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Characterizing the Anxiolytic Potential and Synergistic Efficacy of Cannabidiol and d-limonene

Nathashi Jayawardena, *The University of Western Ontario*

Supervisor: Laviolette, Steven, *The University of Western Ontario*

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Abstract

Emerging evidence has elucidated the anxiolytic properties of the cannabis-derived phytochemicals cannabidiol (CBD) and d-limonene and the 'Entourage Effect' wherein, multiple components of cannabis synergize to produce stronger effects than their pure counterparts. However, no studies have yet explored this effect in combinations of CBD and limonene. Thus, the present thesis investigated the anxiolytic and synergistic potential of concurrently administered intra-nucleus accumbens shell CBD (1 ng/0.5 µl or 5 ng/0.5 µl) and inhaled limonene (200 µl or 2000 µl). Additionally, the role of the 5-HT_{1A} receptor in mediating these effects was examined by co-application of the antagonist NAD299 and by the assessment of downstream molecular biomarkers. Findings from this study demonstrated for the first time that relative to their isolated counterparts, combinations of limonene and CBD more effectively reduces symptoms of anxiety, with the observed reversal of these effects with NAD299 elucidating a role at the 5-HT_{1A} receptor.

Keywords:

Cannabis, Cannabidiol, CBD, d-limonene, limonene, cannabinoid, monoterpene, terpene, Entourage Effect, anxiety, 5-HT_{1A}, NAD299, nucleus accumbens shell, mesocorticolimbic system.

Summary for Lay Audience

Current medications for anxiety disorders are fraught with adverse side-effects such as ongoing drug dependence, withdrawal, and memory loss. Thus, there is a critical need for the development of novel pharmacotherapies with safer and more tolerable profiles. While cannabis use has been associated with an increased risk of psychosis, these effects are associated with the cannabinoid tetrahydrocannabinol (THC). In contrast, extensive evidence has demonstrated that the cannabis-derived phytochemicals, cannabidiol (CBD) and d-limonene, possess anxiolytic properties, with several studies associating the anti-anxiety effects of CBD with specific brain regions associated with reward and mood dysfunction, namely the nucleus accumbens shell (NASh).

The citrus-scented monoterpene, d-limonene, has been shown to reduce anxiety in pre-clinical and clinical assays in a similar biochemical manner to CBD. In addition to their analogous properties, emerging evidence has alluded to a phenomenon known as the 'Entourage Effect' (EE). The Entourage Effect posits that multiple components in the cannabis plant interact to produce a stronger influence than each component in isolation, i.e., a synergistic effect. This effect is documented mainly for THC and other cannabis-derived phytochemicals and there is currently no knowledge of how combinations of CBD and limonene may produce clinically synergistic effects. Thus, this research project examined dose combinations of co-applied intra-NASh CBD and inhaled d-limonene that may provide the greatest anxiolytic efficacy, as well as assess associated changes in protein expression in the brain by pre-clinical modeling of anxiety-related behavioural and molecular assays in rodents.

The results of this study demonstrate for the first time the EE-potentiated anxiolytic effects of concurrently administered CBD and d-limonene. Additionally, by utilizing a specific molecular antagonist (NAD299) and by assessing changes in anxiety-related biomarkers, this thesis elucidated the potential 5-HT_{1A} receptor-mediated signalling brain pathways targeted by these formulations. Ultimately, these findings combined with the evidence that CBD and limonene exhibit remarkably safe pharmacological profiles, highlight the therapeutic potential of cannabis-derived compounds and their prospective use as a natural alternative or adjunct to current anti-anxiety medications.

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Abbreviations

5-HT	5-hydroxytryptamine, Serotonin
5-HT_{1A}/5-HT_{1A}R	5-hydroxytryptamine or Serotonin 1A Receptor
Akt	Protein Kinase B
Akt Ser473	Protein Kinase B Serine-473 Residue
AMG	Amygdala
AMPH	Amphetamine
ANOVA	Analysis of Variance
BLA	Basolateral Amygdala
BNST	Bed Nucleus of the Stria Terminalis
BZ	Benzodiazepine
CB	Cannabinoid
CBD	Cannabidiol
$CB_{1/2}$R	Cannabinoid Receptor Type 1 and 2
CFC	Contextual Fear Conditioning
CNS	Central Nervous System
DA	3,4-dihydroxyphenethylamine or Dopamine
DRN	Dorsal Raphe Nucleus
D1/D2/D3	Dopamine Receptor 1/2/3
EE	Entourage Effect
EPM	Elevated Plus Maze
ERK	Extracellular Signal-Regulated Kinase
ERK1/2	Extracellular Signal-Regulated Kinases 1 and 2
ES	Effect Size
GABA	Gamma-Aminobutyric Acid, γ-aminobutyric acid

$GABA_AR$	Gamma-Aminobutyric Acid Receptor A
$GABA_BR$	Gamma-Aminobutyric Acid Receptor B
GAD	Generalized Anxiety Disorder
GPCR	G-protein Coupled Receptor
GSK3	Glycogen Synthase Kinase 3
$GSK3_\beta$	Glycogen Synthase Kinase 3 Beta
HP	Hippocampus
JNK	c-Jun N-terminal Kinases
JNK1/2	c