeutic interactions, dubbed the “Entourage Effect”, especially in regard to anti-anxiety effects (Russo, 2011). The entourage effect refers to the concept that specific compounds within cannabis, may functionally interact with each other (thus forming an
“entourage” or combined group effect) to synergize or potentiate the pharmacotherapeutic potential of cannabis-derived formulations—particularly in a manner whereby the novel combinatorial formulation achieves a superior psychotropic effect compared to either individual treatment administration alone. For example, do specific terpenes interact with either THC or CBD to modulate or potentiate there pharmacological and/or therapeutic properties? With this important concept in mind, the subsequent literature overview seeks to illustrate the importance of pursuing such alternative anti-anxiety medications and the mechanisms of action which underlie phytocannabinoid and monoterpenes therapeutic effects, with a focus on exploring the potential for the presence of the Entourage Effect in co-administration of CBD and the specific monoterpe, linalool.

1.1 Overview of Anxiety and Anxiety Disorders

1.1.1 Defining Anxiety and Anxiety Disorders

Anxiety is an emotion experienced commonly throughout an individual’s life, often characterized by worried thoughts, feelings of tension and physiological changes such as increased blood pressure (Kazdin, 2000). The presentation of anxiety, according to the theory of cognitive appraisal proposed by Lazarus and Folkman (1984), often stems from threat appraisals generated by individuals in their perception of a stressful situation, or stressor. In responding to these threats, strategies such as emotion-, problem- and meaning-based coping are often utilized; consequently, by addressing these external stressors, internal states of anxiety which developed in response to stressors can be relieved. However, while stress is often acute in nature and subsides following removal of the originating stressor, anxiety can continue to persist even after the external stressor is eliminated. Furthermore, while feeling anxious or experiencing anxiety may be commonplace, increased severity of these emotions, such as frequency and deleterious
impacts to daily functioning, may be indicative of a chronic mental health disorder.

According to the Diagnostic and Statistical Manual of Mental Disorders, 5th Edition (DSM-V) established by the American Psychiatric Association, a key element in the diagnosis of an anxiety disorder in comparison to normal states of anxiety, involves clinical presentations wherein the individual’s mental condition is severely impairing their functioning in one or more activities of daily living; these include but are not limited to, social and occupational functioning across multiple settings. This key requirement is shared amongst the diagnoses of the eight principal anxiety disorders currently listed in the DSM-V: separation anxiety disorder, selective mutism, specific phobia, social anxiety disorder (social phobia), panic disorder, agoraphobia, generalized anxiety disorder and substance/medication-induced anxiety disorder. Unlike individuals experiencing anxiety whose mental condition may improve following therapeutic interventions and the development of effective coping strategies, many individuals diagnosed with anxiety disorders require specialized treatment to adequately manage their conditions and/or improve their long-term mental health outcomes.

1.1.2 Prevalence of Anxiety Disorders and Implications

According to a recent systematic review conducted by the World Health Organization, the global age-standardized prevalence of any anxiety disorder (including post-traumatic stress disorder) is approximately 21.7%. Furthermore, findings suggest that in conflict settings, the prevalence of anxiety increases with age, with prevalence rates over 30% in individuals over 40 years of age (Charlson et al., 2019). When assessing the lifetime prevalence of anxiety disorders based off of large population-based surveys, the prevalence rate was found to be as high as 33.7% (Bandelow et al., 2015). These rates are alarming in light of the fact that the Anxiety and Depression Association of America reported that those with anxiety disorders are three-to-five
times more likely to require a clinical treatment and six times more likely to be hospitalized due
to development of a co-morbid psychiatric disorder than those without anxiety disorders. Given
these findings and coupled with the fact that the United Nations projects the global population to
rise to 8.5 billion by 2030, there exists an urgent need to address the scaling and efficacy of
treatments for this increasing number of individuals with mental health illnesses.

1.1.3 Conventional Treatments for Anxiety Disorders

The primary options for treating anxiety disorders include psychotherapy-based
interventions (such as cognitive-behavioural therapy) and conventional anti-anxiety medications
(including benzodiazepines, which potently modulate GABAergic transmission, and SSRIs
which modulate serotonergic transmission). Previous research has indicated optimal health
outcomes after undergoing individual cognitive behavioral therapy (large effect size of 1.30,
n=93), which can range from 5 to 20 weekly sessions (30 to 60 minutes) to achieve desired
health outcomes (Bandelow et al., 2015; National Health Service). Although Bandelow and
authors found that medications such as benzodiazepines (ES: 2.15; n = 42) and selective
serotonin reuptake inhibitors (ES: 2.09; n = 62) were more effective than cognitive behavioral
therapy (CBT), the issues of potential side effects and unwanted drug interactions potentially
outweighed the effectiveness of the treatments when considering patient safety and longevity of
treatment effects. However, a 2-year follow-up meta-analyses demonstrated that through 93
studies with 185 study arms, the enduring efficacy of both drug medication and CBT treatment
persisted with relatively equal efficiency (Bandelow et al., 2018). When combined with the fact
that relapse rates between drug treatments (8-56%) and CBT (48%) for anxiety disorders was
relatively comparable, the distinction between treatment advantage becomes minimal. Other
factors, such as ease of access, drug costs, as well as patient adherence, become influential
factors in determining treatment type pursuance and long-term efficacy (Bandelow et al., 2008; Donovan et al., 2010; Durham et al., 2005).

Frontline anxiety medications such as SSRIs and benzodiazepines, despite their convenience advantages when compared to continual weekly sessions of psychotherapy, suffer from multiple drawbacks, including potential ineffectiveness and chronic side-effects, such as drug dependence and withdrawal. In a recent study by Kagan et al. (2020) which conducted a 3-to-12 year follow-up after initial treatment in the child/adolescent multimodal extended long-term study (CAMELS Trial), the 40.6% of [total] 318 patients who chose to discontinue anxiety medication use following the trial reported primary concerns of perceived ineffectiveness (31.8%) and side effects (25.5%). To compound issues further, a study conducted by Read and Williams (2018) found that 61% of patients (n=1431) reported experiencing at least 10 adverse effects, including blunting of affect (71%), sexual dysfunction (66%), suicidality (50%), withdrawal symptoms (59%) and addiction concerns (40%). The latter two adverse effects of withdrawal and addiction are of vital concern when addressing the tranquilizer-class anxiety medications, the benzodiazepine family. Despite their acute efficacy, studies suggest that usage of over four months, higher dosages, immediate drug cessation and utilization of short-acting benzodiazepines such as alprazolam, can result in the development of serious withdrawal syndromes (Owen and Tyrer, 1983). Reported withdrawal symptoms include epileptic seizures, tachycardia, gastrointestinal dysfunction, drug tolerance and locomotor abnormalities, often resulting in a transition to higher and higher doses of benzodiazepines (Franck et al., 2004). Long-term problems with cognitive functioning among patients even after six months following drug cessation was reported by Barker (2004). In summary, these multiple issues of both short and long-term side-effects associated with most current anti-anxiety medications, raises
substantial concerns about their viability as the primary go-to treatment of choice for anxiety treatments. More importantly, they underscore the importance in finding alternative pharmacotherapies for the treatment of anxiety disorders that display fewer side-effects, better patient tolerance and long-term safety profiles.

1.2 The Mesocorticolimbic Pathway

Central to an understanding of the mechanisms underlying the presentation of anxiety symptoms is an investigation of the mesocorticolimbic (MCL) pathway—comprised of the mesocortical and mesolimbic neural pathways, it is critically involved in a host of cognitive and affective functions including learning, emotion, memory and motivated behaviours related to reward learning (Helbing et al., 2016; Yamaguchi et al., 2011). It includes the dopaminergic (A10) neurons which project from the ventral tegmental area (VTA) to the prefrontal cortex (PFC), nucleus accumbens (NAc) and olfactory tubercle, with the combination of the latter two structures referred to as the ventral striatum (Ikemoto, 2010). Notably however, the mesolimbic pathway is reciprocal in nature, with GABAergic projections from the nucleus accumbens extending into regions such as the VTA and regulating its activity in turn (Yang et al., 2018).

While the mesolimbic pathway consists of the VTA and ventral striatum, its impact on cognitive functioning revolves around connections with other brain structures. Connections to the amygdala and hippocampus ultimately allow for the development of fear learning and memory formation, while connections with the prefrontal cortex and anterior cingulate cortex allow for mediation of emotional regulation and executive functioning (Everitt and Robins, 2005).

1.2.1 Nucleus Accumbens

The Nucleus Accumbens (NAc) is a site of convergence for many excitatory afferent connections including the VTA, cerebral cortex and thalamus, allowing for subsequent
regulation of behavioural responses towards reward and conditioned learning and memory (Nicola et al, 2000; Wise, 2004; Zahm, 2000). Structurally, the NAc is comprised of two major divisions: the core (NAcc) and the shell (NAcSh). Both divisions consist of mostly medium spiny neurons (MSN) which have been found to have relatively low baseline firing rates, suggesting these neurons are primarily GABAergic in nature (Castro and Bruchas, 2019). Highly regulated through dopamine innervation, the MSNs within the NAc have been identified to contain multiple dopamine receptor variations including D1 and D2 subtypes. These subtypes have been found to partially delineate NAc efferent projections referred as direct (primarily D1; targets: substantia nigra and VTA) or indirect (primarily D2; targets: pallidal and hypothalamic structures) pathways (Castro and Bruchas, 2019).

On a systems-level, the NAc has also been implicated by studies as being critically important brain region in the management of anxiety-related behaviors (Kalin et al., 2005; Lago et al., 2017; Levita et al., 2012). As documented by Levita et al (2012) and Lago et al. (2017), the involvement of the NAc in modulating motivation may not only be limited to risk/reward responses, but also the motivation to avoid danger; as a result, aberrant NAc activity states result in impaired risk avoidance management that underlie the maladaptive avoidance behaviors in the vast majority of anxiety disorders. These interpretations compliment the findings made by Kalin et al. (2005) who utilized PET scanning and demonstrated in rhesus monkey models that increased NAc metabolic activity is positively correlated with excessive freezing time in the human intruder paradigm—effectively emphasizing the importance of the NAc in modulating perceived anxiety.

Furthermore, developments in the field of natural medications have yielded interesting findings implicating the NAc as a region of interest for therapeutic interventions (Bhattacharyya
et al., 2010). Through the use of functional magnetic resonance imaging (fMRI), Bhattacharyya and authors showed that oral administrations of the phytocannabinoid, cannabidiol (derived from the plant species *Cannabis sativa*), were able to reverse aberrant NAc activity and psychotic symptoms induced by exposure to the psychoactive phytocannabinoid, Δ-9-
Tetrahydrocannabinol. Given that abnormal NAc activity has been linked to increased anxiety symptoms (Kalin et al., 2005) and that severe anxiety can lead to the presentation of psychotic symptoms, this collection of aforementioned studies highlights the therapeutic potential for the development of treatments targeting NAc activity in the management of mood/anxiety disorders.

1.2.2 Prefrontal Cortex

Many studies have demonstrated that the prefrontal cortex (PFC) is instrumental in modulating neuronal activity underlying anxiety and depression (Hare and Duman., 2020; Ironside et al., 2019). Comprised of numerous subdivisions including the prelimbic, ventral orbital, ventrolateral orbital and dorsolateral orbital regions, the PFC sends glutamatergic and GABAergic neurons to associated brain regions to elicit downstream effects (Bedwell et al., 2015; Lee et al., 2014; Wanchoo et al., 2009). While the exact mechanisms of action through which the PFC exerts its systems-level effects is still unclear, the PFC has been found to be directly involved in inhibiting aversive memories developed in fear conditioning paradigms, with inactivation measures such as lesions of the PFC leading to decreased extinction while PFC electrical stimulation led to increased extinction of conditioned responses (Ledoux, 2000; Maroun et al., 2012; Morgan et al., 1993; Muller et al., 1997).

While the PFC has been implicated in the processing of emotions, it is arguably best known for its role in cognitive functioning and executive decision-making. A primary area of focus has been its direct and indirect involvement in establishing and maintaining working
memory, as well as its influence on the motor cortices in carrying out locomotor activity (Funahashi, 2017; Lara and Wallis, 2015; Schulz et al., 2019). In light of these various integral roles that the PFC is involved in, as well as the fact that it has been implicated in emotional processing, the PFC presents a significant target of interest in which anxiety—and anxiety-related cognitive changes—can be studied using electrophysiology or drug treatment protocols to elucidate potential mechanisms and intra- or inter-PFC functional connections correlating with observed behavioral phenomena. In fact, recent research in our laboratory has shown that the cannabidiol can potently modulate cognitive and affective functions in the PFC (Szkudlarek et al., 2019). Specifically, Szkudlarek and authors had demonstrated that acute treatment of intracranial CBD infusions into the PFC region was capable in reversing symptoms of cognitive changes that stemmed from aberrant PFC states and THC administration via 5-HT1AR signaling mechanisms, highlighting the potential for therapeutic interventions targeting the PFC region in the management of mood/anxiety disorders.

1.2.3 Functional Connections between the PFC, NAc and Associated Brain Regions

While the two mesocorticolimbic brain regions of the NAc and PFC have been shown to play important roles in affective processing as described in previous sections, a full understanding of the mechanistic intricacies of the mesocorticolimbic pathway involves the study of the relationship between PFC and NAc—more specifically, the various connections between the two brain regions which modulate each other’s activity states. The NAc in particular has been found to receive both glutamatergic and GABAergic projections from the PFC (Lee et al., 2014; Russo and Nestler, 2013; Torregrossa et al., 2008; Wanchoo et al., 2009). While increased activity from PFC glutamatergic projections to the NAc was determined to be antidepressant in nature (Russo and Nestler, 2013), optogenic stimulation of GABAergic
projections from the PFC to the NAc was found to result in increased avoidance behavior by rodent subjects (Lee et al., 2014). Conversely, the NAc is able to indirectly modulate the activity of the PFC through efferent GABAergic projections to the VTA, which in turn regulate dopaminergic release from the VTA to the PFC, effectively modulating PFC neuronal activity (Buchta et al., 2017; Ellwood et al., 2017; Norris et al., 2016; Yang et al., 2018). Collectively, these studies demonstrate the integrative nature in which the NAc and PFC function as a system rather than standalone structures on a molecular level within the mesolimbocortical pathway.

As mentioned above, associated brain structures such as the VTA play an important role in facilitating affective processing within the MCL pathway. Interestingly, in addition to projections to the PFC, the VTA has been documented to reciprocally modulate the NAc through VTA-NAc dopaminergic projections (Groenewegen and Russchen, 1984; Heimer et al, 1991; Nauta et al, 1978; Sesack et al., 2009; Somogyi et al, 1981; Zahm and Heimer, 1993). This is particularly important to note as behavioral studies have documented impaired VTA-NAc circuitry to be responsible for anxiety-related social behavior dysfunction and avoidance (Russo and Nestler, 2013; Van der Kooij et al., 2018), suggesting that the VTA is capable of modulating both PFC and NAc activity states during affective processing.

Furthermore, within the mesolimbocortical pathway, the discovery of PFC-amygdala connections has led to studies documenting the important role of the PFC in regulating amygdala activity, with complete extinction of conditioned aversive responses being achieved in conjunction with increased fMRI activity of the PFC region and decreased activity in the amygdala (Ironside et al., 2019; Pitman et al., 2012). In addition to these findings highlighting brain activity alteration in response to PFC activation/inactivation, it is worth noting that individuals diagnosed with depression and those with an anxiety disorder (including PTSD) have
been found to exhibit hyperactive amygdala activity that is hypothesized to be due to reduced inhibition from the PFC (Etkin et al., 2010; Hamilton et al., 2012; Maroun et al., 2012; Rauch et al., 2000). Interestingly, while this top-down regulation from the PFC to amygdala predominates the literature surrounding the two regions’ connections, GABAergic projections from the amygdala to the PFC presents the notion that the PFC-amygdala connections are reciprocal, rather than unidirectional in nature—adding an element of complexity to determining the individual functionality of each region (Seo et al., 2016).

Taken together, these studies documenting the various functional connections between these mesolimbocortical brain regions suggests clearly that not only do the PFC and NAc play a role in affective processing, but that these regions are influenced by associated brain structures such as the VTA and amygdala—emphasizing the fact that the affective processing abilities of the PFC and NAc are not only modulated by one another, but also by other brain regions within the MCL circuit that should be considered within a comprehensive analysis of MCL affective processing.

1.2.4 The Role of the Mesocortiolimbic Pathway and Receptor Targets in Anxiety Disorders

Due to its integral role in emotion and cognitive processing as previously described, the mesocorticolimbic (MCL) pathway has been implicated in many studies not only assessing reward and motivation learning, but also anxiety disorders as well. A study conducted by Burkhouse et al. (2020) found that the NAc played a critical role during treatment recovery from anxiety disorders, as greater bilateral NAc volumes correlated with significantly greater reductions in anxiety symptoms pre-to-post SSRI and CBT treatment. In conjunction with findings that suggest NAc plays a key role in the communicative circuit between the amygdala and the prefrontal cortex (Mannella et al., 2013), growing evidence suggests NAc may be a key
target in anxiety disorder treatment and in the broader MCL pathway.

As a vital component of the cortical aspect of the MCL pathway, the PFC has been implicated in a host of cognitive and affective functioning roles, including its top-down inhibition of the amygdala by the PFC, yielding in turn a distinct profile of increased PFC regulatory control being linked to decreases in perceived anxiety levels as mentioned earlier (Ledoux, 2000; Maroun et al., 2012; Morgan et al., 1993; Muller et al., 1997). When considered in conjunction with the findings that decreased PFC volumes have been correlated to increased measures of both anxiety and depression (Madonna et al., 2019; Mohlman et al., 2009) and that patients with anxiety and/or depressive disorders have been found to possess circuit abnormalities in amygdala and hippocampal regions—two associated brain regions within the mesocorticolimbic pathway essential in fear memory acquisition and recall—the PFC presents itself as a viable target in the treatment of anxiety, depression or comorbid diagnoses of these two mood disorders (Etkin et al., 2010; Godsil, 2013; Hamilton et al., 2012; Maroun et al., 2012; Rauch et al., 2000).

Expanding upon the VTA-NAc circuitry analysis discussed in section 1.2.3, recent studies examining the VTA have demonstrated the significance of its activity in the presentation of anxiety symptoms. A study conducted by Cha et al. (2014) utilized fMRI to measure the brain activity of participants while undergoing fear generalization tasks, with findings indicating that participants with generalized anxiety disorder (GAD) exhibited heightened VTA activity compared to non-GAD subjects in response to generalized stimuli. VTA hyperactivity was also shown to correlate to manic states in mouse models, characterized by increased dopaminergic activity and resulting locomotor abnormalities (Coque et al., 2011). When considering the DAergic projections from the VTA to the NAc as well as its connections to cortical regions, the
VTA ultimately presents a vital area of interest along with the NAc in the management of anxiety disorders.

Correlations between behavioral and molecular analyses highlight GABA and 5-HT receptors and their associated downstream molecular pathways, as being essential targets when assessing models of anxiety and anxiety symptoms. Using proton magnetic resonance spectroscopy, Strasser et al. (2019) had demonstrated that there existed a significant negative correlation between subjects’ perceived situational stress and NAc GABA levels. This data signifying the importance of NAc GABA levels in regulating anxiety is supported by Lopes et al. (2012), who found that GABA-inhibition within the NAcSh increased anxiety symptoms in the context of rodent feeding behaviour. In a similar manner, increasing evidence points towards decreased intra-PFC GABA levels contributing towards the establishment and maintenance of anxiety and depression disorders (Ghosal et al., 2017), suggesting that internal imbalances in inhibitory control within these brain regions can lead to significant systems-level mood dysfunction. Interestingly, the application of GABA$_A$R antagonists in the VTA has been found to be crucial in reversing drug-induced behavioral changes following their microinfusion administrations into the NAcSh and VTA (Blacktop et al., 2016; Norris et al., 2016)—highlighting the importance of GABAergic transmission between the NAcSh and VTA as well as within VTA local circuitry. Overall, these studies suggest that both GABA levels and GABAergic transmission within the mesocorticolimbic pathway are vital in the management of anxiety, as indicative of the effectiveness of benzodiazepine as a frontline anxiety medication.

Existing evidence suggests that dysregulation of serotonergic transmission also plays a significant role in the development of anxiety disorders, with decreases in 5-HT levels and serotonergic transmission being associated with increased anxiety (Gartside et al., 1995). In
particular, abnormal 5-HT$_{1A}$R activity has been implicated in both the development and treatment (SSRIs) of anxiety disorders (Akimova et al., 2009; Gordon and Hen, 2004; Lesch and Gutknecht, 2004; Strobel et al., 2003). Within the PFC, 5-HT$_{1A}$Rs have been found located on pyramidal glutamatergic neurons as well as interneurons that act on these pyramidal cells; analysis conducted by Albert et al. (2014) have demonstrated anxiety symptoms to present during low and high levels of 5-HT, presenting an interesting notion of non-directional imbalance of 5-HT levels being the etiology of intra-PFC mood dysfunction. In addition to the presence of HT$_{1A}$R and its autoreceptor activity within the PFC and raphe nuclei (Garcia-Garcia et al., 2014), HT$_{1A}$R have been found localized on GABAergic interneurons within the NAcSh (Van Bockstaele et al., 1996). This in turn suggests that serotonergic afferent projections to the NAcSh may directly inhibit these interneurons, allowing for downstream increased activity of striatal projections and modulation of anxiety-processing brain structures.

![Figure 1 Schematic of the Mesocorticolimbic Circuit and Associated Anatomical Connections](image1.png)

*Figure 1 Schematic of the Mesocorticolimbic Circuit and Associated Anatomical Connections.* Non-exhaustive visualization of currently known projections between MCL brain regions and modulatory regions of interest. Arrow projection legend: Blue (glutamatergic), Green (GABAergic), Red (dopaminergic), Purple (serotonergic).
1.2.5 Overview of Notable Molecular Biomarkers in Anxiety Disorders

In terms of downstream molecular signaling pathways associated with anxiety disorders, abnormal levels of intracellular biomarkers such as GSK3β, Akt, ERK and JNK proteins have been associated with anxiety symptoms and mood disorders (Crofton et al., 2017; Engeli et al., 2014; Jope et al., 2006; Willock et al., 2016). While the activity of these proteins have been correlated with presentations of anxiety symptoms which may appear phenotypically similar, the generation of these symptoms through the regulation of specific signaling pathways and downstream effectors documented in the literature is relatively unique between the proteins.

GSK3β, otherwise known as glycogen synthase kinase-3β, is a constitutively active protein whose downregulation has been shown to be correlated with potential anxiolytic effects (Crofton et al., 2017; Mines et al., 2010). Specifically, assessing the function of GSK3β within the NAcSh, Crofton et al. (2017) demonstrated that GSK3β-knockdown in rat models effectively decreased anxiety-like behavior through a potential suppressing mechanism on tonally active interneurons (TANs). In another study, Mines et al. (2010) found that inhibition of GSK3 in Fmr1 knockout mice decreased displays of anxiety during social interaction. As a downstream target of both the Wnt and PI3K-Akt pathways, GSK-3β is readily inhibited through phosphorylation of its Serine-9 residue within the N-terminus domain (Sani et al., 2012).

Akt, often referred to as Protein Kinase B, is a member of the PI3K-Akt pathway involved in various cell activities including survival and proliferation (Qiao et al., 2018). Phosphorylation at the Serine-473 and Threonine-308 residues in the activation loop of the enzyme activates Akt, resulting in the activation of many downstream targets including the inhibition of GSK3β (Bellacosa et al., 1998; Cross et al., 1995). Knockdown and polymorphism analysis of Akt within animal and human subjects demonstrated that alterations of Akt ultimately
resulted in increased susceptibility to chronic stress as well as heightened displays of trait anxiety, suggesting that Akt signaling plays a critical role in mood/anxiety regulation (Engeli et al., 2014; Willock et al., 2016).

Extracellular signal-regulated kinase (ERK) is a member of the mitogen-activated protein kinase (MAPK) pathway responsible for a host of cellular roles, including but not limited to mitogen signaling in cell cycle progression and integration of stress signals (Einat et al., 2003; Zamora-Martinez et al., 2014). Often activated in response to cell stress, increased concentration of phosphorylated ERK (p-ERK) has been associated with increased symptoms of anxiety and depression, as well as overall mood dysregulation in various brain regions including the frontal cortex and hippocampus (Ailing et al., 2008; Einat et al., 2003; Hudson et al., 2019).

JNK, also referred to as c-Jun N-terminal kinase, is also a member of the MAPK family pathway along with ERK; as such, its phosphorylated activation occurs primarily in response to perceived cell stress (Zeke et al., 2016). While the primary active isoform, JNK1, is known for its role in regulating neuronal differentiation and maturation, studies have also implicated JNK1 in facilitating the development of symptoms reflective of anxious and depressive states (Hollos et al., 2018; Mohammad et al., 2018). Interestingly, a proposed mechanism through which JNK1 elicits its effects within the hippocampus is through the prioritization of immature granule cell neurogenesis. These immature cells have been observed to negatively regulate mature dentate granule cell populations through recruitment of synapsing GABAergic inhibitory neurons, resulting in overall decreased hippocampal neuronal activity and abnormal communication with connected brain regions (Marín-Burgin et al., 2012).

While individual analyses of these protein biomarkers have yielded promise in providing potential mechanisms to address in regard to anxiety disorder and treatment, it should be noted
that these proteins ultimately work in conjunction with one another through both direct interactions (e.g. the inhibition of GSK3β by Akt) as well as indirect downstream targets. Furthermore, depending on the brain region being influenced, the resulting activation or inactivation of these enzymes may yield varying behavioral outcomes.

1.3 Cannabis: An Alternative Treatment for Anxiety Disorders

1.3.1 Effects of Cannabis on Mental Health

Despite recorded usage as a medicinal plant for the past millennia, the effects and underlying mechanisms of cannabis have still yet to be fully uncovered (Mechoulam, 1986; Russo, 2007; 2008). Indeed, while cannabis’s effects have been primarily attributed to key natural constituents such as tetrohydrocannabinol (THC) (Gaoni and Mechoulam, 1966), naturally occurring endocannabinoids such as anandamide (AEA) (Devane et al., 1992) and 2-arachidonoylglycerol (2-AG) (Mechoulam et al., 1995; Sugiura et al., 1995), as well as cannabidiol (CBD) and other such phytocannabinoids, the overall physiological effects that the plant Cannabis sativa yields as a whole has yet to be conclusively defined.

Nonetheless, clinical and pre-clinical cannabis research has demonstrated the potential therapeutic use of cannabinoid formulations including administration of plant extracts in the treatment of movement disorders, psychiatric disorders, Alzheimers disease, epilepsy, pain and multiple sclerosis (Montero-Olease et al., 2020). A defining milestone in the development of cannabis-related therapeutic formulations include the United Kingdom’s approval of Nabiximols (CBD-THC extract) in treating pain and spasticity in multiple sclerosis following a clinical study conducted by Novotna et al. (2011). Although whole-plant extracts have yet to be approved by North American regulatory agencies in the treatment of health conditions, cannabis-derived and related products such as the FDA-approved drugs Epidiolex (purified CBD extract treating
syndrome-specific seizures) and Dronabinol (synthetic THC used as an appetite stimulant, antiemetic and sleep apnea remedy) highlight the therapeutic potential of cannabis-related products.

1.3.2 Properties and Mechanisms of Action of Cannabidiol

Cannabidiol is a major phytocannabinoid within *Cannabis sativa* whose psychotropic effects have largely been attributed to its regulatory role in the reduction of psychoactive symptoms associated with THC. Within the endocannabinoid system, it has been demonstrated that while cannabidiol possesses low affinity towards the endocannabinoid receptors CB\(_1\) and CB\(_2\) which THC interacts with, CBD is capable of antagonizing the effects of CB\(_1\) and CB\(_2\) receptor agonists (Pertwee et al., 2002; Thomas et al., 2007). In addition, more recent studies have suggested that CBD can act as a negative allosteric modulator for both CB receptors, supporting the role of CBD as an inverse agonist for both THC and 2-AG receptor interactions within the endocannabinoid system (Laprairie et al., 2015; Martínez-Pinilla et al., 2017; Morales et al., 2016). Furthermore, a pre-clinical study by Hudson et al. (2019) discovered a potential mechanism of action for THC/CBD interactions, demonstrating that increased anxiety-salience observed following intra-ventral hippocampal infusions of THC was due to modulation of extracellular signal-regulated kinase phosphorylation (p-ERK1/2). Subsequently, CBD-THC coadministrations were found to reverse these observed changes through downregulation of p-ERK1/2 activity, providing further evidence for this pathway mechanism. Interestingly however, CBD has also been found to indirectly augment CB receptor activity, potentiating the effects of the CB receptor agonist anandamide (AEA) through its competitive interference in transport protein binding, with clinical trials suggesting the subsequent elevation of AEA levels to be inversely correlated with the presentation of psychotic symptoms in schizophrenia or
schizophreniform patients (Leweke et al., 2012).

Aside from endocannabinoid receptors, CBD acts on several anxiety-related receptor targets within the mesolimbic pathway, including 5-HT and GABA receptors. The effect of CBD on 5-HT$_{1A}$ receptor activation in particular, is well documented, with both behavioral and molecular studies demonstrating its role as an agonist at this subtype (Linge et al., 2016; Norris et al., 2016; Pelz et al., 2017; Resstel et al., 2009; Russo et al., 2005). In terms of the mesolimbic pathway, Norris et al. (2016) reported that administration of a 5-HT$_{1A}$ antagonist, NAD299, into the NAcSh was effective in reversing the anxiolytic effects elicited by CBD within an olfactory fear memory paradigm—suggesting 5-HT$_{1A}$ auto-receptor activation to be potentially integral to the regulation of NAcSh GABA neurons and overall fear memory formation.

The interaction between CBD and GABA receptors is rather distinct between the two receptor subtypes, GABA$_A$ and GABA$_B$. While current evidence suggests that CBD does not act through GABA$_B$ receptor activation (Lopes et al., 2012; Straiker et al., 2018), there exists multiple studies which demonstrate its effects on the GABA$_A$ receptor, where can act as a positive allosteric modulator (Bakas et al., 2017; Lopes et al., 2012; Morano et al., 2016; Ruffolo et al., 2018). In addition, given that both in-vitro and in-vivo studies (Mallet et al., 2005; Tepper et al., 2008) have shown GABA$_A$R activation to inhibit GABA interneurons within the ventral striatum—and more importantly, the subsequent increase in firing activity by NAc projection neurons following such interneuron inhibition—there may exist a potential GABA$_A$R-mediated pathway of modulation in regards to the ability of the NAcSh to inhibit downstream VTA DAergic activity and consequently modulate emotional states.

These mechanisms of action may shed light on the reported therapeutic properties of cannabidiol, including anticonvulsive, anxiolytic, antipsychotic, anti-inflammatory and anti-
nausea effects (Mechoulam et al., 2002). Furthermore, despite its relatively poor bioavailability through oral ingestion, pharmacological significance for both CBD and its metabolites has been found in-vivo (Ujváry et al., 2016); in fact, Bergamaschi et al. (2011) demonstrated that a single 600mg oral dose of CBD was effective in significantly reducing anxiety symptoms of social anxiety disorder (SAD) patients. With consideration of its relatively safe profile as a non-psychoactive phytocannabinoid, CBD may ultimately constitute a natural alternative in place of or assisting conventional medications in the treatment of neuropsychiatric conditions, especially anxiety disorders, given CBD’s ability to influence the activity of 5-HT and GABA_A receptors that play a key role in the pathophysiology of such disorders.

1.3.3 Properties and Mechanisms of Action of the Mono-Terpene, Linalool

Linalool is an odorous, acyclic monoterpene found within many plants including *Lavandula angustifolia* (Lavender) and *Cannabis sativa* (Milanos et al., 2017). As a component of various essential oils, the use of linalool includes not only its incorporation in the perfume industry but also in the treatment of various pathological conditions. For example, numerous studies have reported that exposure to linalool can produce various effects, including but not limited to cardiovascular effects (Anjos et al., 2013), anti-inflammatory effects (Huo et al., 2013) and anxiolytic effects (Bradley et al., 2007; Harada et al., 2018; Linck et al., 2010).

Despite differing modes of administration (intraperitoneal, oral, odorous), various studies have proposed that the underlying mechanisms of action for the physiological effects of linalool and *Lavandula angustifolia* (which primarily consists of linalool and linalool metabolites) involve the activation of GABA_A and 5-HT_1A receptors (Harada et al., 2018; Kessler et al., 2012, 2014; Malcolm and Tallian, 2018; Milanos et al., 2017), as well as competitive inhibition of NMDA receptors (Brum et al., 2001; Elisabetsky et al., 1995, 1999). In addition, in-vitro studies
have demonstrated that linalool administration to SH-SY5Y neuronal cells significantly decreases expression of adenylate cyclase I, p-ERK and PKA, all of which are associated with anxiety-related phenotypes, highlighting another potential mechanism manner in which linalool may exert its psychotropic effects (Caputo et al., 2016; 2017; 2018).

Of particular interest for clinical applications is the non-invasive, olfactory route of linalool administration. While a major aspect of the odorous effects of linalool were originally thought to involve the inhalation and subsequent dissolving of linalool into the bloodstream, Harada et al. (2018) have shown that linalool exposure in anosmic mouse models produces anxiolytic effects via functional olfactory pathways. Given that the olfactory nervous system connects to brain regions associated with memory, emotions and arousal such as the entorhinal cortex and amygdala (Christen-Zaech et al., 2003), activation of these systems presents a plausible explanation for anxiety symptom reduction following olfactory linalool administration.

Similar to CBD, given the relative safe profile of linalool and its metabolites (Malcolm et al., 2018) as well as its documented anxiolytic effects exhibited in both animal and human studies following non-invasive odor administration (Harada et al., 2018; Linck et al., 2010; Takahashi et al., 2011; Zhang et al., 2016), there is potential usage for linalool as an anxiolytic compound or in co-administration with conventional anti-anxiety medications or anxiolytic cannabinoids, such as CBD.

1.3.4 The Entourage Effect

The Entourage Effect refers to the proposed mechanism by which combinations of separate phytocannabinoid extracts might yield psychoactive effects that are more potent than their individual administration (Ben-Shabat et al., 1998; Mechoulam et al., 1999; Russo, 2011). Wagner and Ulrich-Merzenich (2009) have proposed that the potential mechanisms through
which the Entourage Effect might be observed include multi-target receptor effects, increased pharmacokinetic efficiency, improved bactericidal effects as well as modulation of adverse events—with the latter being well established in the case of CBD’s ability to attenuate THC-induced psychosis (Davies and Bhattacharyya, 2019; Hahn, 2018).

Moreover, studies have demonstrated that whole-plant extracts of *Cannabis sativa* have yielded greater therapeutic effectiveness in comparison to pure component administrations. For example, Johnson et al. (2010) observed in a randomized controlled trial of advanced cancer patients that while treatment with pure-THC extract did not yield significant anti-nociceptive effects compared to placebo, patients taking CBD:THC combined extracts reported significantly less pain compared to placebo-treated patients. In the context of animal studies, Gallily et al. (2015) reported that while increasing dosage administrations of pure-CBD extracts in mouse models yielded a biphasic effect on the mediation of inflammation, the dose-response curve observed following whole-plant extract treatment resulted in a starkly linear correlation between increased dosage and decreased presence of inflammatory cytokines.

While the therapeutic effects of linalool are well documented as previously described, research specifically examining the existence of a potential Entourage Effect involving linalool-cannabinoid combinations is relatively scarce. Interestingly however, Takahashi et al. (2011) discovered that there existed significant differences in anxiolytic efficiencies between *Lavandula* plant species depending on their percentage composition of linalool (LO) and linalool acetate (LA). While correlational analysis indicates lack of significant correlation between linalool vs. anxiolysis ($r = +0.54$) and linalool acetate vs. anxiolysis ($r = +0.72$), there was a significant correlation ($P < 0.05$) between combined LO + LA odorous treatment and anxiolytic efficiency ($r = +0.82$). This study suggests the mechanistic potential for interaction dynamics between
linalool and other cannabis compounds in achieving anxiolytic outcomes.

As it currently stands in the literature surrounding cannabis research, there exists a lack of studies documenting the potential for Entourage Effects between the monoterpenes and phytocannabinoids (Russo, 2011). However, given previously described similarities in both receptor targets (GABA<sub>A</sub>R, 5-HT<sub>1A</sub>R, p-ERK downregulation), common modulation of neuronal pathways and anxiolytic outcomes between linalool and CBD, there exists an interesting possibility for the existence of an Entourage Effect between the two compounds. This exploration forms the foundation for my research project and thesis.

1.4 Research Aims and Hypothesis

The overarching hypothesis of my thesis is that combinations of CBD with linalool yield greater reductions in anxiety-related behaviors and anxiety-related molecular biomarkers compared to individual administrations of either treatment due to an Entourage Effect between the two compounds.

In have addressed this general research question with the following specific experimental aims:

Aim 1: Determine if CBD-linalool co-administration in the NAcSh on anxiolytic measures of spatial cognitive functioning, emotional memory formation and processing within the baseline cohort exhibit an Entourage Effect by potentiating the behavioural anti-anxiety effects of either compound alone.

Aim 2: Characterize the pharmacological mechanisms associated with any potential linalool-CBD entourage effects by determining the potential roles of the anxiety-related biomarkers GABA<sub>A</sub> or ERK-1-2 signalling pathways in these effects. Subsequently, characterize
the localized molecular signaling effects of CBD, linalool or their combination, on anxiolytic actions directly in the NAcSh in baseline cohorts.

**Aim 3:** Determine whether the anxiolytic effects elicited by linalool-CBD combinations are able to reverse anxiety symptoms induced by exposure to chronic unpredictable stress within the chronic stress cohort.

2. Methods

2.1 Animals and Housing

Adult male Sprague-Dawley rats (300-400 grams) were acquired from Charles River Laboratories (Quebec, Canada) and used in accordance with guidelines outlined by the Canadian Council for Animal Care (CCAC) and University of Western Ontario Animal Care Committee (AUS protocol 2018-053). Upon arrival, rats were paired and housed together under controlled conditions (constant temperature and humidity, food and water provided ad libitum, 12-hour light/dark cycle) monitored by both ACVS and laboratory technicians in the designated animal care facility at Western University. Rat housing specifications include a plexiglass rectangular box filled with approved corn bedding and environmentally enriched objects (such as wood chewing blocks, and paper nesting material).

2.2 Surgical Procedures

Surgical procedures for adult male Sprague-Dawley rats were initiated after at least 7 days following their arrival to the animal care facility at Western University. Rat subjects designated for surgery were anesthetized with a 2 : 1 mixture ratio of Ketamine (80 mg/kg;
Vetoquinol) and Xylazine (6 mg/kg; Bayer) via intraperitoneal injection. Following confirmation of the lack of a pain reflex response, subjects were then treated with meloxicam (1 mg/kg) to further decrease any pain perception as well as inflammation; in addition, a second dosage was administered during the first day post-surgery. Rats were positioned in stereotaxic apparatus for cannula implantation, with a warm heat pad positioned below a disposable urine pad to maintain constant body temperature; to this end, the subject’s temperature was taken both pre- and post-surgery. Following surgical preparation, eight-millimeter stainless steel guide cannulas (22 G; Plastics1) were implanted into the NAcSh bilaterally at a 12° angle using these stereotaxic coordinate positions at a 12° angle (mm from bregma): anterior-posterior (AP) ± 1.8, lateral (LAT) ± 2.6, and ventral (V) – 7.4 from the subject’s dural surface. All coordinates were acquired from the Rat Brain Atlas created by Paxinos and Watson (2005). Guide cannulas were then secured in place through the use of miniature screws attached to the skull and dental acrylic cement.

![Figure 2 Visualization of intra-NAcSh surgical guide cannulations.](image)

Representative slide (A) is shown alongside a copy with an overlay of the relevant rat brain atlas (Paxinos and Watson, 2005) from a cortical perspective (B). White arrowheads point towards the injector tips located within the NAcSh region.
2.3 Drug Preparation and Administration

Linalool (97%; Acros Organics), CBD, EPA (eicosapentanoic acid, ERK activator) and Flumazenil (Tocris) were utilized in this study. Regarding intra-cranial drug infusions, vehicle solutions comprised of dimethyl sulfoxide (DMSO), cremophor and 0.9% pH 7.4 saline (1:1:18). CBD-only, EPA-only and Flumazenil-only drug solutions were created from dissolving the respective drugs in the DMSO-Cremomophor-Saline solvent (referred to in brief as the “PBS solution”), with a mixture ratio of 1:1:18 to 5:5:30. Combination administrations, namely the CBD-EPA and CBD-Flumazenil treatment mixtures, were created using the 1:1:18 and 5:5:30 ratio compositions respectively. Nitrogen gas was used to evaporate the ethanol solvent from the original EPA stock solution. Target concentrations of drugs within each mixture were as follows: 5 ng/0.5µL CBD (sub-threshold dosage; Norris et al., 2016), 1 mM EPA (effective dosage in attenuating CBD-THC anxiolytic effects; Hudson et al., 2019) 5 µg/µL flumazenil (effective dose from an aggregate of studies; Da Cunha et al., 1999, Dos Reis and Canto-de-Souza, 2008, Herzog et al., 1996, Jardim et al., 2001).

To prepare for odorous drug administration, the day prior to beginning behavioral testing, rat subjects were placed within a transparent, rectangular plexiglass cage with a filter top (to prevent odours from entering or leaving the cage) and four spice jars (one in each corner, secured by Velcro adhesive). The spice jars each contained a small weigh boat which contained distilled water (2000 µL); this cage apparatus would serve as the medium for the odorous drug delivery. Rat subjects were allowed to habituate to this novel environment for 30 minutes before being placed back into their housing cages. On test day, subjects were placed within the same odor-exposure box for a 30-minute exposure session; however, based on their treatment group designations, they would be exposed to either distilled water (2000 µL) or linalool (200 µL or
2000 μL). Subjects exposed to distilled water—and thus essentially air—were labelled with the term “V_Air”, while those exposed to linalool were given the prefix “L200-” or “L2000” depending on whether they were exposed to 200 μL or 2000 μL (per spice jar) respectively.

Immediately following this odor treatment session, intracranial drug deliveries were conducted. Intra-NAcSh microinfusions were conducted using microinjectors attached to a Hamilton syringe, performed over a 1-minute time period. Microinjectors were kept in place for an additional 1-minute time period following infusion to allow for maximum diffusion from the injector tips. Subjects treated with PBS solutions were designated with the term “V_PBS”, while those given bilateral administration of 5 ng/0.5μL CBD were labelled with the term “-CBD.” Following this, animal subjects were then immediately exposed to designated test procedures.

![Visualization of odor exposure apparatus](image)

**Figure 3 Visualization of odor exposure apparatus.** Shown above on the left is the rectangular Plexiglas cage (filter top not shown) that housed the subject during olfactory exposure; on the right is representation of an individual spice jar found in the Plexiglas cage containing a weight boat filled with either water or linalool depending on treatment designation.
2.4 Chronic Unpredictable Stress Protocol

The Chronic Unpredictable Stress (CUS) Protocol was a protocol designed by previous lab member Dr. Justine Renard to simulate the acquirement of chronic stress in designated rat subjects. The protocol involved 14 consecutive days of unpredictable stress activities, including supra-threshold footshocks (0.6 mA), food and water deprivation, wet bedding, the tilting of rat plexiglass home cages at a 45° angle, and sudden light deprivation. Following the 14-day protocol, food, water, and housing conditions returned to previous controlled conditions and animal behavioral testing began immediately. Subjects exposed to the CUS protocol were labelled with the prefix “CUS-” to specify their chronically stressed nature; these subjects were then compared with treatment group interventions to determine whether these interventions were effective in reversing chronic-stress induced cognitive changes acquired from exposure to the CUS protocol.
2.5 Experimental Cohorts and Treatment Group Designations

Aside from the aforementioned baseline and chronic stress cohorts (the latter employing the CUS protocol to induce chronic stress within subjects), separate experimental cohorts received intra-NAcSh flumazenil and EPA administration to test whether GABA<sub>A</sub>R and/or ERK activity were potentially correlated with behavioral effects associated with CBD-Linalool administration. The final group designation summary is illustrated in the table below.

<table>
<thead>
<tr>
<th>Experimental Cohorts</th>
<th>Treatment Group Designations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acute Treatment (Groups: 6)</td>
<td>( V_{Air,PBS}, V_{Air,CBD}, L200-V_{PBS}, L2000-V_{PBS}, L200-CBD, L2000-CBD )</td>
</tr>
<tr>
<td>Chronic Stress (Groups: 4)</td>
<td>( V_{Air,PBS}, CUS-V_{Air,PBS}, L2000-CBD, CUS-L2000-CBD )</td>
</tr>
<tr>
<td>Flumazenil Challenge (Groups: 4)</td>
<td>( V_{Air,PBS}, V_{Air,PBS}+\text{Flumazenil}, L2000-CBD, L2000-CBD+\text{Flumazenil} )</td>
</tr>
<tr>
<td>EPA Challenge (Groups: 4)</td>
<td>( V_{Air,PBS}, V_{Air,PBS}+\text{EPA}, L2000-CBD, L2000-CBD+\text{EPA} )</td>
</tr>
</tbody>
</table>

*Figure 4 Experimental Cohorts and Associated Treatment Group Designations.* Combinations of treatment prefixes and terms are representative of treatment group designations.
2.5 Behavioral Testing

2.5.1 *Open Field Test.*

This task is a sensorimotor test which measured overall locomotor activity and thigmotaxis of rat subjects. The test apparatus consists of a transparent plexiglass box that assesses various motor movements using a grid system of laser interference detection (San Diego Instruments). Center and total distance travelled (in cm) by rat test subjects were recorded during 30-minute test sessions for data analyses, with the first 5 minutes of behavioral observations being assessed for locomotor and thigmotaxic measures.

2.5.2 *Light Dark Box Test.*

This task measured the bright-space anxiety exhibited by rats, based upon the rats’ instinctive tendency to avoid bright-lit environments. The test apparatus comprised of a two-compartment non-transparent plexiglass box (50 x 25 x 37 cm), with a white-coloured, uncovered compartment and a black-coloured, lidded compartment. A 10 by 10 cm open doorway separated these different compartments, allowing rat subjects to easily traverse between the two compartments. The white-coloured compartment was brightly lit by an overhead lamp located approximately 125 cm above the floor of this respective compartment. To begin the experimental session, the rat was placed in the white-coloured compartment with its back facing the open doorway. Rats were then observed for a total of 5 minutes, with measurements such as total time spent in the light, risk assessment, latency to first compartmental and second compartmental transitions, as well as total number of transitions during the test session. Transitions between compartments were deemed complete when all four feet of the rat were placed in the new compartment. Risk assessment measures included times when the rat was in the dark compartment and placed at a minimum its nose into the light compartment, with the
maximum inclusion criteria being three of four paws in the light compartment. Test sessions were recorded and analyzed offline (Behaview software; www.pmbogusz.net).

2.5.3 Social Motivation and Recognition

The Social Motivation and Recognition test, otherwise known as the Three-Chamber Sociability and Social Memory test, is a three-stage test used to assess the degree of sociability and social recognition memory, as described by Loureiro et al. (2015). The first stage of habituation was a 13-minute session whereby the subject could explore the test apparatus and become familiar with the environment. The next day, within a span of 24 hours, the subject was placed again within the center chamber for a 5-minute session with the chamber gates down. After this 5-minute session, the second stage of social motivation and third stage of social recognition were conducted in successive order. The second stage involved placing an empty small wired cage and another one filled with a stranger male rat within the left and right chambers; chamber gates were then lifted, and the test subject’s time spent interacting with each cage was recorded for 8 minutes. Immediately after the second stage, a novel male rat was introduced into the empty wired cage, rendering the cage already with a rat from the first stage the “familiar rat”; the subject’s total interaction time with each cage was then recorded for another 8-minute session. Throughout the testing protocol, the empty wired cage and stranger rat wired cage in the second stage of the test were counterbalanced between the left and right chambers of the test apparatus. Subjects’ recorded interaction times with the cages during the task was analyzed offline (Behaview software; www.pmbogusz.net).

2.5.4 Elevated Plus Maze

This task involved a black acrylic maze apparatus with four arms (10 x 50 cm), extending
from a 10 x 10 cm base platform elevated 50 cm above the floor. Two opposite arms were shielded with 40 cm-high walls, while the remaining two opposing arms were unshielded except for a 1 cm-high ledge that served to prevent subjects from falling off the platform while exploring. The elevated plus maze task measures open-space anxiety, with primary outcomes including total time spent in open arms, risk assessment (total time spent by the subject placing at a minimum its nose, or at a maximum three of its paws in the open arm area) and total number of arm transitions. Rat subjects were placed on the center platform facing an open arm and allowed to explore the maze apparatus for 10 minutes. Exploration behavior was recorded and analyzed offline (Behaview software; www.pmbogusz.net).

2.5.5 Context-Dependent Fear Conditioning

As an associative learning and memory task, context-dependent fear conditioning measures the degree of freezing behavior exhibited by rats following the formation or recall of a previously acquired associative fear-memory between a footshock stimulus and a specific sensory or contextual cue. Using the same test apparatus described previously (Norris et al. 2016), the task involved two stages: conditioning and testing. Conditioning (Stage 1) involved pairing contextual stimuli (in the form of a black-and-white striped walled, lidless box) to supra-threshold footshocks (0.8 mA, 1 second in duration, 10 total administered at randomized intervals over a 25 minute session) delivered via a metallic grid shock floor located at the base of the box enclosure. The next day, within 24 hours, rat subjects were re-introduced to the same test environment (Stage 2) and their subsequent freezing behavior recorded. Treatment effects for the acquisition phase of fear memory was assessed by administering treatment immediately prior to the conditioning phase; in contrast, to examine the effects of drug interventions to modulate the recall of fear memory formation was tested by administering treatment immediately prior to the
testing (recall) phase of the procedure. Recorded data was then analyzed in an offline manner (Behaview software; www.pmbogusz.net).

2.5.6. **Spontaneous Alternation**

This test assesses the rats’ spatial working memory and examines the ability of the rat to hold short term memory (i.e. having just visited a specific maze arm) during the performance of the task. Normally, healthy rats will alternate between the test arms but will continue to re-enter the just experienced arms when there is a working memory deficit. A Y-maze (black acrylic, 3 arms separated by angles of 120° from one another; each arm length was 50 cm with a wall height of 40 cm) was placed on the floor and rats were placed at the end of a designated starting arm facing the wall. Subjects were then allowed to explore the maze apparatus and alternate between arms during a 15-minute session. A successful arm entry was classified as all four paws of the rat had either entered or left an arm, and a successful set of alternations was defined by three consecutive entries into unique arms (e.g. entry into arm A, then B, then C). Subjects’ exploratory behaviors were recorded and analyzed offline (Behaview software; www.pmbogusz.net).

2.5.7 **Novel Object Recognition**

The novel object recognition test examines a rat’s ability to discern between novel and familiar objects, representative of short or long-term memory (depending on the retention interval). Healthy rats will recall an object previously encountered, preferring the choice of a novel object when given a choice to explore a recently encountered vs. novel object in the test chamber. This ability is impaired during memory impairments, whereby rats will show deficits in discerning between the previously encountered vs. novel object during the object exploration test. The test apparatus comprised of an 80 x 80 cm lidless box (black acrylic). Subjects were
exposed to the box for a 20-minute session one day prior to the actual test to allow them to habituate to this novel environment. Testing day consisted of two stages, each 3 minutes in length. During the first stage, subjects were placed in the test box that contained two identical objects in two corners (15 cm away from each box wall, with both corners being shared by a single side). Following the 3-minute session, subjects were returned to their housing cages. One hour later, subjects were re-introduced to the box with one of the former objects replaced at the same previous corner location. Designations for novel-familiar object alternations were counterbalanced between subjects. The time subjects spent sniffing the object (deemed as exploratory behavior) was recorded and analyzed offline (Behaview software; www.pmbogusz.net).

2.5.8 Testing Timelines

Shown below are the different test cohorts and their associated behavioral assays, organized in a chronological manner and beginning from day 1 of testing or protocol exposure:

**Figure 5 Timeline of Various Test Cohorts.** Not scaled proportionately; an abstract overview.
2.6 Molecular Analyses

At the end of experimental tests prior to euthanasia, rats were given one last drug and odour administration (as previously described) based upon their respective treatment group designations during behavioral testing. Subjects were then injected intra-peritoneally with an overdose of sodium pentobarbital (240 mg/kg, i.p., Euthanyl™) and decapitated. Brains were extracted and then flash frozen at –80°C. Coronal sections (99 µm) of the brains containing the PFC, and NAcSh were sliced using a cryostat and mounted on glass slides; bilateral microdissections were then taken from the specified brain regions (NAcSh microdissections were taken from around the infusion site to avoid any regions with active gliosis). Tissue samples were homogenized using a Dounce homogenizer and proteins were then isolated using lysis buffer containing phosphatase and protease inhibitors. Protein quantification was conducted using the bicinchoninic acid (BCA) assay.

The western blotting procedure was conducted in the same manner as previously described by Lyons et al. (2013), with protein samples being denatured in Laemmli buffer and diluted to ensure all concentrations were equal and each well loaded with 25 µg of protein. Samples underwent SDS polyacrylamide gel electrophoresis (SDS-PAGE) at 125V for 1.5 hours in 10% acrylamide gels, followed by transference to nitrocellulose membranes using the Trans-Blot Turbo Transfer System (Bio-Rad) at 2.5A for 10 minutes. After blocking with 2.5% bovine serum albumin (BSA) in TBS-T for one hour, membranes were then placed in blocking solution containing the following primary antibodies with their respective host species and dilutions as follows: α-tubulin (mouse; 1:10,000; Sigma-Aldrich), phosphorylated GSK3α/β ser21/9 (p-GSK3α/β; rabbit; 1:1000; Cell Signaling Technology), total GSK3α/β (t-GSK3α/β; mouse; 1:250; Santa Cruz Biotechnology), phosphorylated AktSer473 (p-AktSer473; rabbit; 1:1000;
Cell Signaling Technology), phosphorylated AktThr308 (p-AktThr308; rabbit; 1:750; Cell
Signaling Technology), total Akt (t-Akt; mouse; 1:1000; Cell Signaling Technology),
phosphorylated ERK1/2 (p-ERK; rabbit; 1:1000; Cell Signalling Technology), total ERK1/2 (t-
ERK; mouse; 1:2000; Cell Signalling Technology), phosphorylated JNK (p-JNK; rabbit; 1:1000;
Cell Signalling Technology), total JNK (t-JNK; rabbit; 1:1000; Cell Signalling Technology).

Membranes were finally probed with species appropriate fluorophore-conjugated
secondary antibodies (LI-COR IRDye 680RD and IRDye 800CW; Thermo Scientific) at a
dilution of 1:10000. LI-COR Odyssey Infrared Imaging System and Image Studio analysis
software were then used to scan and obtain densitometry measurements respectively,
normalizing the intensity of each sample’s target protein band to its respective α-tubulin band
intensity.

2.7 Histology

Rat subjects were euthanized, with their brains extracted and sliced as previously
mentioned in section 2.6. Following tissue extraction procedures as described in section 2.6
regarding western blotting, slides with PFC and NAcSh coronal sections were then stained with
cresyl violet dye as detailed by Loureiro et al. (2015). Sections with visible cannula tip sites
viewed under a microscope were photographed and correlated with their respective locations as
according to the rat brain atlas by Paxinos and Watson (2005) for placement accuracy.

2.8 Statistical Analyses

All statistical analyses were performed using SPSS (IBM SPSS Statistical Package
Version 26). Preliminary analyses for normal distribution were conducted for both behavioral
and molecular data (normalized densitometry measurements) using the Kolmogorov-Smirnov
test, followed by either Two-way t-tests or One-way ANOVA analyses where appropriate; *post-hoc* analyses were conducted utilizing Fischer’s LSD test. All statistical significances were denoted by p < 0.05, with a 95% confidence interval.
3. Results: Behavioral Assays

3.1 Baseline Cohort Behavioral Results

3.1.1 Open Field Test

The open field test was conducted primarily to assess potential alterations in locomotor activity, and secondarily as an open-space anxiety measure (Figure 6). One-way ANOVA tests demonstrated that there were no significant differences in total distance travelled between treatment groups in the baseline cohort ($F_{(5, 57)} = 1.332, p = 0.264$; figure 6B) ultimately suggesting no significant differences in locomotor ability between treatment groups in their respective test analyses.

In summary, these baseline cohort results from the open field test demonstrated that no significant locomotor abnormalities resulted from treatment interventions in the cohort—effectively eliminating the potential of locomotor abnormalities confounding the results of all subsequent behavioral assays which inherently involve locomotion to some degree.
**Figure 6 Open Field Test, total locomotion: baseline treatment cohort.** Pictogram A represents a schematic of the open field test apparatus. B represents total locomotion exhibited by treatment groups in the baseline treatment cohort, with no significant differences present between groups. One-way ANOVA, \( p \leq 0.05 \). CBD dosages represent bilateral dosages in ng/\( \mu \)L; linalool dosages represent dosages in \( \mu \)L. Error bars represent standard error of means (SEMs). Error bars represent standard error of means (SEMs). Sample Sizes: \( V_{Ac} V_{PBS} \) (N=9), \( V_{Ac} \text{CBD} \) (N=9), \( L200-V_{PBS} \) (N=8), \( L2000-V_{PBS} \) (N=11), \( L200-\text{CBD} \) (N=10), \( L2000-\text{CBD} \) (N=10).
3.1.2 Light Dark Box Test

The light dark box test was used to assess bright-space anxiety exhibited by treatment groups. As shown in figure 7, when measuring total time spent in the light compartment, both L200-CBD and L2000-CBD presented significantly increased time spent exploring the light chamber compared to all other treatment groups (p ≤ 0.01 for all group comparisons except when comparing L200-CBD with L2000-CBD) in the baseline cohort (F(5, 56) = 6.752, p ≤ 0.001; figure 7B).

The secondary assessment of bright-space anxiety, the total number of transitions made by test subjects, also differed significantly between treatment groups in the baseline cohort (F(5, 56) = 5.775, p ≤ 0.001; figure 7C). Interestingly, while the L2000-CBD treatment group exhibited significantly higher number of transitions between compartments compared to all other groups (p ≤ 0.05) except L200-CBD (p = 0.074), the L200-CBD treatment group only exhibited significantly higher number of transitions when compared to the V_Air V_PBS and V_Air V_CBD groups (p ≤ 0.05). In addition, the L2000-V_CBD group presented significantly higher total number of compartmental transitions compared to the V_Air V_PBS group (p = 0.016).

These LDB test results strongly suggest the presence of an Entourage Effect (EE) between CBD and linalool which was supra-additive in its anxiolytic effects relative to sub-threshold doses of either alone, given that the L2000-CBD group exhibited significantly reduced bright-space anxiety compared to the V_Air V_PBS group, as well as its component vehicle groups V_Air CBD and L2000-V_PBS in both anxiety assessment measures (total time in light, total number of transitions). Results also suggest that this EE-potentiated bright-space anxiolysis is present in a lesser degree following L200-CBD treatment, as the L200-CBD group exhibited significantly reduced bright-space anxiety compared to the V_Air V_PBS group, V_Air CBD and L200-V_PBS groups.
in the primary anxiety measure (total time in light) but not the secondary anxiety measure (total number of transitions).
Figure 7 Light Dark Box Test, time spent in the light compartment and total compartmental transitions: baseline treatment cohort. A) Light Dark Box test schematic. Pictogram B represents the total time in the light compartment spent by groups in the baseline treatment cohort, with L200-CBD and L2000-CBD groups presenting significantly higher time in light than all other treatments. C represents the total number of compartmental transitions made by groups in the baseline treatment cohort; L2000-CBD group presented significantly higher transition counts than all other treatments except its lower dose L200-CBD counterpart. L200-CBD transition count was only significantly higher in comparison to VₐᵣVᵦₚ and Vᵦᵦ CBD treatment groups, while L2000-Vₐᵣ yielded significantly increased number of transitions when compared to the VₐᵣVᵦₚ group. One-way ANOVA, p ≤ 0.05. CBD dosages represent bilateral dosages in ng/μL; linalool dosages represent dosages in μL. *P ≤ 0.05, **P ≤ 0.01. Error bars represent standard error of means (SEMs). Sample Sizes: VₐᵣVᵦₚ (N=8), VᵦᵦCBD (N=9), L200-Vᵦₚ (N=10), L2000-Vᵦₚ (N=13), L200-CBD (N=10), L2000-CBD (N=12).
3.1.4 Social Motivation and Recognition Test

To assess sociability and social recognition memory as a pre-clinical model of social anxiety disorder, the social motivation and recognition test was employed with results as shown in figure 8. Sociability and social recognition memory were respectively quantified as social motivation index (SMI) and social recognition index (SRI) values. SMI values were expressed as the normalized absolute difference of time spent sniffing the two cages in stage 2 (social motivation), while SRI values were expressed as the normalized absolute difference of time spent sniffing the two cages in stage 3 (social recognition).

Ultimately, significant differences in stage 2 social motivation index and stage 3 social recognition index values were not found between groups in the overall ANOVA analysis of the baseline cohort (stages 2 and 3 respectively: $F(5, 44) = 1.127, p = 0.360$ [Figure 8B]; $F(5, 44) = 0.714, p = 0.616$ [Figure 8C]).

Taken together, these SMI and SRI findings of non-significance in the baseline cohort suggest respectively that there are no significant differences in social novelty preference and social recognition memory between treatment groups—and consequently that the combination treatments of linalool and cannabidiol do not elicit an EE-potentiated change in social cognition, at least in the baseline cohort. This result is not particularly surprising however as previous studies in our lab have demonstrated that social anxiety induced cognitive alterations generally only occur following chronic stress exposure or other neurodevelopmental toxic insults (e.g. Renard et al., 2017).
Figure 8 Social Motivation and Social Recognition Test, SMI and SRI: baseline treatment cohort. Pictograms A represents a schematic of the Social Motivation and Social Recognition Test. B represents the baseline treatment cohort in the assessment of SMI (social motivation index) values exhibited by different treatment groups; Pictogram C represents the baseline treatment cohort respectively in the context of SRI value assessment, with no significant differences present between groups in the baseline treatment cohort. One-way ANOVA, p ≤ 0.05. CBD dosages represent bilateral dosages in ng/μL; linalool dosages represent dosages in μL. Error bars represent standard error of means (SEMs). Sample Sizes: V_Air PBS (N=10), V_Air CBD (N=7), L200-VPBS (N=9), L2000-VPBS (N=8), L200-CBD (N=8), L2000-CBD (N=8).
3.1.3 Elevated Plus Maze Test

The open space anxiety of test subjects was assessed using the Elevated Plus Maze behavioral assay. Regarding the time spent in open arms which represents the primary measure of open space anxiety in this test, as shown in figure 9, one-way ANOVA analysis revealed no significant differences between groups in the baseline cohort ($F(5, 53) = 1.690, p = 0.153$; figure 9B).

Within the secondary open space anxiety measure of total arm transitions, no significant differences in transition counts between open and closed arms were discovered between groups in the baseline cohort ($F(5, 53) = 0.386, p = 0.856$; figure 9C).

Taking into account the findings of non-significance in both the EPM assessments of total in open arms and total arm transitions in the baseline cohort, this suggests that there were no differences between groups in the perception of open-space anxiety in the more sensitive EPM apparatus (as compared to the OFT apparatus); consequently, these findings present the lack of EE-potentiated open-space anxiety within the context of the EPM test paradigm following combination treatments of linalool and cannabidiol.
**Figure 9 Elevated Plus Maze Test, open arm exploration time and total transitions between open and closed arms:**

**Baseline treatment cohort.** Pictograms A represents a schematic of the Elevated Plus Maze test. B) Baseline treatment cohort in the assessment of open arm exploration time, with no significant differences present between groups. Pictograms C represents the baseline treatment cohort in the assessment of total arm transitions, with a similar trend to open arm exploration time assessment and ultimately no significant differences present between groups. One-way ANOVA, p ≤ 0.05. CBD dosages represent bilateral dosages in ng/μL; linalool dosages represent dosages in μL. Error bars represent standard error of means (SEMs). Sample Sizes: Vₐₐₐₐنبي (N=9), VₐₐₐₐCBD (N=8), L200-Vنبي (N=10), L2000-Vنبي (N=12), L200-CBD (N=10), L2000-CBD (N=10).
3.1.5 Context-Dependent Fear Conditioning

The context-dependent fear conditioning (CFC) behavioral assay assessed context-dependent anxiety observed through freezing time presentation. As demonstrated in figure 10, in the context of the CFC-Acquisition (as previously described), significant differences in freezing time were found in the baseline cohort (\(F_{(5, 47)} = 3.195, p = 0.015\); figure 10B). The L200-CBD group exhibited significantly lower freezing time when compared to solely the \(V_{Air}V_{PBS}\) and L200-\(V_{PBS}\) groups (\(p \leq 0.05\)); the L2000-CBD group however demonstrated significantly lower freezing time compared to all treatment groups (\(p \leq 0.05\) except its lower dose L200-CBD counterpart (\(p = 0.668\)).

Significant differences were also found between groups in the baseline cohort when assessing CFC-Recall (\(F_{(3, 34)} = 6.360, p = 0.002\); figure 10C). The L2000-CBD group was found to exhibit significantly lower freezing time compared to all other groups (\(p \leq 0.05\)).

In summary, these CFC assay results demonstrate that the L2000-CBD treatment was able to significantly reduce the context-dependent anxiety induced by the CFC apparatus in both the CFC-Acquisition and CFC-Recall challenges. This in turn suggests that the EE-potentiating anxiolysis induced by L2000-CBD in this behavioral assay is effective in attenuating both fear memory formation and recall, as shown respectively by the results from the CFC-Acquisition and CFC-Recall challenges, strongly suggesting a strong entourage effect for the combinatorial effects of CBD and linalool, relative to either compound alone.
Figure 10: Context-Dependent Fear Conditioning—Acquisition and Recall: baseline treatment cohort. 

Panel A represents the CFC behavioral assay schematic. Panel B represents the baseline treatment cohort in the CFC-Acquisition challenge; the L200-CBD group exhibited significantly decreased freezing time compared to the V₃₀₀-PBS and L2000-PBS groups, while the L2000-CBD group demonstrated significantly lower freezing time than all other groups except its lower dose L200-CBD counterpart. Panel C represents the baseline treatment cohort in the CFC-Recall challenge; the L2000-CBD group presented significantly lower freezing time compared to all other groups. One-way ANOVA, p ≤ 0.05. CBD dosages represent bilateral dosages in ng/µL; linalool dosages represent dosages in µL. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001. Error bars represent standard error of means (SEMs). Sample Sizes: [Acquisition] V₃₀₀-PBS (N=9), V₃₀₀-CBD (N=8), L200-PBS (N=8), L2000-PBS (N=10), L200-CBD (N=9), L2000-CBD (N=9); [Recall] V₃₀₀-PBS (N=9), V₃₀₀-CBD (N=10), L200-PBS (N=9), L2000-PBS (N=9), L2000-CBD (N=10).
3.2 Flumazenil Challenge Behavioral Results

In light of the results from the baseline cohort suggesting the presence of an EE-potentiated anxiolysis following L2000-CBD treatment, the flumazenil challenge—involving groups that received intra-cranial flumazenil alongside L2000-CBD treatment—was conducted to determine whether this EE-potentiation involved GABA\(_{A}\)R activity modulation, given that previous studies have shown that linalool and CBD may generate anxiolysis via GABA\(_{A}\)R mechanisms of action (Bakas et al., 2017; Harada et al., 2018; Lopes et al., 2012; Milanos et al., 2017).

3.2.1 Open Field Test

The OFT was used to identify potential alterations in locomotor activity and secondarily as an open-space anxiety measure in the flumazenil cohort (Figure 11). One-way ANOVA tests demonstrated that there were no significant differences in total distance travelled between treatment groups in the flumazenil challenge cohort (\(F(3, 26) = 0.339, p = 0.798; \text{figure 11A}\)), ultimately suggesting no significant differences in locomotor ability between treatment groups.

When assessing thigmotaxis anxiety measures of center-to-total locomotion ratios (expressed as a percentage), ANOVA analysis revealed significant differences between treatment groups (flumazenil: \(F(3, 32) = 10.06, p \leq 0.01; \text{figure 11B}\)); post hoc analyses showed that the L2000-CBD treatment group exhibited significantly higher center/total locomotion (%) compared to all other treatment groups (\(p \leq 0.05\)).

These flumazenil challenge OFT results demonstrated that no significant locomotor abnormalities resulted from treatment interventions in the cohort, removing concerns of locomotor abnormalities confounding interpretations of results from subsequent behavioral assays which all involve locomotion. Furthermore, the center/total locomotion (%) results
suggest that intra-cranial flumazenil administration is effective in removing the EE-potentiated open-space anxiolysis exhibited by L2000-CBD group, as demonstrated by the significantly reduced center/total locomotion (%) value following co-administration of L2000-CBD and flumazenil.

A
OFT (Flumazenil Challenge): Total Locomotion

B
OFT (Flumazenil Challenge): Center/Total Locomotion (%)

Figure 11 Open Field Test, total locomotion and center/total locomotion (%): flumazenil challenge cohort. Pictogram A demonstrates total locomotion exhibited by treatment groups in the flumazenil challenge cohort, with no significant differences present between groups. Pictogram B however, representing center/total locomotion (%) in the flumazenil challenge cohort, presents significant differences between groups with the L2000-CBD group exhibiting significantly higher center/locomotion (%) values than all other groups. One-way ANOVA, $p \leq 0.05$. CBD dosages represent bilateral dosages in ng/µL; linalool dosages represent dosages in µL. ***$P \leq 0.001$, Error bars represent standard error of means (SEMs). Error bars represent standard error of means (SEMs). Sample Sizes: $V_{12}V_{755}$ (N=7), $V_{12}V_{755}$+Flumazenil (N=8), L2000-CBD (N=10), L2000-CBD+Flumazenil (N=8).
3.2.2 Light Dark Box Test

The LDB test was used to assess bright-space anxiety exhibited by treatment groups using the same primary and secondary anxiety measures assessed in the baseline cohort (total time in light compartment, total compartmental transitions). Within the flumazenil challenge cohort, significant differences in total time spent in the light compartment between groups was discovered through one-way ANOVA analysis ($F_{(3, 31)} = 18.89$, $p \leq 0.001$; figure 12A), with the L2000-CBD group presenting significantly greater time in the light compartment than all other treatment groups ($p \leq 0.001$).

Furthermore, one-way ANOVA analysis revealed significant differences in terms of total transition counts between treatment groups in the flumazenil challenge cohort ($F_{(3, 31)} = 18.19$, $p \leq 0.001$; figure 12B). Specifically, post hoc analyses revealed the number of total transitions made by the L2000-CBD to be significantly higher than all other treatment groups ($p \leq 0.001$).

These flumazenil challenge LDB test results demonstrated that intra-cranial flumazenil administration was able to effectively eliminate the EE-potentiated anxiolysis elicited following L2000-CBD treatment, as shown with the significant reduction in both total time in the light compartment and total compartmental transitions exhibited by the co-treated L2000-CBD+flumazenil group.
Figure 12 Light Dark Box Test, total time in light compartment and total compartmental transitions: flumazenil challenge cohort. Pictogram A represents the total time in the light compartment spent by groups in the flumazenil challenge cohort; the L2000-CBD group presenting significantly higher time in light than all other treatments. B represents the total number of compartmental transitions made by groups in the flumazenil challenge cohort, the L2000-CBD group presented significantly higher compartmental transition counts than all other treatments. One-way ANOVA, \( p \leq 0.05 \). CBD dosages represent bilateral dosages in ng/µL; linalool dosages represent dosages in µL. **\( p \leq 0.001 \). Error bars represent standard error of means (SEMs). Sample Sizes: \( V_{ab}V_{PBS} \) (N=8), \( V_{ab}V_{PBS}+\text{Flumazenil} \) (N=8), \( L2000-\text{CBD} \) (N=12), \( L2000-\text{CBD}+\text{Flumazenil} \) (N=7).
3.2.3 Context-Dependent Fear Conditioning (Recall)

Utilizing the same CFC test apparatus employed in baseline cohort testing, context-dependent anxiety (specifically with treatment given immediately prior to stage 2 testing) observed through freezing time presentation was assessed in the flumazenil challenge cohort. As shown through one-way ANOVA and subsequent post-hoc analyses in **figure 13**, the L2000-CBD group also presented significantly lower freezing time compared to all other treatment groups ($F_{(3, 32)} = 19.283, p \leq 0.001$).

Conversely, when taking account the non-significance in freezing time between the L2000-CBD+flumazenil group and the two vehicle groups (V\text{Air} V\text{PBS}, V\text{Air}+Flumazenil) as well as the significantly higher freezing time presented by the L2000-CBD+flumazenil group when compared to the flumazenil-free L2000-CBD group ($p \leq 0.05$), these results suggest that intracranial flumazenil administration is able to prevent the presentation of EE-potentiated fear-memory anxiolysis induced by L2000-CBD treatment in the CFC-Recall challenge condition.

![CFC-Recall Challenge](image)

**Figure 13 Context-Dependent Fear Conditioning—Recall: flumazenil challenge cohort.** This figure represents the flumazenil challenge cohort in the CFC-Recall challenge; the L2000-CBD group exhibited significantly decreased freezing time compared to all other treatment groups. One-way ANOVA, $p \leq 0.05$. CBD dosages represent bilateral dosages in ng/μL; linalool dosages represent dosages in μL. $***p \leq 0.001$. Error bars represent standard error of means (SEM). Sample Sizes: [Recall] $V\text{Air} V\text{PBS}(N=9), V\text{Air} V\text{PBS}+Flumazenil(N=8), L2000-CBD(N=10), L2000-CBD+Flumazenil(N=9)$.
3.3 EPA Challenge Behavioral Results

Given that baseline cohorts indicated the presence of EE-potentiated anxiolysis following L2000-CBD treatment, the EPA challenge—involving groups that received intra-cranial EPA (ERK activator) alongside L2000-CB treatment—was carried out to determine whether this EE-potentiation involved ERK activity modulation, as previous studies have suggested that linalool and CBD may yield anxiolysis through regulation of pERK activity (Caputo et al., 2016; 2017; 2018; Hudson et al., 2019).

3.3.1 Open Field Test

Employing the OFT assay used in the flumazenil challenge cohort, total distance travelled and thigmotaxis activity of treatment groups were assessed to respectively determine the presence of any differences in locomotion and open-space anxiety between treatment groups in the EPA challenge cohort.

OFT results from the EPA challenge cohort demonstrated no significant differences between treatment groups in the assessment of total locomotion (F(3, 34) = 0.993, p = 0.409; figure 14A); however, significant differences between groups were found when assessing center/total locomotion (%), with the L2000-CBD treatment exhibiting significantly increased center/total locomotion (%) values (p ≤ 0.05) in comparison to all other groups (F(3, 34) = 10.688, p ≤ 0.01; figure 14B).

These OFT findings presented by the EPA challenge cohort demonstrate that no significant locomotor abnormalities resulted from treatment interventions in the cohort, indicating that there were no locomotor abnormalities present that would potentially confound interpretations of results from subsequent behavioral assays involving locomotion. In addition,
the center/total locomotion (%) results suggest that intra-cranial EPA administration is effective in removing the EE-potentiated open-space anxiolysis exhibited by L2000-CBD group, as shown by the significantly reduced center/total locomotion (%) value following co-administration of L2000-CBD and flumazenil in conjunction with the non-significance between the L2000-CBD+flumazenil and the two vehicle groups (VAirVPBS, VAir+EPA).

Figure 14 Open Field Test, total locomotion and center/total locomotion (%): EPA challenge cohort. Pictogram A represents total locomotion exhibited by treatment groups in the EPA challenge cohort, with no significant differences present between treatment groups. Pictogram B represents center/total locomotion (%) in the flumazenil challenge cohort; significant differences were found present between groups, with the L2000-CBD group exhibiting significantly higher center/locomotion (%) values than all other groups. One-way ANOVA, p ≤ 0.05. CBD dosages represent bilateral dosages in ng/μL; linalool dosages represent dosages in μL. ***P ≤ 0.001. Error bars represent standard error of means (SEMs). Error bars represent standard error of means (SEMs). Sample Sizes: VAirVPBS (N=7), VAirVPBS+EPA (N=9), L2000-CBD (N=10), L2000-CBD+EPA (N=9).
3.3.2 Light Dark Box Test

Utilizing the LDB test in the same manner as in the baseline and flumazenil challenge cohorts, bright-space anxiety exhibited by EPA challenge treatment groups was assessed through the primary measure of total time in the light compartment and secondarily through the measure of total compartmental transitions.

Within the EPA challenge cohort, one-way ANOVA analysis revealed significant differences in total time spent in the light compartment between treatment groups ($F_{(3, 35)} = 7.644, p = 0.001$; **figure 15A**), with post hoc analyses demonstrating that the L2000-CBD group presented significantly greater time spent in the light compartment compared to all other groups ($p \leq 0.05$). A similar trend was observed in regard to total compartmental transitions, with one-way ANOVA analysis demonstrating significant differences between treatment groups ($F_{(3, 35)} = 11.921, p \leq 0.01$; **figure 15B**) and post hoc analyses showing that the L2000-CBD group exhibited a significantly increased amount of compartmental transitions compared to all other groups ($p \leq 0.05$).

The significantly decreased total time spent in the light compartment and compartmental transitions exhibited by the L2000-CBD+EPA treatment group when compared to the EPA-free L2000-CBD group, concurrent with non-significance when compared to the $V_{\text{Air}}V_{\text{PBS}}$ and $V_{\text{Air}}+\text{EPA}$ vehicle groups suggests that intra-cranial EPA administration is capable of preventing the EE-potentiated bright-space anxiolysis induced by L2000-CBD treatment.
Figure 15 Light Dark Box Test, total time in light compartment and total compartmental transitions: EPA challenge cohort. Pictogram A depicts the total time in the light compartment spent by treatment groups in the EPA challenge cohort; the L2000-CBD group presented significantly higher time in light than all other treatments. Pictogram B represents the total number of compartmental transitions made by groups in the EPA challenge cohort, with the L2000-CBD group presenting significantly higher compartmental transition counts than all other treatments. One-way ANOVA, p ≤ 0.05. CBD dosages represent bilateral dosages in ng/µL; linalool dosages represent dosages in µL. **P ≤ 0.01, ***P ≤ 0.001. Error bars represent standard error of means (SEMs). Sample Sizes: V_A, V_PBS (N=8), V_A, V_PBS+EQP (N=8), L2000-CBD (N=12), L2000-CBD+EQP (N=8).
3.3.3 Context-Dependent Fear Conditioning (Acquisition)

Employing the same CFC test apparatus used in previous cohort testing, context-dependent anxiety—specifically with treatment given prior to the stage 1 conditioning phase—was observed through freezing time presentation was assessed in the EPA challenge cohort.

As demonstrated in figure 16, one-way ANOVA analysis revealed significant differences between treatment groups in the assessment of freezing time ($F_{(3, 34)} = 5.548, p = 0.004$; figure 16); post hoc analyses further showed that the L2000-CBD treatment group exhibited significantly greater time spent frozen when compared to all other groups ($p \leq 0.05$).

Notably, given that the L2000-CBD+EPA treatment group exhibited non-significant differences in freezing time when compared to vehicle groups ($V_{\text{Air}}, V_{\text{PBS}}, V_{\text{Air}+\text{EPA}}$) and that it presented significantly increased freezing time in comparison to its EPA-free L2000-CBD treatment counterpart, these results suggest that intra-cranial EPA administration is effective in preventing the presentation of EE-potentiated fear-memory anxiolysis elicited by L2000-CBD treatment in the CFC-Acquisition challenge condition.
Figure 16 Context-Dependent Fear Conditioning—Acquisition: EPA challenge cohort. This figure represents the EPA challenge cohort in the CFC-Acquisition challenge; the L2000-CBD group exhibited significantly decreased freezing time compared to all other treatment groups. One-way ANOVA, p ≤ 0.05. CBD dosages represent bilateral dosages in ng/μL; linalool dosages represent dosages in μL. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001. Error bars represent standard error of means (SEMs). Sample Sizes: V_{Ac}V_{PBS} (N=9), V_{Ac}V_{PBS+EPA} (N=8), L2000-CBD (N=9), L2000-CBD+EPA (N=9).
3.4 Chronic Stress Behavioral Results

The previous studies explored the ability of CBD + linalool combinations to produce acute anxiolytic effects in otherwise healthy rat cohorts. However, given that most chronic anxiety disorders are experienced following prolonged exposure to environmental stressors, we next explored the potential effects of CBD + linalool combinations in mitigating anxiogenic effects induced by chronic stress exposure (see methods). Following the findings from the baseline cohort suggesting that L2000-CBD treatment is capable of eliciting an EE-potentiated anxiolysis in various contexts, chronic stress models were generated using the CUS protocol employed in this study and incorporated into a chronic stress cohort to determine whether L2000-CBD treatment was capable of reversing alterations induced by chronic stress—and secondarily if such a reversal was potent enough to induce the originally observed Entourage Effect seen in the baseline cohort.

3.4.1 Open Field Test

Utilizing the OFT assay employed in previous cohorts, the locomotion and center/total locomotion (%) of chronic stress cohort treatment groups were assessed to respectively determine if any significant differences in locomotor or open-space anxiety were present between groups.

One-way ANOVA analysis of total locomotion ($F_{(3, 29)} = 0.833, p = 0.487$; figure 17A) and center/total locomotion (%) ($F_{(3, 32)} = 1.115, p = 0.359$; figure 17B) revealed no significant differences between treatment groups in the chronic stress cohort. In addition, a pre-planned two-tailed t-test comparison between the $V_{Air}$-$V_{PBS}$ and CUS-$V_{Air}$-$V_{PBS}$ treatment groups revealed no significant differences in center/total locomotion (%) ($t_{(14)} = 1.022, p = 0.325$; figure 17B—not visualized due to insignificance).
Collectively, these results indicate that there were no significant locomotor differences between groups that could confound the interpretation of results from subsequent behavioral assays involving an element of locomotion. Furthermore, these findings suggest that the chronic stress resulting from exposure to the 2-week CUS protocol employed in this study is not effective in achieving significant stress-induced open-space anxiety in the OFT paradigm (as shown by the non-significance between the $V_{Air/V_{PBS}}$ and $V_{Air/V_{PBS}}$ treatment groups).

**Figure 17** Open Field Test, total locomotion and center/total locomotion (%): Chronic stress cohort. Pictogram A represents total locomotion exhibited by treatment groups in the chronic stress cohort, with no significant differences present between treatment groups. Pictogram B represents center/total locomotion (%) in the chronic stress cohort; significant differences were not found present between treatment groups. One-way ANOVA, $p \leq 0.05$; two-tailed t-test, $p \leq 0.05$. CBD dosages represent bilateral dosages in ng/µL; linalool dosages represent dosages in µL. Error bars represent standard error of means (SEMs). Error bars represent standard error of means (SEMs). Sample Sizes: $V_{Air/V_{PBS}}$ (N=8), $V_{Air/V_{PBS}}$ (N=8), L2000-CBD (N=9), CUS-L2000-CBD (N=8).
3.4.2 Spontaneous Alternation

The spontaneous alternation (SA) behavioral paradigm was used to assess potential working memory deficiencies between treatment groups in the chronic stress condition, with results as shown in figure 18B. When assessing normalized alternation counts (expressed as a percentage value in relation to total entry counts), no significant differences were found between groups ($F_{(3, 29)} = 1.986, p = 0.138$; figure 18B). A pre-planned two-tailed t-test comparison between the $V_{Air}V_{PBS}$ and CUS-$V_{Air}V_{PBS}$ treatment groups presented no significant differences ($t_{(14)} = 1.627, p = 0.126$; figure 18B—comparison not visualized due to non-significance).

The insignificance in normalized alternation counts (%) found between groups in the SA test ultimately suggests that there is lack of comparable memory deficits between said treatment groups. Furthermore, pre-planned comparison results between $V_{Air}V_{PBS}$ and CUS-$V_{Air}V_{PBS}$ treatment groups suggests that the 2-week CUS protocol used in this study was not sufficient in generating a chronic stress-induced model of cognitive impairment. Nevertheless, an important implication from these results is that the combination of CBD + linalool (at doses which are highly anxiolytic), did not produce any cognitive impairments in and of themselves, relative to VEH controls, suggesting that the potential therapeutic properties of CBD + linalool are free of negative cognitive side effects, which, as previously discussed, are serious clinical limitations to most commonly used anxiolytic medications.
Figure 18 Spontaneous Alternation, normalized alternation counts (%). Pictogram A represents a schematic of the spontaneous alternation test. B) Assessment of normalized alternation counts in the chronic stress cohort; no significant differences were present between treatment groups. One-way ANOVA, p \( \leq 0.05 \). CBD dosages represent bilateral dosages in ng/μL; linalool dosages represent dosages in μL. Error bars represent standard error of means (SEMs). Samples Sizes: V₅₄-V₅₈S (N=8), CUS-V₅₄-V₅₈S (N=8), L2000-CBD (N=9), CUS-L2000-CBD (N=8).
3.4.3 Novel Object Recognition

Long-term memory capacity was assessed through observations of object discrimination by test subjects from the chronic stress cohort in the novel object recognition assay.

Significant differences in the novelty preference score, which measured novelty preference in relation to overall exploration time of assigned objects, were not found between treatment groups in the chronic stress cohort following one-way ANOVA analysis ($F_{(3, 26)} = 0.693, p = 0.565$; figure 19B). Furthermore, pre-planned two-tailed test comparisons between the $V_{Air}V_{PBS}$ and CUS-$V_{Air}V_{PBS}$ treatment groups presented no significant differences ($t_{(13)} = 0.489, p = 0.647$; figure 19B—comparison not visualized due to non-significance).

This lack of significant differences observed in the NOR paradigm suggests that there were no relative deficiencies in long-term memory cognition between treatment groups in the chronic stress cohort. In addition, lack of significant differences between the $V_{Air}V_{PBS}$ and CUS-$V_{Air}V_{PBS}$ treatment groups following pre-planned comparison analysis suggests that the 2-week CUS protocol used in this study was not sufficient in generating a chronic stress-induced model of cognitive impairment. Again, similar to results observed with spontaneous alternation, these findings suggest that the potential therapeutic properties of CBD + linalool for anxiolysis are free of negative cognitive side effects.
Figure 19 Novel Object Recognition, novelty preference score (%). Pictogram A represents a schematic of the novel object recognition test. B) Depiction of the novelty preference scores exhibited by treatment groups in the chronic stress cohort; no significant differences were found present between groups. One-way ANOVA, \( p \leq 0.05 \). CBD dosages represent bilateral dosages in ng/μL; linalool dosages represent dosages in μL. Error bars represent standard error of means (SEMs). Samples Sizes: \( V_{AdV_{PBS}} \) (N=8), CUS-\( V_{AdV_{PBS}} \) (N=7), L2000-CBD (N=9), CUS-L2000-CBD (N=6).
3.4.4 Light Dark Box Test

The LDB assay was used to assess for differences in bright-space anxiety between treatment groups within the chronic stress cohort, utilizing the same primary measure of total time spent in the light compartment and secondary measure of total compartmental transitions employed in previous cohorts.

One-way ANOVA and subsequent post hoc analysis of chronic stress cohort data revealed significant differences between treatment groups, with both the CUS-L2000-CBD and non-stressed L2000-CBD treatment groups exhibiting significantly increased exploration time in the light compartment (p ≤ 0.05) compared to V\text{Air}V\text{PBS} and CUS-V\text{Air}V\text{PBS} (F(3, 27) = 5.824, p ≤ 0.003; \textbf{figure 20A}). In addition, a pre-planned comparison (significance denoted in green) between the V\text{Air}V\text{PBS} and CUS-V\text{Air}V\text{PBS} treatment groups used to assess the validity of the chronic stress model in the context of bright-space anxiety demonstrated significant differences between the two groups, with the V\text{Air}V\text{PBS} group found to spend significantly greater time in light than its CUS-V\text{Air}V\text{PBS} counterpart (t(13) = 2.431, p = 0.030).

In regard to the secondary measure of bright-space anxiety, the number of total transitions made by treatment groups differed significantly (F(3, 27) = 4.218, p ≤ 0.014; \textbf{figure 20B}), with the CUS-L2000-CBD group presenting significantly greater number of total transitions than the CUS-exposed and non-stressed V\text{Air}V\text{PBS} groups (p ≤ 0.05), while the non-stressed L2000-CBD group presented only significantly higher total transition counts when compared to the CUS-V\text{Air}V\text{PBS} treatment group (p ≤ 0.05).

Taken together, the significant reduction in anxiety symptoms presented by the CUS-L2000-CBD group when compared to the CUS-V\text{Air}V\text{PBS} group suggests that L2000-CBD administration is effective in attenuating bright-space anxiety induced by chronic stress.
Furthermore, the non-significance between L2000-CBD and CUS-L2000-CBD groups and significant difference between $V_{\text{Air}} V_{\text{PBS}}$ and CUS-L2000-CBD groups suggests that the EE-potentiated bright-space anxiolysis observed in the baseline cohort is also achieved under chronic stress conditions.

**Figure 29 Light Dark Box Test, total time in light compartment and total compartmental transitions: chronic stress cohort.** Pictogram A: Total time in the light compartment spent by treatment groups in the chronic stress cohort. L2000-CBD and CUS-L2000-CBD groups spent significantly higher time in light than all other groups; pre-planned comparison (significance denotation in green) between $V_{\text{Air}} V_{\text{PBS}}$ and CUS-$V_{\text{Air}} V_{\text{PBS}}$ groups revealed the latter to exhibit significantly less time in the light compartment. B) Total number of compartmental transitions made by chronic stress cohort groups. The CUS-L2000-CBD group presented significantly higher transition counts than all other groups (except L2000-CBD); the L2000-CBD group displayed significantly higher transition counts compared to the CUS-$V_{\text{Air}} V_{\text{PBS}}$ group. One-way ANOVA, $p \leq 0.05$. CBD dosages represent bilateral dosages in ng/µL; linalool dosages represent dosages in µL. *$p \leq 0.05$, **$p \leq 0.01$. Error bars represent standard error of means (SEMs). Sample Sizes: $V_{\text{Air}} V_{\text{PBS}}$ (N=8), CUS-$V_{\text{Air}} V_{\text{PBS}}$ (N=7), L2000-CBD (N=8), CUS-L2000-CBD (N=8).
3.4.5 Social Motivation and Recognition Test

Utilizing the same SMRT assay employed in the baseline cohort, the sociability and social recognition memory of treatment groups in the chronic stress cohort were respectively assessed through the previously used measures of SMI and SRI.

While one-way ANOVA analysis revealed no significant differences between treatment groups in stage 2 SMI values ($F(3, 26) = 1.631, p = 0.206$; figure 21A), a pre-planned two-tailed t-test comparison conducted within the chronic stress cohort between the $V_{Air} V_{PBS}$ and CUS-$V_{Air} V_{PBS}$ groups revealed the CUS-treated model to elicit significantly lower stage 2 SMI values ($t(12) = 2.441, p = 0.031$; figure 21A—significance comparison shown in green).

In regard to stage 3 of the SMRT assay, treatment groups in the chronic stress cohort demonstrated significantly different SRI values ($F(3, 26) = 5.615, p = 0.004$; figure 21B). Post hoc analyses revealed that the SRI value exhibited by the stressed $V_{Air} V_{PBS}$ group was significantly lower when compared to all other treatment groups ($p \leq 0.05$).

In summary, these SMRT assay results from the chronic stress cohort suggest that the CUS protocol employed in this study was effective in generating chronic stress-induced social cognition impairments, as exemplified by the pre-planned comparison in figure 21A and the one-way ANOVA analysis in figure 21B. Furthermore, stage 3 SRI value analysis suggests that L2000-CBD treatment was effective in reversing the social recognition memory deficits induced by chronic stress exposure, as demonstrated by the significantly higher SRI values of the CUS-L2000-CBD group compared to the CUS-$V_{Air} V_{PBS}$ group.
Figure 21 Social Motivation and Recognition Test, SMI and SRI: chronic stress cohort. Pictograms A represents the chronic stress cohort in the assessment of SMI (social motivation index) values exhibited by different treatment groups; while a pre-planned t-test comparison between $V_{Ar\ V_{PBS}}$ and CUS-$V_{Ar\ V_{PBS}}$ revealed the CUS-$V_{Ar\ V_{PBS}}$ group to exhibit a significantly lower SMI value (significance comparison denoted in green), no significant differences were detected in the general ANOVA analysis of the chronic stress cohort. Pictogram B represents the chronic stress cohort in the context of SRI value assessment; significant differences were found present between groups, with the CUS-$V_{Ar\ V_{PBS}}$ group exhibiting a significantly lower SRI value compared to all other groups. One-way ANOVA, $p < 0.05$; two-tailed t-test, $p < 0.05$. CBD dosages represent bilateral dosages in ng/µL; linalool dosages represent dosages in µL. *$P < 0.05$. Error bars represent standard error of means (SEMs). Sample Sizes: $V_{Ar\ V_{PBS}}$ (N=7), CUS-$V_{Ar\ V_{PBS}}$ (N=7), non-stressed L2000-CBD (N=8), CUS-L2000-CBD (N=8).
3.4.6 Elevated Plus Maze

The EPM assay was utilized in the chronic stress cohort in the same manner as done previously with the baseline cohort, assessing open-space anxiety through the primary measure of time spent in open arms and the secondary measure of total arm transitions.

One-way ANOVA analysis revealed significant differences in open arm exploration time between treatment groups in the chronic stress cohort ($F_{(3, 28)} = 3.391, p = 0.032$; figure 22A), with subsequent post hoc analysis showing that the L2000-CBD and CUS-L2000-CBD groups exhibited significantly greater open arm exploration time in comparison to the CUS-$V_{Air}V_{PBS}$ group ($p \leq 0.05$). A pre-planned comparison conducted between $V_{Air}V_{PBS}$ and CUS-$V_{Air}V_{PBS}$ groups assessing open arm exploration time (in an attempt to investigate the ability of the CUS protocol to generate chronic-stress induced open-space anxiety) yielded no significant differences ($t_{(14)} = 1.872, p \leq 0.082$; figure 22A—significance comparison not visualized due to non-significance). Nevertheless, there were strong trends towards the CUS inducing significant anxiogenic effects relative to non-stressed controls.

With regards to the secondary measure of open-space anxiety in the EPM assay, one-way ANOVA analysis demonstrated significant differences in arm transition counts between treatment groups in the chronic stress cohort ($F_{(3, 28)} = 3.433, p = 0.030$; figure 22B). Post hoc analyses specifically revealed a significant increase in arm transitions committed by both the L2000-CBD and CUS-L2000-CBD groups when compared to the CUS-$V_{Air}V_{PBS}$ group ($p \leq 0.05$).

These EPM assay results from assessment of the chronic stress cohort ultimately suggest that while the CUS protocol was ineffective in inducing significantly increased open-space anxiety in the EPM behavioral paradigm, L2000-CBD treatment was nonetheless effective in
reversing moderate open-space anxiety induced by chronic stress.

**Figure 22 Elevated Plus Maze Test, open arm exploration time and total transitions between open and closed arms:**

c**hronic stress cohort.** Pictograms A and B represent the chronic stress cohort in the assessment of open arm exploration time and total arm transitions respectively; in regard to these assessments, the L2000-CBD and CUS-L2000-CBD groups were found to exhibit both greater exploration time in open arms and increased total arm transition counts when compared to the CUS-VaVpS group. One-way ANOVA, \( p \leq 0.05 \). CBD dosages represent bilateral dosages in ng/µL; linalool dosages represent dosages in µL. *\( p \leq 0.05 \), **\( p \leq 0.01 \). Error bars represent standard error of means (SEM). Sample Sizes: VaVpS (N=8), CUS-VaVpS (N=8), L2000-CBD (N=8), CUS-L2000-CBD (N=8).
4. Results: Molecular Assays (Baseline Cohort)

Within the context of the baseline cohort, the molecular biomarkers previously described in the introduction (GSK-3β, Akt, ERK and JNK) were quantified to in an attempt to elucidate potential mechanisms of action correlating with various degrees of anxiolytic efficiency displayed in behavioral assays. Two sets of ANOVA analyses were conducted for each protein quantified, with one focused on comparing linalool vehicle groups to the double-control group \((V_{Air}V_{PBS})\) within the NAcSh region (referred to as the “control analysis”), while the other focused on comparing both L200-CBD and L2000-CBD to \(V_{Air}V_{PBS}\) and \(V_{Air}CBD\) in both the NAcSh and PFC regions (referred to as the “experimental analysis”). Both analyses expressed levels of protein in comparison to the control group \((V_{Air}V_{PBS})\) as percentage values.
4.1 GSK-3β Protein Analysis

As demonstrated in figure 23, one-way ANOVA analysis revealed no significant differences in the control analysis (p-GSK-3β: $F_{(2, 10)} = 2.173$, $p = 0.1646$ [figure 23A]; T-GSK-3β: $F_{(2, 10)} = 0.9494$, $p = 0.4193$ [figure 23B]; p/T-GSK-3β: $F_{(2, 11)} = 0.274$, $p = 0.7654$ [figure 23C]). Within the experimental analysis, significant differences were found in the NAcSh region when assessing p/T-GSK-3β values ($F_{(3, 20)} = 4.526$, $p = 0.0141$; figure 23F); analysis of differences between groups in p-GSK-3β ($F_{(3, 20)} = 1.956$, $p = 0.1531$; figure 23D) and T-GSK-3β ($F_{(3, 20)} = 1.339$, $p = 0.29$; figure 23E) demonstrated non-significance. Post hoc analysis of p/T-GSK-3β values revealed that the $V_{Air}$CBD group exhibited significantly increased levels in comparison to both $V_{Air}V_{PBS}$ and L2000-CBD groups ($p \leq 0.05$). Significantly different levels of GSK-3β were also exhibited by treatment groups in PFC samples when analyzing p/T-GSK-3β ($F_{(3, 27)} = 3.642$, $p = 0.0251$; figure 23I) and p-GSK-3β ($F_{(3, 27)} = 3.554$, $p = 0.0274$; figure 23G) values; no significance was found when assessing levels of T-GSK-3β ($F_{(3, 27)} = 0.3919$, $p = 0.7598$; figure 23H). In both the post hoc assessments of p/T-GSK-3β and p-GSK-3β values, the $V_{Air}$CBD group exhibited significantly increased levels in comparison to both $V_{Air}V_{PBS}$ and L2000-CBD groups ($p \leq 0.05$).

Collectively, given the non-significance observed between the L2000-CBD and $V_{Air}V_{PBS}$ treatment in all GSK3β molecular assays conducted in as shown in the experimental analyses, these findings suggest that L2000-CBD achieves its EE-potentiated anxiolysis (observed in the behavioral tests) without significant modulation of GSK3β activity from baseline levels. Furthermore, the lack of effect present following linalool treatment in the control analysis suggests that linalool likely does not modulate GSK3β activity on its own; importantly however, in light of the significant reduction of p/T-GSK3β levels following L2000-CBD treatment.
(compared to $V_{Ah}$CBD) in both experimental analyses within the PFC and NAcSh regions, these findings suggest that linalool is potentially involved in allosteric modulation of GSK3\(\beta\) activity above a certain threshold level—and that this allosteric intervention may serve as a component mechanism of action for previously observed EE-potentiated anxiolysis.
Figure 23 Effects of Treatment Conditions on p-GSK3β, T-GSK3β, p/T-GSK3β levels. Pictograms A, B, C represent control analyses of intra-NAcSh p-GSK3β, T-GSK3β, p/T-GSK3β levels respectively; no significant differences were found. D, E, F represent respectively experimental analyses of intra-NAcSh p-GSK3β, T-GSK3β, p/T-GSK3β levels; VₐAc-CBD group exhibited significantly increased p/T-GSK3β levels compared to VₐAc-PBS and L2000-CBD groups (F). G, H, I represent experimental analyses of intra-PFC p-GSK3β, T-GSK3β, p/T-GSK3β levels respectively; VₐAc-CBD group exhibited significantly increased p-GSK3β (G) and p/T-GSK3β expression (I) compared to VₐAc-PBS and L2000-CBD groups. J) Representative banding of p-GSK3 isoforms, T-GSK3 isoforms and associated α-tubulin in control and experimental analyses. One-way ANOVA, p ≤ 0.05. CBD dosages represent bilateral dosages in ng/μL; linalool dosages represent dosages in μL. *P ≤ 0.05, **P ≤ 0.01. Error bars represent standard error of means (SEMs). Sample Sizes: [Control Analysis, NAcSh] VₐAc-PBS (N=4), L200-PBS (N=5), L2000-PBS (N=4); [Experimental Analysis, NAcSh] VₐAc-PBS (N=7), VₐAc-CBD (N=7), L200-CBD (N=5), L2000-CBD (N=6); [Experimental Analysis, PFC] VₐAc-PBS (N=10), VₐAc-CBD (N=9), L200-CBD (N=6), L2000-CBD (N=9).
4.2 Akt Protein Analysis

As shown in figure 14, no significant differences were found in the control analysis when focusing on either the Ser473 or Thr308 sites following a one-way ANOVA test assessment (p-AktSer473: F(2, 12) = 1.614, p = 0.2395 [figure 24A]; T-Akt: F(2, 12) = 3.802, p = 0.0526 [figure 24B]; p/T-AktSer473: F(2, 12) = 1.399, p = 0.2843 [figure 24C]; p-AktThr308: F(2, 12) = 0.8696, p = 0.4439 [figure 24D]; T-Akt: F(2, 12) = 3.664, p = 0.0573 [figure 24B]; p/T-AktThr308: F(2, 12) = 1.862, p = 0.1975 [figure 24C]).

While no significant differences were found in the NAcSh region when assessing AktThr308 sites (p-AktThr308: F(3, 20) = 0.5868, p = 0.6304 [figure 24E]; T-Akt: F(3, 30) = 1.148, p = 0.3456 [figure 24F]; p/T-AktThr308: F(3, 20) = 0.3236, p = 0.8082 [figure 24G]), p-AktSer473 (F(3, 21) = 0.5449, p = 0.6570; figure 24E) or T-Akt in the context of AktSer473 quantification (F(3, 21) = 0.5727, p = 0.6392; figure 24F), significantly different levels of p/T-AktSer473 were present in the brain region (F(3, 22) = 5.19, p = 0.0073; figure 24G). Specifically, both the VAirCBD group and L200-CBD groups exhibited significantly increased p/T-AktSer473 values in comparison to the VAirV PBS and L2000-CBD treatment groups (p ≤ 0.05).

Similar trends were found in the PFC region (figure 14I/J/K); while no significant differences were present following ANOVA analysis of AktThr308 site quantifications (p-AktThr308: F(3, 21) = 0.5868, p = 0.6304 [figure 24I]; T-Akt: F(3, 30) = 1.148, p = 0.3456 [figure 24J]; p/T-AktThr308: F(3, 21) = 0.3236, p = 0.8082 [figure 24K]) or the T-Akt assessment of the AktSer473 cohort (F(3, 30) = 2.642, p = 0.0674; figure 24J), expression levels of p-AktSer473 and p/T-AktSer473 were significantly different between groups (respectively: F(3, 30) = 12.2, p ≤ 0.001 [figure 24I]; F(3, 30) = 4.536, p = 0.0098 [figure 24K]). With regards to the assessment intra-PFC p-AktSer473 levels, the VAirCBD group demonstrated significantly higher levels than
all other treatment groups (p ≤ 0.05); when assessing p/T-AktSer473 group comparisons, the V\textsubscript{Air}CBD group exhibited significantly increased expression levels compared to V\textsubscript{Air}V\textsubscript{PBS} and L2000-CBD treatment groups (p ≤ 0.05), while the L200-CBD group presented significantly higher p/T-AktSer473 levels when compared to the V\textsubscript{Air}V\textsubscript{PBS} group (p ≤ 0.05).

Altogether, these Akt molecular assay findings suggest that the EE-potentiated anxiolysis elicited following L2000-CBD treatment observed previously in behavioral paradigms likely does not involve significant modulation of Akt activity from baseline levels (as evidenced by the non-significance in p/T-AktSer473 and p/T-AktThr308 levels between L2000-CBD and V\textsubscript{Air}V\textsubscript{PBS} treatment groups in both PFC and NAcSh experimental analyses). Furthermore, similar to the previous GSK3β analysis, the lack of significant differences between treatment groups following linalool administration in the control analysis suggests that linalool likely does not modulate Akt activity on its own; however the significant reduction of p/T-Akt levels following L2000-CBD treatment (compared to V\textsubscript{Air}CBD) in both PFC and NAcSh experimental analyses present the notion that linalool is potentially involved in allosteric modulation of Akt activity above a certain threshold level. Unlike the GSK3β assay however, L200-CBD treatment achieved significantly higher p/T-AktSer473 levels in the NAcSh experimental analysis (as well as a moderate increase in PFC regional analysis) when compared to the L2000-CBD group, suggesting that a dose-dependent attenuation of p/T-AktSer473 levels induced through increasing dosages of linalool olfactory exposure may be a component mechanism of action through which L2000-CBD treatment achieves its EE-potentiated anxiolysis.
Experimental Analysis: Intra-NAcSh

H

Experimental Analysis: Intra-NAcSh

p-AktSer473 T-Akt α-tubulin
CBD: --- 10 10 10
Linalool: --- 200 2000

80
Figure 24 Effects of Treatment Conditions on p-Akt, T-Akt, p/T-Akt levels. Pictograms A, B, C represent control analyses of intra-NAcSh p-Akt, T-Akt, p/T-Akt levels respectively; no significant differences were found. E, F, G represent respectively experimental analyses of intra-NAcSh p-Akt, T-Akt, p/T-Akt levels; the VₐδCBD and L200-CBD groups exhibited significantly increased p-T-AktSer473 levels compared to VₐδPBS and L2000-CBD groups (G). I, J, K represent experimental analyses of intra-PFC p-Akt, T-Akt, p/T-Akt levels respectively; L200-CBD group presented significantly higher p/T-AktSer473 expression compared to its associated VₐδPBS group (K), while the VₐδCBD group exhibited significantly increased p/T-AktSer473 levels compared to both VₐδPBS and L2000-CBD groups (K) as well as demonstrating significantly higher p-AktSer473 expression compared to all other groups (I). D, H, L represent banding of p-Akt, T-Akt and associated α-tubulin in control (D) and experimental analyses (H, L). One-way ANOVA, \( p \leq 0.05 \); separate analyses were conducted as demarked by line breaks on the x-axis. CBD dosages represent bilateral dosages in ng/μL; linalool dosages represent dosages in μL. \( \ast P \leq 0.05, \ast\ast P \leq 0.01, \ast\ast\ast P \leq 0.001 \). Error bars represent standard error of means (SEM). Sample Sizes: [Control Analysis, NAcSh] VₐδPBS (N=4), L200-PBS (N=5), L2000-PBS (N=6); [Experimental Analysis, NAcSh] VₐδVₐδ (N=7 for AktSer473, N=6 for AktThr308), VₐδCBD (N=7), L200-CBD (N=5), L2000-CBD (N=6); [Experimental Analysis, PFC, AktSer473] VₐδVₐδ (N=10), VₐδCBD (N=9), L200-CBD (N=6), L2000-CBD (N=9); [Experimental Analysis, PFC, AktThr308] VₐδVₐδ (N=8), VₐδCBD (N=7), L200-CBD (N=4 for p- and p/T-AktThr308), L2000-CBD (N=6).
4.3 ERK Protein Analysis

As exhibited in Figure 15, no significant differences were found in the control analysis when assessing quantification measures for either the ERK1 isoform (p-ERK1: \( F_{(2, 12)} = 1.922, p = 0.1888 \) [figure 25A]; T-ERK1: \( F_{(2, 12)} = 2.709, p = 0.1069 \) [figure 25B]; p/T-ERK1: \( F_{(2, 12)} = 1.117, p = 0.3589 \) [figure 25C]) or ERK2 isoform (p-ERK2: \( F_{(2, 12)} = 1.088, p = 0.3681 \) [figure 25A]; T- ERK2: \( F_{(2, 12)} = 2.727, p = 0.1056 \) [figure 25B]; p/T- ERK2: \( F_{(2, 12)} = 0.9841, p = 0.4020 \) [figure 25C]).

Within the context of the NAcSh region, while no significant differences were found in T-ERK in either ERK1 (\( F_{(3, 19)} = 1.884, p = 0.1666 \); figure 25E) or ERK2 (\( F_{(3, 19)} = 1.138, p = 0.3589 \); figure 25E), there were significantly different intra-NAcSh levels of p-ERK1 (\( F_{(3, 19)} = 3.561, p = 0.0337 \); figure 25D), p/T-ERK1 (\( F_{(3, 19)} = 4.329, p = 0.0174 \); figure 25F), p-ERK2 (\( F_{(3, 19)} = 4.298, p = 0.0179 \); figure 25D) and p/T-ERK2 (\( F_{(3, 19)} = 4.09, p = 0.0213 \); figure 25F) between groups. Post hoc analyses revealed similar trends between phosphorylated levels of ERK isoforms versus control and phosphorylated/total levels of ERK isoforms versus control; the VAirCBD group presented significantly increased p-ERK1, p-ERK2, p/T-ERK1 and p/T-ERK2 in comparison to VAirV PBS and L2000-CBD treatment groups (\( p \leq 0.05 \)). In addition, solely in regard to analysis of p/T-ERK1 and p/T-ERK2 levels, the L200-CBD treatment group displayed significantly higher levels of protein ratios when compared to its higher dosage counterpart L2000-CBD treatment group (\( p \leq 0.05 \)).

Similar to the NAcSh region, within the PFC region (Figure 15G/H/I) no significant differences were found in regards to T-ERK in both ERK1 (\( F_{(3, 22)} = 0.2756, p = 0.8424 \); figure 25H) and ERK2 (\( F_{(3, 22)} = 0.564, p = 0.6445 \); figure 25H); however, significant differences between treatment group quantifications of p-ERK1 (\( F_{(3, 22)} = 8.194, p \leq 0.001 \); figure 25G), p/T-
ERK1 ($F_{(3, 22)} = 4.073, p = 0.0192$; figure 25I), p-ERK2 ($F_{(3, 22)} = 8.656, p \leq 0.001$; figure 25G) and p/T-ERK2 ($F_{(3, 22)} = 5.549, p = 0.0054$; figure 25I) were found. The same pattern of significant differences between treatment groups was present in the PFC region, with the V_AirCBD demonstrating significantly increased p-ERK1, p-ERK2, p/T-ERK1 and p/T-ERK2 levels in comparison to all other treatment groups ($p \leq 0.05$).

Taken together, these findings from the ERK molecular assay suggest that the EE-potentiated anxiolysis elicited following L2000-CBD treatment observed previously in behavioral paradigms likely does not involve significant modulation of ERK activity from baseline levels (as presented by the non-significance in p/T-ERK1/2 levels between L2000-CBD and V_Air, V_PBS treatment groups in both PFC and NAcSh experimental analyses). Furthermore, similar to the previous GSK3β and Akt analyses, the lack of significant differences (albeit in the presence of a trend) between treatment groups following linalool administration in the control analysis suggests that linalool likely does not modulate ERK activity on its own; however the significant reduction of p/T-ERK levels following L2000-CBD treatment (compared to V_AirCBD) in both PFC and NAcSh experimental analyses present the notion that linalool is potentially involved in allosteric modulation of ERK activity above a certain threshold level. In addition, the fact that L200-CBD treatment achieved significantly higher p/T-ERK1/2 levels in the NAcSh experimental analysis suggests that a dose-dependent attenuation of intra-NAcSh p/T-ERK1/2 levels resulting from olfactory exposure to increasing dosages of linalool may be a mechanism of action through which L200-CBD treatment elicits its EE-potentiated anxiolysis observed previously in the behavioral assays.
Figure 25 Effects of Treatment Conditions on p-ERK, T-ERK, p/T-ERK levels. Pictograms A, B, C represent control analyses of intra-NAcSh p-ERK, T-ERK, p/T-ERK levels respectively; no significant differences were present. D, E, F respectively represent experimental analyses of intra-NAcSh p-ERK, T-ERK, p/T-ERK levels; the V₅₂₀CBD group exhibited significantly increased p-ERK1/2 (D) and p/T-ERK1/2 levels (F) compared to V₅₂₀VPBS and L2000-CBD groups, while the L200-CBD group presented significantly higher p/T-ERK1/2 levels compared to its higher combination dose counterpart, L2000-CBD group (F). G, H, I represent experimental analyses of intra-PFC p-ERK, T-ERK, p/T-ERK levels respectively; the V₅₂₀CBD group was found to exhibit significantly higher p-ERK (G) and p/T-ERK expression (I) compared to all other treatment groups in its test analyses. Pictogram J represents banding of p-Akt, T-Akt and associated α-tubulin in control and experimental analyses. One-way ANOVA, \( p \leq 0.05 \); separate analyses were conducted as demarked by line breaks on the x-axis. CBD dosages represent bilateral dosages in ng/μL; linalool dosages represent dosages in μL. *\( P \leq 0.05 \), **\( P \leq 0.01 \), ***\( P \leq 0.001 \). Error bars represent standard error of means (SEMs). Sample Sizes: [Control Analysis, NAcSh] V₅₂₀VPBS (N=4), L200-VPBS (N=5), L2000-VPBS (N=6); [Experimental Analysis, NAcSh] V₅₂₀VPBS (N=6), V₅₂₀CBD (N=6), L200-CBD (N=5), L2000-CBD (N=6); [Experimental Analysis, PFC] V₅₂₀VPBS (N=6), V₅₂₀CBD (N=8), L200-CBD (N=4), L2000-CBD (N=8).
4.4 JNK Protein Analysis

In a similar manner to the GSK-3β, Akt, and ERK protein analyses, no significant differences were found between treatment groups in the control analysis of either the JNK1 isoform (p-JNK1: F(2, 11) = 0.7413, p = 0.4989 [figure 26A]; T- JNK1: F(2, 10) = 3.133, p = 0.0878 [figure 26B]; p/T- JNK1: F(2, 10) = 0.1874, p = 0.8320; [figure 26C]) or JNK2 isoform (p- JNK2: F(2, 11) = 0.3476, p = 0.7139 [figure 26A]; T- JNK2: F(2, 10) = 2.752, p = 0.1116 [figure 26B]; p/T- JNK2: F(2, 10) = 0.1727, p = 0.8439 [figure 26C]).

Interestingly, when observing experimental analysis results within the NAcSh region as shown in figure 16D/E/F, significant differences between treatment groups were only found within the quantification of p-JNK1 (F(3, 19) = 3.827, p = 0.0267; figure 26D) and not within any other assessment of JNK1 protein levels (T- JNK1: F(3, 19) = 3.052, p = 0.0536 [figure 26E]; p/T- JNK1: F(3, 19) = 2.899, p = 0.0618 [figure 26F]) or JNK2 protein levels (p- JNK2: F(3, 19) = 2.872, p = 0.0634 [figure 26D]; T- JNK2: F(3, 19) = 2.779, p = 0.0693 [figure 26E]; p/T-JNK2: F(3, 19) = 1.569, p = 0.2298 [figure 26F]). Post hoc analyses of p-JNK1 quantification revealed significantly increased expression levels by the V_Air CBD group when compared to its respective V_Air PBS and L2000-CBD treatment groups (p ≤ 0.05).

ANOVA analyses of PFC region JNK quantification revealed a stark contrast to NAcSh region results (figure 16G/H/I). While non-significance was found between treatment groups when assessing T-JNK1 (F(3, 29) = 0.7696, p = 0.5204; figure 26H) and T-JNK2 (F(3, 29) = 0.5617, p = 0.6446; figure 26H), significant differences were found in the analysis of p-JNK1 (F(3, 29) = 5.746, p = 0.0033; figure 26G), p/T- JNK1 (F(3, 29) = 6.781, p = 0.0013; figure 26I), p-JNK2 (F(3, 29) = 4.885, p = 0.0072; figure 26G) and p/T-JNK2 levels (F(3, 29) = 3.824, p = 0.0201; figure 26I) between treatment groups. In both p-JNK1 and p-JNK2 post hoc analyses, the V_Air CBD group
demonstrated significantly increased expression levels when compared to \( V_{\text{Air}} \) \( V_{\text{PBS}} \) and L2000-CBD treatment groups (\( p \leq 0.05 \)); unlike \( p\)-JNK1 however, \( p\)-JNK2 analysis also demonstrated the L200-CBD group to exhibit significantly higher quantification than the L2000-CBD group (\( p \leq 0.05 \)). Between \( p/T\)-JNK1 and \( p/T\)-JNK2 post hoc analyses however a pattern of significant differences between groups was found, with both the \( V_{\text{Air}} \)CBD and L200-CBD groups expressing higher quantification levels compared to \( V_{\text{Air}} \) \( V_{\text{PBS}} \) and L2000-CBD treatment groups in their respective cohorts of analysis (\( p \leq 0.05 \)).

Similar to previous molecular analyses, these findings from the JNK molecular assay suggest that the EE-potentiated anxiolysis elicited following L2000-CBD treatment observed previously in behavioral paradigms likely does not involve significant modulation of JNK activity from baseline levels (as presented by the non-significance in \( p/T\)-JNK1/2 levels between L2000-CBD and \( V_{\text{Air}} \) \( V_{\text{PBS}} \) treatment groups in both PFC and NAcSh experimental analyses). Furthermore, the non-significance (albeit in the presence of a trend) between treatment groups following linalool administration in all measures within the control analysis suggests that linalool likely does not modulate JNK activity on its own. However, the significant reduction of intra-PFC \( p\)-JNK1/2 levels (as well as intra-PFC \( p/T\)-JNK1/2 levels) following L2000-CBD treatment (compared to \( V_{\text{Air}} \)CBD) suggests the notion that linalool is likely involved in allosteric modulation of JNK activity above a certain threshold level. In addition, similar to patterns found in Akt and ERK molecular assays, the fact that L200-CBD treatment achieved significantly higher \( p/T\)-JNK1/2 levels in the PFC experimental analysis suggests that a dose-dependent attenuation of intra-PFC \( p/T\)-JNK1/2 levels resulting from olfactory exposure to increasing dosages of linalool may serve to be a component mechanism of action through which L2000-CBD treatment elicits its EE-potentiated anxiolysis (observed in the behavioral assays).
Figure 26 Effects of Treatment Conditions on p-JNK, T-JNK, p/T-JNK levels. Pictograms A, B, C represent control analyses of intra-NAcSh p-JNK, T-JNK, p/T-JNK levels respectively; no significant differences were found. D, E, F respectively represent experimental analyses of intra-NAcSh p-JNK, T-JNK, p/T-JNK levels; the VAcCBD group was found to exhibit significantly increased p-JNK1 levels compared to VAcVPMS and L2000-CBD groups (D); no other significant differences between treatments were found within intra-NAcSh experimental analyses. G, H, I represent experimental analyses of intra-PFC p-JNK, T-JNK, p/T-JNK levels respectively; the VAcCBD group was found to exhibit significantly higher p-JNK1/2 (G) and p/T-JNK1/2 expression (I) compared to VAcVPMS and L2000-CBD groups in its respective analyses; furthermore, L2000-CBD treatment was found to result in significantly higher p-JNK2 compared to L2000-CBD treatment (G) and the L2000-CBD group overall displayed significantly greater expression of p/T-JNK1/2 compared to VAcVPMS and L2000-CBD groups (I). J)

Representative banding of p-JNK, T-JNK and associated α-tubulin in control and experimental analyses. One-way ANOVA, p ≤ 0.05; separate analyses were conducted as demarked by line breaks on the x-axis. CBD dosages represent bilateral dosages in ng/μL; linalool dosages represent dosages in μL. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001. Error bars represent standard error of means (SEMs). Sample Sizes: [Control Analysis, NAcSh] VAcVPMS (N=4), L200-Vpms (N=5), L2000-Vpms (N=4); [Experimental Analysis, NAcSh] VAcVPMS (N=6), VAcCBD (N=6), L200-CBD (N=5), L2000-CBD (N=6); [Experimental Analysis, PFC] VAcVPMS (N=9), VAcCBD (N=9), L200-CBD (N=6), L2000-CBD (N=9).
Discussion

The findings of this thesis demonstrate for the first time the potential presence of the Entourage Effect between linalool and CBD in establishing a greater anxiolytic effect than their individual administrations. While previous studies had demonstrated the CBD and linalool’s modes of action through positive allosteric modulation of GABA<sub>A</sub>Rs and p-ERK downregulation, this study highlighted the importance of these mechanisms specifically following administration within the Nucleus Accumbens Shell (NAcSh) region. In light of the hypothesis set forth which proposed EE-potentiated anxiolytic effects present in both symptoms and biomarkers, these findings which demonstrate that significantly increased anxiolytic effects of the combinatorial treatment (linalool and CBD) observed in behavioral assays were negatively correlated with GABA<sub>A</sub>R inhibition and p-ERK activation, suggests that the linalool-CBD anxiolytic interaction dynamic likely involves a unique mediation of neuronal activity and cellular MAPK pathways not achieved by their individual administrations.

Baseline Treatment Cohort: EE-Potentiated Anxiolytic Effects in Behavioral Assays

<table>
<thead>
<tr>
<th><strong>Behavioral Assay</strong></th>
<th><strong>Result</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Open Field Test</td>
<td>No differences in locomotion</td>
</tr>
<tr>
<td>Light Dark Box Test</td>
<td>↓ Anxiety</td>
</tr>
<tr>
<td>Social Motivation and Recognition Test</td>
<td>No differences in sociability or recognition</td>
</tr>
<tr>
<td>Elevated Plus Maze</td>
<td>No differences in anxiety</td>
</tr>
<tr>
<td>Context-Dependent Fear Conditioning</td>
<td>↓ Anxiety (Reduced Fear Formation and Recall)</td>
</tr>
</tbody>
</table>

Figure 27 Summary of Baseline Treatment Cohort Results from Behavioral Assays.
Regarding bright-space and fear-memory related anxiety, the EE-potentiated anxiolytic effect of linalool and CBD in comparison to their individual administrations was readily apparent. Within the light dark box and contextual fear conditioning assays, the combined 2000µL linalool and 5ng/0.5µL CBD treatment yielded much greater decreases in anxiety symptoms than did their individual administrations (2000µL linalool or 5ng/0.5µL CBD), suggesting that the observed EE-potentiated anxiolysis is superiorly effective in alleviating bright-space and fear memory-related anxiety. These findings are consistent with those published currently in the literature surrounding the anxiolytic potential of individual cannabidiol (Fogaca et al., 2014; Gomes et al., 2010; Lemos et al., 2010; Long et al., 2010; Resstel et al., 2006) and linalool treatments (Coelho et al., 2018; Harada et al., 2018) in these test paradigms. Furthermore, in the context of the CFC behavioral assay, this EE-potentiated anxiolysis exhibited by the L2000-CBD treatment was present in both the acquisition and recall scenarios, suggesting that the treatment is potentially effective in both the prevention of anxiety stemming from fear memory consolidation and the recall of such fear-related memory events. When taking into account the observation that locomotor differences between groups were absent as documented by Linck et al. (2010) and Long et al. (2010) following acute linalool and CBD administrations respectively, this in turn presents the notion that this EE profile may uniquely affect the limbic region (known for affective processing) without altering motor cortex functionality (Ali et al., 2015; Guimaraes, 2004).

Interestingly, while the L2000-CBD treatment presented EE-potentiated anxiolysis within these behavioral test paradigms, the lesser linalool-CBD combination treatment of L200-CBD did not elicit significantly different freezing time compared to either of its component vehicle groups (VAirCBD or L200-VPBS) and only expressed significant differences to its VAirCBD
vehicle counterpart in the secondary bright-space anxiety measure of total transitions. Yet, despite this contrast in EE-potentiated anxiolysis between L200-CBD and L2000-CBD treatments, no significant differences were found between the two groups in all test paradigms—suggesting the lack of a conclusive dose-dependent response in the combinatorial administration of linalool and CBD (with respect to comparisons between 200 μL and 2000 μL). These findings of non-significance but increasing trends of anxiolytic efficiency with greater linalool dosage administrations has been documented by Coelho et al. (2018), presenting the notion that there may be a moderate dose-dependent curve in linalool anxiolytic efficiency.

The overall lack of significant differences between treatment groups when assessing social cognition measures within the social motivation and recognition test suggests that there is no EE-potentiation in social cognitive abilities or associated anxiety following linalool-CBD dual treatment. Although this observation stands in contrast to the finding established by Linck et al. (2010) whereby linalool induced greater social interaction time (equivalent to greater social motivation values), these findings align with the non-significance found from administration of CBD-alone treatments (Long et al., 2010; Malone et al., 2009). In addition, while an olfactory administration of linalool was used in the context of this thesis, Linck et al had employed intraperitoneal linalool administrations; given that Takahashi et al. (2011) had found linalool acetate to be essential in eliciting anxiolytic effects observed in the EPM test paradigm, this suggests that the interaction between linalool and its formed metabolites following systemic metabolism of pure linalool may be responsible for this difference in observed social behaviors.
Linalool-CBD combinations demonstrated anxiolysis in the EPM assay only following CUS exposure

Although results from the chronic stress cohort demonstrated significant differences between CUS-exposed V_Air V_PBS and L2000-CBD treatment groups in the EPM assay, this was not the case in the baseline cohort. Indeed, while the LDB and CFC assays suggested the presence of an EE-potentiated anxiolysis following L2000-CBD treatment, this phenomenon was not found in the EPM test anxiety measures within the baseline cohort as currently observed in the literature surrounding CBD and linalool’s individually observed anxiolytic effects (Bradley et al., 2007; Campos and Guimarães, 2008; Harada et al., 2018). It should be noted, however, that different modes of administration (intracranial versus intraperitoneal, dorsolateral periaqueductal gray versus NAcSh) as well as duration of drug administration (acute versus chronic) have been employed to achieve these results in the aforementioned studies; thus, significant differences between observed results and literature findings may stem from differences in testing methodologies. Furthermore, when taking into consideration studies incorporating intracranial infusion of CBD into brain regions within the mesolimbocortical pathway (PFC, hippocampus) and within closer anatomic proximity to the NAcSh (PFC), no significant differences were found in anxiety measures assessed in the elevated plus maze test and other open-space anxiety test assays (Hudson et al., 2019; Szkudlarek et al., 2019).

Another potential basis for these findings may be attributable to the notion that the EE-potentiated anxiolysis of L2000-CBD attenuates different forms of anxiety to differing degrees, especially depending on the behavioral paradigm used; specifically, while this potentiated anxiolysis may be present in the reduction of bright-space and fear-memory anxieties as observed respectively in the LDB and CFC assays, it may not be as relevant in the context of
open-space anxiety assessed in the EPM assay. This line of inquiry has been supported by Miyakawa et al (2003), who found that rodent models expression of anxiety measures between the LDB and EPM tests were not always consistent—suggesting either differences in environmental conditions or measures of different forms of anxiety being the cause of this observed phenomenon (Takao and Miyakawa, 2006). Furthermore, even in the consideration of schizophrenic rodent models (C57BL/6J) where CBD treatment is proposed to have a significant reduction in anxiety, the same anxiolytic profile was not observed between the two tests following CBD treatment (Long et al., 2010; Schleicher et al., 2019).

Taking into account the fact that post hoc analyses of open-field thigmotaxis (via center/total locomotion ratio assessments) conducted in the flumazenil and EPA challenge cohorts revealed the L2000-CBD group to yield significantly reduced open-space anxiety when compared to the V_Air V_PBS control group, this ultimately suggests that the non-significance within baseline cohort assessments involving EPM anxiety measures is likely attributable to a combination of differences in methodology and varying sensitivity between assays in assessing specific forms of anxiety—and that these variations in environmental conditions or sensitivity to such fluctuations which may play a role in the generation of non-significance essentially did not influence CUS-exposed models in the same manner.
**GABA<sub>A</sub>R inactivation reversed EE-potentiated anxiolytic effects**

<table>
<thead>
<tr>
<th>Behavioral Assay</th>
<th>Result (L2000-CBD vs. vehicle counterparts)</th>
<th>Result (L2000-CBD+Flumazenil vs. L2000-CBD counterpart)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Open Field Test</td>
<td>No differences in locomotion; ↓ Anxiety</td>
<td>No differences in locomotion; ↑ Anxiety</td>
</tr>
<tr>
<td>Light Dark Box Test</td>
<td>↓ Anxiety</td>
<td>↑ Anxiety</td>
</tr>
<tr>
<td>Context-Dependent Fear</td>
<td>↓ Anxiety (Reduced Fear Recall)</td>
<td>↑ Anxiety (Reduced Fear Recall)</td>
</tr>
</tbody>
</table>

**Figure 28 Summary of Flumazenil Challenge Cohort Results from Behavioral Assays.**

Significant increases in LDB and CFC anxiety measures exhibited by L2000-CBD subjects following co-administration of the GABA<sub>A</sub>R antagonist flumazenil suggests that GABA<sub>A</sub>R activation is essential in the EE-potentiated anxiolysis induced through L2000-CBD treatment. While assessment of L2000-CBD effects is a novel venture, the lack of significant differences between control and flumazenil vehicle groups in these tested assays and flumazenil attenuation of BDZ-site agonist-induced anxiolysis have been documented in the existing literature (Clément et al., 2009; Herzog et al., 1996; Hussain et al., 2015; Rex et al., 1996). In addition, the observed lack of locomotor differences between co-administrations of flumazenil with L2000-CBD and L2000-CBD only treatments found here are in line with previous findings in the baseline cohort—reinforcing the notion that L2000-CBD treatment likely does not impair motor function, and thus flumazenil would not be expected to elicit any alternations in locomotor activity. Interestingly however, the assessment of OFT open-space anxiety introduced in the flumazenil challenge cohort demonstrated that while locomotor abilities of test subjects did not significantly differ between treatment groups, L2000-CBD treatment generated significantly
reduced open-space anxiety symptoms as evidenced by the significantly greater center/total locomotion percentage ratios in comparison to the $V_{Air}/V_{PBS}$ group. In addition, the significantly increased open-space anxiety exhibited by the L2000-CBD+flumazenil group when compared to the flumazenil-free L2000-CBD group counterpart suggests that GABA\(_A\)R antagonism plays a key role in attenuating L2000-CBD induced open space anxiety in the OFT assay.

Delving further into the EE-potentiated anxiolysis presented in the LDB and CFC assays, it is important to mention that given the absence of significant differences between control vehicles and flumazenil treated control vehicles in conjunction with significantly increased anxiety exhibited by the L2000-CBD+flumazenil group (in contrast to its L2000-CBD group counterpart) in these two assays, this suggests that flumazenil attenuations of L2000-CBD anxiolysis may stem from mostly antagonist-exogenous compound interactions rather than antagonist-endogenous compound interactions. A plausible explanation for this phenomenon includes the possibility that flumazenil competitive inhibition of GABA\(_A\)Rs following intra-NAcSh administration in control vehicles induces changes in neuronal activity that are not significant enough to be translated to behavioral changes—but that in the presence of an increased number of neuronal populations being hyperpolarized due to L2000-CBD treatment, flumazenil antagonism of L2000-CBD induced molecular mechanisms is significant enough to translate into a reversion of observed behavior in L2000-CBD treated subjects.

Given that the literature has documented the greatest change in GABA EC\(_{50}\) to be elicited by CBD following allosteric modulation at \(\alpha2\) subunit configurations (Bakas et al., 2017) and primarily for linalool at \(\alpha1\) subunit configurations (Kessler et al., 2014; Milanos et al., 2017), this differential modulation of GABA\(_A\)R subunits provides support for EE-potentiated anxiolysis of L2000-CBD—and given that flumazenil has been found to primarily target \(\alpha1/\alpha2/\alpha3/\alpha5\) subunit
configurations (Huang et al., 1998; Wieland and Lueddens, 1994), its administration and subsequent competitive inhibition at these sites would be expected to result in the behavioral observations seen in this experimental challenge cohort. This hypothesized mechanism of action would especially help explain the L2000-CBD induced reduced fear recall that was reversed by flumazenil treatment, as Schmitz et al. (2017) had demonstrated decreased hippocampal GABA levels to result in dysfunctional suppression of memory recall. Furthermore, in consideration of the connections between the olfactory bulb, the entorhinal cortex and hippocampus as previously mentioned (Christen-Zaech et al., 2003; Vanderwolf, 1992), this GABA$_A$R-mediated anxiolytic theory provides a potential explanation for downstream effects elicited by activation of the olfactory system upon linalool inhalation.

Although promising, further in-vitro investigations assessing co-administrations of linalool and CBD on GABA EC$_{50}$ however are required to substantiate correlational claims between potential synergism surrounding EE-potentiated GABA current induction and the antagonistic effects elicited by flumazenil on the molecular and systems level.
**Activation of p-ERK reversed EE-potentiated anxiolytic effects**

<table>
<thead>
<tr>
<th>Behavioral Assay</th>
<th>Result (L2000-CBD vs. vehicle counterparts)</th>
<th>Result (L2000-CBD + EPA vs. L2000-CBD counterpart)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Open Field Test</strong></td>
<td>No differences in locomotion</td>
<td>No differences in locomotion</td>
</tr>
<tr>
<td></td>
<td>↓ Anxiety</td>
<td>↑ Anxiety</td>
</tr>
<tr>
<td><strong>Light Dark Box Test</strong></td>
<td>↓ Anxiety</td>
<td>↑ Anxiety</td>
</tr>
<tr>
<td><strong>Context-Dependent Fear</strong></td>
<td>↓ Anxiety (Reduced Fear Acquisition)</td>
<td>↑ Anxiety (Reduced Fear Acquisition)</td>
</tr>
</tbody>
</table>

Figure 29 Summary of EPA Challenge Cohort Results from Behavioral Assays

The attenuation of EE-potentiated anxiolytic effects following EPA coadministration observed in the EPA challenge cohort suggests that p-ERK activation is a key regulator of mechanistic pathways utilized by L2000-CBD treatment to elicit its observed anxiety reduction in test subjects. Non-significance findings between control vehicles and EPA-treated vehicles in these assays are representative of the currently established literature (Einat et al., 2003; Sierra-Fonseca et al., 2019). Furthermore, regarding interactions between p-ERK and CBD, Hudson et al. (2019) had found (utilizing the same mode of acute EPA administration via intra-vHipp) that EPA administration—and consequently, increased p-ERK expression—was able to significantly attenuate the anxiolysis induced by the combinatorial administrations of THC and CBD within the LDB and CFC [acquisition] assays. Lastly in line with previous test cohorts, no significant differences in locomotion was detected between groups, suggesting the lack of locomotor impairments as a result of EPA treatment and the confirmation of no potential confounder in subsequent assays as a result of locomotor differences.

This finding of non-significance in locomotor differences is particularly interesting in
light of the fact that co-treatment of intra-cranial EPA and L2000-CBD resulted in the
elimination of OFT open-space anxiolysis, which was found present following L2000-CBD
treatment as shown by stark contrasts in center/total locomotion (%) performance—
demonstrating a similar pattern of results to the OFT anxiety assessment conducted by Hudson et
al. (2019). Although Hudson and authors utilized group comparisons which are not directly
comparable with the results in this study (as THC+CBD and EPA+THC+CBD groups were used
as opposed to L2000-CBD and L2000-CBD+EPA groups), the significantly increased open-
space anxiety observed following the addition of EPA to THC+CBD combination treatments is
especially notable due to the fact that it provides a comparative assessment p-ERK effects on
EE-potentiation; as despite these obvious differences in EE combination components (CBD &
THC versus CBD & linalool), the study provides at least a rudimentary foundation to refer to in
the pursuit of correlations between p-ERK levels and novel EE combinations, such as the one
between CBD and linalool explored in this thesis. In addition, while elevated p-ERK expression
is known to result in downstream gene expression alterations over a long-term basis, the acute
findings in this thesis and those made by Hudson et al. (2019) suggests that the observed
attenuations in anxiety likely stems from fast-acting targets downstream of increased p-ERK
activation. This aligns with observations made in a review conducted by Peng et al. (2010)
highlighting the correlation between increased p-ERK and two receptors involved in long term
potentiation (LTP) memory receptors, the NMDA and AMPA receptors. Given the results in the
CFC assay which involves fear memory formation and subsequent recall, the increased freezing
time exhibited by test subjects who received co-administrative L2000-CBD and EPA treatment
suggests that the observed EE-potentiated anxiolysis likely involves either inactivation or fast-
acting downregulation of these target receptors and their subsequent synaptic alterations.
As previously mentioned, correlations between ERK activation and linalool have also been studied by Caputo et al. (2016; 2017; 2018) who discovered through in-vitro assays that linalool was able to inhibit ADCY1, p-ERK and PKA expression within the SH-SY5Y cell line. PKA has been shown to be essential in a host of cellular activities, including the regulation of NMDARs and stimulation of downstream CREB-dependent transcription, both of which are implicated in memory formation (Impey et al., 1998). ADCY1, which catalyzes the formation of cAMP and thus is innately involved in the subsequent activation of PKA, has been found upregulated in the nucleus accumbens of subjects with fear memory and associated anxiety (Du et al., 2019); even more relevant in its study is the discovery that the lack of ADCY1 has been directly linked to lowered sustainability of contextual memories, such as those acquired in the CFC assay (Shan et al., 2008). Given the imaging findings of ERK and PKA co-activation during LTP structural changes by Tang and Yasuda (2017) as well as the fact that ERK-mediated signaling is a downstream target of ADCY1 activation, artificial p-ERK activation via EPA administration provides a plausible explanation for the loss of behavioral effects previously seen following linalool administration.

While these initial findings suggest ERK phosphorylation levels to be vital in regulating EE-potentiated anxiolysis induced by L2000-CBD, it should be mentioned that the effects observed following EPA administration undoubtedly involves ERK interaction with other proteins and their associated signaling pathways, such as the PI3K (phosphoinositide 3-kinase) pathway known to be implicated in crosstalk activity as well as regulation of the MAPK/ERK pathway (Steelman et al., 2011; Won et al., 2012). Thus, while these initial findings provide insight into the gross relations between EPA administration and associated reductions in EE-potentiated anxiolytic efficiency, further investigations studying associated crosstalk proteins...
(alone and in tandem) are required to elucidate the exact molecular mechanisms underlying these behavioral correlations.

**Chronic Unpredictable Stress: EE-Potentiated effects in behavioral assays**

<table>
<thead>
<tr>
<th><strong>Behavioral Assay</strong></th>
<th><strong>Result</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>Spontaneous Alternation Test</td>
<td>No differences in working memory</td>
</tr>
<tr>
<td>Novel Object Recognition Test</td>
<td>No differences in long-term memory</td>
</tr>
<tr>
<td>Open Field Test</td>
<td>No differences in locomotion or anxiety</td>
</tr>
<tr>
<td>Light Dark Box Test</td>
<td>↓ Anxiety</td>
</tr>
<tr>
<td>Social Motivation and Recognition Test</td>
<td>No differences in social motivation</td>
</tr>
<tr>
<td>Elevated Plus Maze Test</td>
<td>↑ Social Recognition</td>
</tr>
<tr>
<td></td>
<td>↓ Anxiety*</td>
</tr>
</tbody>
</table>

**Figure 30 Summary of Chronic Stress Cohort Results from Behavioral Assays.** *Moderately effective chronic stress model.*

Utilizing the novel chronic unpredictable stress (CUS) protocol established by former laboratory researcher Dr. Justine Renard, no significant differences in locomotor activity were found while significant differences were observed between treatment groups in the light dark box and three-chamber social interaction test that suggest the presence of EE-potentiated effects. Although employing different variants of the CUS protocol, various studies have reported similar findings of relatively equal locomotor activity but increased perceived anxiety by CUS-exposed subjects when compared to non-stressed controls (Kaufmann and Brennan, 2018; Monteiro et al., 2015). Interestingly, the inability of L2000-CBD to reverse the observed anxiety in the CUS-
VAirVPBS group stands in contrast to these literature findings and previous results of open-space anxiolysis observed between L2000-CBD and VAirVPBS groups in the challenge cohorts. While longer duration (4-8 weeks) and variations in CUS protocols, as well as use of mouse models may explain the differences in OFT results between those found in the literature (Kaufmann and Brennan, 2018; Monteiro et al., 2015) and this study, discrepancies concerning significant differences comparing L2000-CBD and VAirVPBS vehicle groups between challenge cohorts and the chronic stress cohort are very likely to be attributable to vast variations in animal handling duration prior to testing. Specifically, the fact that the L2000-CBD and VAirVPBS groups experienced increased handling durations (an extra 14 days prior to testing due to CUS protocol procedures) compared to their equivalents in the challenge cohort likely resulted in a significant reduction of baseline anxiety levels; combined with the fact that the OFT assay was also conducted relatively early on in the testing timeline following minimally stressful behavioral assays (SA and NOR), it is entirely plausible that the reduced baseline anxiety levels remained present during open field testing—effectively attenuating significant differences in open-space anxiety previously observed in the challenge cohorts between the L2000-CBD and VAirVPBS groups.

Studies employing CUS protocol variants have also documented chronically stressed rodent models exhibiting significant increases in anxiety symptoms compared to their non-stressed control counterparts within the light dark box (Beery et al., 2012; Cancela et al., 1995; Nasca et al., 2015) and elevated plus maze behavioral assays (Kaufmann and Brennan, 2018; Monteiro et al., 2015; Rudyk et al., 2019). These established literature findings are consistent with the observed anxiety measures in the LDB test, where a pre-planned t-test comparison between VAirVPBS and CUS-VAirVPBS groups revealed the non-stressed group to exhibit
significantly greater exposure in the light compartment. Interestingly however, this correlation between vehicle groups was only moderate in nature within the EPM assay ($t_{(14)} = 1.872, p = 0.082$; data not shown); this reduction of stress model efficiency may be attributable to the fact that the employed CUS protocol was shorter in nature (2 versus 3 weeks exposure to CUS in the aforementioned studies) and that the EPM test was conducted 14 days after cessation of the CUS protocol (designated last in the battery of tests) in comparison to the LDB test which was carried out 7 days following protocol termination.

Despite this lag in testing, the L2000-CBD treatment was found to result in significantly reduced anxiety within both the LDB and EPM assays, as was observed with the significantly increased exploration in the light compartment and open arms exhibited by the CUS-L2000-CBD group in comparison to its CUS-$V_{Air}$-$V_{PBS}$ counterpart—suggesting that in the context of chronic stress conditions, L2000-CBD treatment may be effective in alleviating both augmented levels of bright-space and open-space anxieties. When considering the establishment of EE-potentiated anxiolysis between L2000-CBD and its vehicle counterparts ($V_{Air}$-$V_{PBS}$, $V_{Air}$-CBD, L2000-$V_{PBS}$) in the baseline cohort as well as assessing the similarities in observed behavioral phenomenon between chronic control and experimental groups, the inference of EE-potentiation within these chronic stress behavioral assays stands to be of merit. While Campos et al. (2013) and Saiyudthong et al. (2016) have demonstrated individual administrations of CBD or linalool to be effective in reversing chronic stress-induced alterations observed in anxiety and depression behavioral assays respectively, their use of daily intraperitoneal administrations starkly contrasts the single pre-test intracranial drug infusions employed within this thesis. Thus, to date, the persistence of conceivable EE-potentiated anxiolysis within chronic stress settings remains a plausible line of inquiry.
Although there were clear differences in the degree of anxiety symptoms presented between CUS-exposed and non-stressed treatment groups in anxiety assessments, results from pre-planned t-test comparisons (data not shown due to non-significance) and ANOVA analysis of SA and NOR tests suggest that working and long-term memory were not impaired in chronically stressed vehicle models. The non-significance found in these memory cognition tests are consistent with findings from Sadowski et al. (2009), Bowman et al. (2006) and Foyet et al. (2017), with the latter employing a CUS protocol most similar in nature to the one used in this thesis. However, despite the lack of memory impairments in CUS rat models, CUS-VAirV PBS subjects were found to exhibit significantly lower SMI and SRI values versus non-stressed V AirV PBS in stages 2 and 3 respectively of the social motivation and recognition test. This deficiency in both social motivation and recognition is consistent with the existing literature assessing effects of chronic stress on equivalent social interaction measures between non-stressed and chronically stressed vehicle groups (Van der Kooij et al., 2014; 2014a; Zain et al., 2019).

The fact that no apparent memory deficits were found between groups within the chronic stress cohort suggests that the significant SMI and moderate SRI deficits presented by CUS-exposed control subjects (in comparison to stress-free V AirV PBS subjects) likely stem from affective processing dysfunction rather than diminished memory capabilities. This finding is particularly interesting in light of the literature surrounding the impact of chronic stress on different brain regions. For while the hippocampus, which is well-known for its involvement in the recall of episodic memory (Ross et al., 2018), can endure several weeks of chronic stress before undergoing structural alterations (Mcewen, 2004), atrophy of dendrites within the PFC has been found to result after a one week duration of CUS or even a single exposure event to
such stressful conditions (Brown et al., 2005; Izquierdo et al., 2006). Given that the PFC is one of the key components within the mesolimbocortical pathway involved in affective processing and executive decision making, structural atrophy of this region—and in particular, degradation of its connections to the amygdala—would help explain the decreased sociability and social novelty exhibited by CUS-treated vehicle groups, as the loss of PFC regulation over the amygdala would provide a likely explanation for this observed phenomenon in the absence of memory impairments (Liu et al., 2020).

Given that L2000-CBD treatment was able to reverse SMI and SRI deficits to a moderate and significant degree respectively in CUS-treated subjects, this in turn suggests that the consequentially inferred EE-potentiation likely involved altered activation of the PFC and associated brain regions in eliciting the changes in observed behavioral phenomena. Taking into consideration that CBD and linalool have both been well documented to act on 5-HT$_{1A}$ and GABA$_{A}$ receptors which are essential in the regulation of neuronal activity, the fact that vast numbers of these receptors reside within brain regions such as the PFC and amygdala lends credence to potential mechanisms of action underlying observed EE-potentiated effects (Albert et al., 2014; Ghosal et al., 2017; Liu et al., 2014). Ultimately however, further investigations will need to be pursued to address this mechanistic line of inquiry pertaining to EE-potentiation and brain region activation.
Molecular Profiles underlying EE-potentiated Anxiolysis

When assessing the possibility of an EE-potentiated anxiolysis between CBD and linalool (2000μL), it is important to not only observe effects on a systems behavioral level but also on the molecular level to potentially determine any underlying mechanisms that may correlate between the two phenomena. While certain trend patterns were observed, the lack of significant differences in all p/T protein expressions between V_{Air}V_{PBS} and the linalool vehicles (L200-V_{PBS} and L2000-V_{PBS}) suggests that odorous linalool administration on its own, whether at the 200 or 2000μL dosage, does not yield notably different protein activation profiles compared to vehicle

<table>
<thead>
<tr>
<th>Groups</th>
<th>Biomarkers and associated p/T ratios vs. vehicle</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GSK-3β</td>
</tr>
<tr>
<td>V_{Air}V_{PBS}</td>
<td>—</td>
</tr>
<tr>
<td>L200-V_{PBS}</td>
<td>—NacSh</td>
</tr>
<tr>
<td>L2000-V_{PBS}</td>
<td>—NacSh</td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th>Groups</th>
<th>Biomarkers and associated p/T ratios vs. L2000-CBD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GSK-3β</td>
</tr>
<tr>
<td>V_{Air}V_{PBS}</td>
<td>—PFC, —NacSh</td>
</tr>
<tr>
<td>V_{Air}CBD</td>
<td>↑PFC, ↑NacSh</td>
</tr>
<tr>
<td>L200-CBD</td>
<td>—PFC, —NacSh</td>
</tr>
<tr>
<td>L2000-CBD</td>
<td>—</td>
</tr>
</tbody>
</table>

Figure 31 Summary of Phosphorylated/Total Protein Ratio Results from Western Blot Assays. All values are based upon % vs vehicle (V_{Air}V_{PBS}). “PFC” denotes the pre-frontal cortex, while “NacSh” denotes the nucleus accumbens shell region. “—” denotes lack of significance, while “↑” denotes significantly increased protein expression compared to its respective treatment comparison (V_{Air}V_{PBS} as represented by sub-figure A, or L2000-CBD as represented by sub-figure B); one-way ANOVA, P ≤ 0.05. All significant differences are highlighted in red for ease of viewing.
conditions. Notably, this molecular observation correlates with the relative lack of significant differences between linalool vehicles and the $V_{Air}V_{PBS}$ treatment group found in the majority of one-way ANOVA analyses evaluating behavioral assay results.

In contrast, while no significant differences in p/T protein expression were found between the $V_{Air}V_{PBS}$ and L2000-CBD groups, the $V_{Air}$CBD group presented significantly different protein levels compared to the L2000-CBD as shown in Figure 31B (these differences are highlighted in red within the summary table). When considering the results from behavioral assays conducted, this in turn suggests that linalool likely acts in a manner that either 1) interacts with CBD at the receptor level via allosteric modulation or receptor-complex formation, 2) alters the effects of downstream signaling originally induced by CBD, 3) targets proteins and their associated pathways outside the scope of the current molecular investigations or 4) results in a combination of all these potential mechanisms. While addressing these mechanistic potentials however, the results of each protein will be discussed in relation to one another as these proteins ultimately interact with one another in either a direct or indirect manner.

Through in-vivo investigations utilizing behavioral assays following the knockdown of GSK3β, Crofton et al. (2017) demonstrated that decreased GSK3β expression was correlated with reductions in anxiety-like behavior in rodent models. In relation to the thesis molecular findings, this is particularly interesting given the fact that while there were no significant differences found in p/T-GSK3β expression between the L2000-CBD or L2000-$V_{PBS}$ treatment and the $V_{Air}V_{PBS}$ group, the $V_{Air}$CBD group exhibited significantly increased levels in comparison to both the $V_{Air}V_{PBS}$ group and L2000-CBD treatment groups. The increase in p/T-GSK3β expression following $V_{Air}$CBD administration and the subsequent decline in following the introduction of linalool into CBD treatment formulations suggests that linalool likely acts in
some manner as an inhibitor of GSK3β phosphorylation—in turn suggesting that L2000-CBD treatment achieves behavioral anxiolysis by inducing molecular changes within both the PFC and NAcSh that either utilize a GSK3β-independent pathway and/or target downstream GSK3β effectors.

The results of the VAirCBD treatment group are supported by the current advancements in literature, which document CBD to noticeably increase p-GSK3β expression (Fogaça et al., 2014; Libro et al., 2016; Vallée et al., 2017); furthermore, Libro et al. (2016) and Renard et al. (2016) had specifically noted correlations between Akt activation and observed p-GSK3β levels. The findings of Libro et al. (2016), who utilized in-vitro assays of mesenchymal stem cells and found CBD administration to result in increased expression of p-GSK3β and p-Akt, provide greater support for the observed increases in these respective phosphorylated,total protein levels following VAirCBD administration; unfortunately however, due to lack of specifications regarding Ser- and Thr-residue phosphorylation, site-specific correlations cannot be referenced to. Interestingly however, Renard et al. (2016), whom utilized the same intra-NAcSh delivery of CBD employed in this thesis, discovered significantly decreased p/T-GSK3β levels concurrent with significantly decreased p/T-Akt levels in CBD-treated versus vehicle-treated AMPH-sensitized rats, further supporting the findings of Libro et al. (2016). This exception to the classical antagonistic relationship between the two proteins has in fact been documented by Beurel et al. (2015), who noted that Akt serine-phosphorylation does not result in absolute inhibition of GSK3 activity—and that in some cases such as the combined inhibitory phosphorylation of AMP-activated kinase (AMPK) by GSK3 and Akt, the two proteins can work in a cooperative rather than antagonistic manner. While the AMPH-sensitization of rat models used by Renard and authors does not provide an ideal comparison to the models utilized in this
thesis, the combined findings of Libro et al. (2016), Renard et al. (2016) and Beurel et al. (2015) nonetheless suggest that CBD may elicit its effects through a mechanism of action that bypasses the Akt/GSK-3 signaling pathways, providing an interesting finding that contrasts traditional associations of inversely-correlated protein activity between GSK3β and Akt (Sani et al., 2012), as well as a potential component mechanism of action through which L2000-CBD elicits EE-potentiated anxiolysis.

In contrast to the documented increase in inhibition of GSK3β following CBD treatment, the molecular literature surrounding linalool focuses on Akt, and thus in extension, its downstream regulation of GSK3β. An in-vitro study conducted by Pan and Zhang (2019) observed in a clear-cut manner that linalool exposure (μM range) resulted in a dose-dependent decrease in both phosphorylated PI3K and Akt, while expression of non-phosphorylated PI3K and Akt remained unchanged. Although no significant differences between linalool-vehicles and the control condition were observed in-vivo in this thesis when assessing either p/T-GSK3β or p/T-Akt, the results of Pan and Zhang help to explain the significant reduction in both protein expressions observed in the L2000-CBD treatment group (when compared to the V_Air CBD group)—ultimately suggesting that linalool may antagonize CBD-mediated effects on GSK3β and Akt through inhibition of the PI3K/Akt pathway. Interestingly however, Rodenak-Kladniew et al. (2018) had found that when employing a higher concentration of linalool in-vitro (in the range of mM rather than μM), there was a negative correlation between phosphorylated Akt levels and linalool concentration trending towards non-significance between the vehicle and 2.0 mM (highest dosage) linalool exposure groups; nonetheless, 1.0 and 1.5 μM of linalool concentration exposures were sufficient to elicit significant declines in phosphorylated Akt levels compared to control conditions. While this discrepancy in findings between the two in-vitro
studies may be attributable to differences in the cell line utilized (OECM-1 vs. HEPG2 respectively), it potentially proposes the fascinating concept of a biphasic effect profile for linalool on the molecular level that presents at specific dosages and via particular administration routes. Consequently, given that these referenced studies involved direct contact of pure linalool with cell cultures, further investigation utilizing intraperitoneal injections of linalool with rat animal models can help clarify whether this dose-dependent response is replicable within in-vivo models and in turn confirm whether there are significant differences in molecular activity profiles between odorous and intraperitoneal exposures to linalool.

When analyzing the potential mechanistic pathways through which these proteins elicit their effects, it is important to consider the duration of treatment administration (which was acute, or at most arguably sub-chronic in nature). Thus, while alterations in GSK3β and Akt activity are known to induce long-term changes through nuclear localization of downstream target effectors and subsequent changes in mRNA production (Steelman et al., 2011), short-term changes are crucial to consider in the context of acute/sub-chronic treatment protocols. A relatively acute mode of action to consider is the ability of GSK3β to inhibit NMDAR-dependent LTP and induce NMDAR-dependent LTD (long-term depression) as demonstrated by Peineau et al. (2007); Peineau and authors had found in particular that inhibition of GSK3β during LTP via activation of the PI3K-Akt pathway prevented synapses from undergoing LTD for up to 1 hour. In addition, Leibrock et al. (2013) had found that while Akt2 knockout mice displayed increased anxiety, they did not present cognitive impairments. Taking these two findings together into consideration, it is plausible that reduced Akt activation—and consequently increased GSK3β and LTD processes—observed in the L2000-CBD group resulted in decreased NMDAR activity and associated NMDAR-dependent LTP that disrupted the formation of synaptic strengthening
that would otherwise augment the consolidation of anxiety-related affections and associated memories in anxiety assays (most notably the CFC test). This hypothesized, fast-acting NMDAR-dependent mode of action itself is substantiated by studies demonstrating both decreased depression and anxiety following NMDAR antagonism (Holubova et al., 2014; Vose and Stanton, 2017). Given the roles of PFC and the NAcSh in working memory and associated affective processing respectively, these observed findings of reduced p/T-GSK3β and p/T-Akt levels elicited by L2000-CBD treatment in comparison its vehicle counterparts present a case for acute resilience to environmental stressors, providing in turn an interesting line of inquiry to pursue in further investigations assessing mechanisms underlying EE-potentiated anxiolysis induced through L2000-CBD treatment.

In addition to GSK3β and Akt expression levels, ERK and JNK proteins have been found to be integral in the modulation of affective processing; specifically, their increased activation has been associated with increased anxiety-like behavior (Ailing et al., 2008; Hollos et al., 2018; Mohammad et al., 2018; Pucilowska et al., 2015; Xiang et al., 2017; Zhao et al., 2017). When it comes to the individual EE-components, CBD exposure (20μM) to in-vitro cultures involving glioblastoma cells has been documented by Ivanov et al. (2017) to result in significantly upregulated levels of p-JNK1/2, while downregulating p-ERK1/2 and p-Akt expression; however, these p-ERK molecular results stand in contrast to the findings of McAllister et al. (2011) who observed CBD treatment (1.5μM) to significantly increase p-ERK expression while not affecting T-ERK expression in in-vitro cultures of human breast cancer cells (MDA-MB231). While this contrast in findings may be explained by the differing nature of the cells used, the change from upregulation to downregulation of p-ERK1/2 following an increase from 1.5 to 10 μM CBD exposure can potentially be attributed to the biphasic effects of CBD
observed by Gallily et al. (2015) following intraperitoneal administration. Specifically, the discovery that this biphasic effect was found present in serum TNFα levels (u-curve correlation between increased CBD dosage and serum TNFα expression) provides support for this potential mechanism of action, as TNFα is capable of activating ERK and JNK kinases (which would in turn phosphorylate ERK and JNK to their active forms) (Sabio and Davis, 2014). Furthermore, this may not only help explain the discrepancy in molecular activation profiles between the studies conducted by McAllister et al. (2011) and Ivanov et al. (2017), but could also provide a basis for differences in p-ERK activation following bilateral intracranial infusions utilized in this thesis and by Hudson et al. (2019)—as Hudson and authors had documented no significant differences in p-ERK expression between control and CBD treatment groups following 100 ng/0.5 μL CBD administration (in comparison to the 5 ng/0.5 μL used in this thesis).

Collectively, these findings not only highlight the potential for biphasic effects on the molecular level following CBD administration but also bring to the forefront possible considerations of TNFα being a key component in CBD’s modulatory effects on affective processing.

Contrasting the observed increase in p-ERK and p-JNK following V_AirCBD administration, the addition of linalool to CBD formulations was shown to elicit a moderate dose-dependent decrease in p-ERK and p-JNK, with a significant reduction being observed following the addition of 2000μL linalool as represented by the L2000-CBD treatment group. These reductions in p-ERK and p-JNK are consistent with the current advancements in literature regarding the molecular effects of linalool administration (Caputo et al., 2018; Gunaseelan et al., 2017). Closer examination reveals that Gunaseelan et al. (2017) recorded in particular the inhibition by linalool of UVB-induced (ultraviolet B rays) ROS formation and associated UVB-induced phosphorylation of ERK and JNK proteins, whilst also inhibiting the formation of
biomarkers including TNFα (presenting TNFα-mediated inhibition of ERK and JNK pathways as a combined target for linalool + CBD treatment). While UVB-induced ROS was evidently not employed in this thesis, the key cellular event of ROS formation that leads to increased levels of p-ERK and p-JNK has been observed to occur in animal models following acute (≤ 1 hour) or chronic stress exposure (Huber et al., 2017; Nadeem et al., 2006; Zafir and Banu, 2009). As a result, these findings correlating ROS activity and decreased p-ERK and p-JNK expression following linalool exposure are arguably applicable to the thesis molecular observations, as these protein analyses were ultimately conducted following acute/sub-chronic stress exposures of rodent models.

Expanding on the work of Caputo and authors, in addition to inhibitions of p-ERK in the SH-SY5Y neuroblastoma cell line, studies conducted (2016; 2017; 2018) had shown that ADCY1 and PKA expression were also reduced in SH-SY5Y cells following linalool administration. Given that the activation cascade of ADCY1/cAMP/PKA leads to the initiation and maintenance of CREB alterations on the genomic level that are instrumental in the prognosis of mood disorders (Dwivedi and Pandey, 2008) as well as the fact that PKA activity has shown to be crucial in initiating neurite extension (Sánchez et al., 2004), some of the contributory effects of linalool towards EE-potentiated anxiolysis may be attributable to the previously mentioned regulation of LTP/LTD events in affective formation and processing. The linalool-induced downstream modulation of PKA activity is particularly interesting to note given that in-vitro studies conducted by Poisbeau et al. (1999) demonstrated that PKA activation in adult hippocampal neurons decreased GABAAR current amplitudes, providing a correlation between the findings of Caputo et al. (2016; 2017; 2018) and the significantly reduced anxiolysis exhibited by the L2000-CBD+flumazenil in the flumazenil challenge cohort. Altogether, these
studies highlight a mechanism of action unique to linalool that potentially differentiates the lack of anxiolysis observed following $V_{Air}$CBD treatment from the presence of EE-potentiated anxiolysis following L2000-CBD treatment. It is interesting to note however that while significant differences between treatment groups were present in regard to p/T-JNK1/2 and p/T-ERK1/2 levels, these differences were relatively region-specific. Increasing dosages of linalool exposure beginning with the linalool-free $V_{Air}$CBD treatment led first to reductions in PFC p/T-JNK1/2 levels, with even greater dosage exposure of 2000 μL linalool yielding reductions in both the PFC and NAcSh regions (when compared to $V_{Air}$CBD group data); furthermore, p/T-ERK1/2 levels exhibited by $V_{Air}$CBD and L200-CBD groups were only significantly higher than those observed following L2000-CBD treatment in the PFC, and not the NAcSh region. Given that the drug delivery methods did not include intra-PFC administrations, these unique patterns of phosphorylation suggest a few inter-region mechanisms of interest including NAcSh-mediated inhibition of PFC MAPK/ERK signaling and an associated PFC-mediated reciprocal inhibition of NAcSh MAPK/ERK signaling at higher doses of linalool (2000 μL)—both of which are reflective of the GABAergic and glutamatergic connections between the two structures of the corticostriatal pathway.

Altogether, these studies assessing the individual effects of CBD and linalool on ERK activity propose a variety of mechanisms which may underlie correlations between phosphorylated ERK levels and the EE-potentiated anxiolysis observed in the baseline cohort, including CBD’s attenuation of TNFα levels, linalool’s reduction of ROS formation and PKA activation (Caputo et al., 2016; 2017; 2018; Gunaseelan et al., 2017; Sabio and Davis, 2014)—all of which have been described to result in decreased ERK activation. In addition, studies have demonstrated that GSK3β and ERK activity are inversely correlated, with ERK-mediated
inhibition of GSK3β activity being noted to particularly involve Akt activation (Pal et al., 2017; Wang et al., 2006). This inverse correlation between the two proteins helps to further explain the significant differences in molecular activation between the $V_{AI}, V_{PBS}$ and L2000-CBD baseline cohort groups, as the latter has been documented to uniquely involve the inhibition of ERK, and consequently non-inhibition of GSK3β through direct upstream PKA modulation in a linalool-dependent manner whilst antagonizing CBD-mediated effects on GSK3β and Akt through PI3K/Akt pathway inhibition as described previously (Caputo et al., 2016; 2017; 2018; Pan and Zhang, 2019). Although western blot analysis of EPA challenge cohort data is required to confirm this interpretation, when considering these literature findings in conjunction with the results of reduced THC+CBD treatment anxiolysis following EPA co-administration in the study conducted by Hudson et al. (2019) and the attenuation of L2000-CBD anxiolytic efficacy when concurrently administered with EPA found in this study, the combined results of baseline cohort molecular analysis and EPA challenge behavioral analysis ultimately suggests that ERK protein activation serves as a likely antagonist of L2000-CBD mechanistic interactions that result in EE-potentiated anxiolysis.

While these proposed individual and concerted mechanisms of action for GSK3β, Akt, ERK and JNK provide insight into the generation of EE-potentiated anxiolysis, it is also important to consider the influence of these proteins on cell-surface receptor targets—in particular the GABA-A receptor, to address potential correlations between systems-level, receptor-level and intracellular activity present in the flumazenil challenge cohort data. Interestingly, GABA$_A$R activity has been found to be negatively correlated to GSK3β activity through a D2-receptor mediated suppression mechanism (Beurel et al., 2015; Li et al., 2012). Furthermore, this suppression mechanism appears to be a product of a larger signaling cascade.
involving Akt, termed the Akt/GSK3β signaling cascade (Beaulieu et al., 2007; Li et al., 2012). Although these literature findings documenting the collective inhibitory regulation of Akt and GSK3β on GABA<sub>A</sub>R activity correlate with p/T-Akt data following L2000-CBD treatment observed in this thesis, there exists lack of a significant increase in p/T-GSK3β following L2000-CBD treatment. While this prevents a complete correlation from being established between these literature findings and the molecular data observed in this thesis, the exact mechanisms of action through which the Akt/GSK3β cascade modulates GABA<sub>A</sub>R activity—whether it be through direct modulation of phosphorylation sites or downstream effectors—remains unclear. In light of this complexity and the research conducted by Beurel et al. (2015) detailing that certain GSK3β protein complexes may be unaffected by inhibitory serine-phosphorylation, while the effects of the Akt/GSK3β signaling cascade may not be directly correlated to observed thesis data, the cascade mechanism presents nonetheless an interesting line of inquiry to pursue in investigating the relationship between these proteins, the GABA<sub>A</sub> receptor and the EE-potentiated anxiolysis found following L2000-CBD treatment in the baseline cohort.

In addition to Akt and GSK3β, the two MAPK proteins—ERK and JNK—have also been found to impact GABA<sub>A</sub>R activity. Through in-vitro investigations using the HEK293 cell line, Bell-Horner et al. (2006) demonstrated that an ERK phosphorylation site (T375) on the GABA<sub>A</sub> receptor was essential in facilitating ERK-mediated inhibition of GABA<sub>A</sub>R activity; furthermore, administration of UO126 (inhibitor of MEK1/2, which is an upstream activator of ERK) resulted in the reduction of ERK phosphorylation/activation and consequently yielded increased GABA<sub>A</sub>R peak current amplitudes. Supporting this finding is the in-vitro research published by Brady et al. (2018), where enhancement of GABA<sub>A</sub>R activity via application of the GABA<sub>A</sub>R agonist muscimol resulted in delayed ERK activation. Interestingly, GABA<sub>A</sub>R agonism via
muscimol administration was also found to affect JNK pathway activity (Ma et al., 2016); a deeper investigation specifically revealed that increased GABAAR activity as a result of GABA or muscimol treatment resulted in significantly reduced JNK1/2 signaling activity compared to controls and that subsequent inhibition of GABAAR activity restored JNK1/2 activity.

Collectively, these in-vitro studies (Bell-Horner et al., 2006; Brady et al., 2018; Ma et al., 2016) demonstrate an opposing relationship between GABAAR activity and the two MAPK proteins, ERK and JNK, which help to explain the significant decrease in p/T-ERK and p/T-JNK levels exhibited by the L2000-CBD group when compared to the VAirVPBS group in the baseline cohort and the corresponding differences in anxiolysis achieved by the two treatments. Lastly, although western blot analysis of the flumazenil challenge cohort is required to substantiate these interpretations, the correlation in findings between GABAAR activity, molecular profiles supported by the literature and anxiolytic differences between the L2000-CBD and control treatment groups suggests that flumazenil—which effectively attenuated the anxiolytic efficiency of the L2000-CBD treatment to vehicle levels—and consequently GABAAR antagonism are able to effectively reverse EE-potentiated anxiolysis elicited by L2000-CBD treatment.
**Figure 32 Summary of Linalool, CBD Cellular Targets and Molecular Pathways Associated with GABA$_A$R-mediated anxiolysis.** Visual representation of cellular targets impacted by linalool and CBD (denoted by green activating or red inhibitory mechanisms of action) to exert anxiolysis detailed in the literature covered in this thesis, including the GABA$_A$ receptor complex (primarily through the $\alpha$-subunit), ADCY1, RTK, TNFR via modulation of TNF$\alpha$ levels, as well as the downstream targets of these proteins/receptors which affect GABA$_A$R activity. “?” and dotted lines denote uncertainty surrounding mechanism through which molecules exert their effects on the GABA$_A$ receptor (JNK, Akt/GSK3$\beta$ cascade). “Cellular Signaling Processes” represent the crosstalk between Akt and ERK pathways which may be of interest in future investigations. ERK T375 Site ($\alpha_1$) denotes the GABA$_A$R $\alpha_1$ subunit target for ERK phosphorylation. ADCY1, adenylate cyclase 1; Akt, protein kinase B; cAMP, cyclic adenosine monophosphate; CBD, cannabidiol; ERK, extracellular signal-regulated kinase; JNK, $\epsilon$-Jun N-terminal kinase; PI3K, phosphoinositide 3-kinase; PKA, protein kinase A; ROS, reactive oxygen species; RTK, receptor tyrosine kinase; TNF$\alpha$, tumour necrosis factor alpha; TNFR, tumour necrosis factor receptor.
While these collections of studies help to elucidate the EE-potentiated mechanism of CBD-linalool treatments, conclusive interpretations remain relatively difficult to establish without further investigation. A primary concern is that although these fascinating results and associated mechanisms may show merit, the current thesis findings and supporting studies discussed can only at best establish a correlational impact between olfactory nervous system activation and downstream effects on the entorhinal cortex and hippocampus (and thus episodic memory formation). For while the literature reports that lavender oil has been shown to modulate autonomic nerve activity and relevant signaling pathways in the CNS (Koulivand et al., 2013), without electrophysiological investigations assessing hippocampal activity following linalool administration, only assumptions can be made between behavioral phenomena, biomarker levels and associated brain activity.

There also remain systems-level and molecular-level complexities to consider in the analysis of behavioral and molecular observations following L2000-CBD treatment. On the systems-level, given that the NAcSh forms an integral component of the greater mesolimbocortical pathway and that the interactions between the PFC, amygdala and nucleus accumbens as a result of their circuit connections have been deemed essential in affective processing (Jackson and Moghaddam, 2001), this suggests that the observed molecular profiles within the PFC region played a key role in producing the behavioral outcomes recorded following treatment exposures. However, the manner in which mesocorticolimbic regions such as the PFC and VTA respond to molecular changes and subsequent reciprocal regulation of associated brain regions—notably in conjunction to the NAcSh—following L2000-CBD administration remains unclear. As mentioned in section 1.2.3, this consideration of associated mesocorticolimbic regions is especially important when it comes to the VTA, as there exists a
prominent feedback system between the NAcSh and VTA that regulates dopaminergic activation of the NAc (and associated structures) as described previously; in addition, VTA glutamatergic inputs have shown to play a regulatory role of intra-NAc GABAergic interneurons, which subsequently in turn modulate the inhibitory projections/ability of the NAc (Qi et al., 2016).

On the molecular level, an important complexity to consider is that significant differences in Akt protein expression were solely found in relation to the Ser473—and not Thr308—site of Akt. This is particularly interesting since Thr308 site-specific phosphorylation was originally thought to be conclusive evidence of Akt activation—and yet emerging evidence suggests that not only can the two sites regulate one another, but that downstream effects differ depending on site-specific phosphorylation and that both sites are required to be phosphorylated for full activation of Akt (Vadlakonda et al., 2013; Yung et al., 2011). Ultimately, further investigations incorporating the use of electrophysiology, as well as specifically disconnection studies between NAcSh and these associated brain regions following the use of protein-specific inhibitors (including phosphorylation site-specific activators/inhibitors in the case of Akt) may help to elucidate the correlation between molecular alterations and neuronal activity both within these regions, and in relation to the NAcSh.

Last but not least, there exists the fact that within this thesis there existed a lack of significant differences between the V_{Air}V_{PBS} control and L2000-CBD treatment groups on the molecular level which did not correlate with differences observed in behavioral assays. While this may appear as an obstacle in understanding the observed EE-potentiated anxiolysis on the behavioral level, this interesting phenomenon can be explained by unique molecular interactions outside of the scope of this thesis investigation as well as the possibility that there may exist unique molecular profiles exhibited in other mesocorticolimbic associated regions (such as the
amygdala, hippocampus) induced by L2000-CBD treatment that are different in nature when compared to $V_{\text{Air}} V_{\text{PBS}}$ treatments. Subsequently, these differences in molecular profiles may be associated with unique modulations of neuronal activity within the mesocorticolimbic system that can help elucidate the contrasting degrees of anxiolysis observed following the two treatment administrations.

Nonetheless, despite these mysteries which remain unanswered and the endless molecular interactions which potentially underlie the observed molecular profiles following treatment administration, this exploratory thesis ultimately establishes a case for the presence of the entourage effect between cannabidiol and linalool that augments their collective anxiolytic potential.

**Conclusions and Future Directions**

As mainstream adoption of cannabis and cannabis-related products grow, there exists a growing need for information surrounding the properties and associated mechanisms of cannabis to ensure safe usage, whether recreational or medicinal. Furthermore, recent advances in the past two decades regarding cannabis research has characterized the existence of various phytocannabinoids such as CBD and THC, as well as a multitude of terpenes which have demonstrated therapeutic properties when administered in an isolated manner. Increasingly however, explorations are being made in the field regarding combinatorial formulations of phytocannabinoid-phytocannabinoid and the potential for a superior therapeutic effect not elicited by either component substance alone; this effect, known as the Entourage Effect, has yet to be thoroughly explored in regard to phytocannabinoid-terpene formulations.

This thesis explored the potential presence of an Entourage Effect between linalool and
cannabidiol, demonstrating for the first time using behavioral assays that a combinatorial administration of these two compounds can yield EE-potentiated bright-space and fear-memory induced anxiolysis in acute treatment and chronic stress settings, as well as a significant reduction in open-space anxiety within specific contexts. In addition, this Entourage Effect was found to be critically modulated by GABA_{A}R regulation and ERK activation, highlighting two potential mechanisms underlying the observed EE-potentiated anxiolysis following concurrent administration of odorous 2000 μL linalool and intra-NAcSh 5 ng/0.5μL cannabidiol treatment. While the lower dosage combination treatment involving 200 μL linalool and 5 ng/0.5μL cannabidiol resulted in some instances of EE-potentiated anxiolysis, the presence of the Entourage Effect was not as consistent as the higher linalool dosage combinatorial treatment; in conjunction with the absence of significant differences between these two treatments, dose-dependent effects cannot be concluded at this time.

Observations of molecular profiles demonstrated differential activations of GSK3β, Akt, ERK and JNK across treatment groups when standardized against control group expression levels. While the lack of significant differences in protein expression between V_{Air}V_{PBS} and L2000-CBD groups strongly suggests there exists other associated proteins and brain regions involved in characterizing the molecular profile elicited following L2000-CBD exposure, the preliminary findings of this thesis suggest that the EE-potentiated anxiolysis exhibited by this treatment group involves a unique interaction between the studied proteins and their associated pathways not observed in either component group alone.

Although questions ultimately remain regarding the underlying mechanisms of the observed EE-potentiated anxiolysis induced by L2000-CBD treatment, the establishment of the Entourage Effect between the two compounds and their associated dosages opens up a new
chapter in the therapeutic potential of cannabis and cannabis-related products—as in addition to linalool, there exists several more terpenes with anxiolytic profiles theorized to yield EE-potentiated effects that have yet to be explored. Given the relatively safe profile of these natural compounds when compared to conventional medications and their associated side effects, these natural medicines present a potential complimentary treatment, or even replacement in the treatment of anxiety and anxiety-related disorders. Furthermore, this thesis helps support emerging evidence suggesting that the NAcSh plays an integral role in affective memory formation and processing, thus giving greater credence to the brain region as a potential target for treatments towards mood disorders. Thus, while future investigations involving electrophysiological analysis, disconnection studies and associated brain regions in the mesocorticolimbic pathway are required to further substantiate any correlations between molecular and behavioral observations, this thesis nonetheless demonstrates the untapped therapeutic potential of cannabis—effectively laying the groundwork for future innovations in cannabis-related medical alternatives.
References


Campos, A. C., Ortega, Z., Palazuelos, J., Fogaça, M. V., Aguiar, D. C., Díaz-Alonso, J., ...


128


Hare, B. D., & Duman, R. S. (2020). Prefrontal cortex circuits in depression and anxiety: contribution of discrete neuronal populations and target regions. *Molecular psychiatry, 10.1038/s41380-020-0685-9*. Advance online publication. doi: 10.1038/s41380-020-0685-9


reviews. Neuroscience, 13(11), 769–787. doi: 10.1038/nrn3339


Saiyudthong, S., Meksepralard, C., & Srijittapong, D. (2016). PS120. Effects of linalool on...


Curriculum Vitae

Name: Richard Leu

Post-secondary Education and Degrees:

Western University
London, Ontario, Canada
2018-2020 MSc.

Western University
London, Ontario, Canada

Honours and Awards:

Natural Sciences and Engineering Research Council of Canada (NSERC) – Alexander Graham Bell Canada Graduate Scholarship 2019-2020

Dean’s Honor List
2014, 2016, 2017

Western University Four Year Continuing Admission Scholarship
2014, 2015, 2017

Related Work Experience:

Teaching Assistant
Western University
2018-2020

Clinical Research Assistant
St. Michael’s Hospital
2016, 2018

Clinical Researcher
University Health Network
2014-2015

Publications:
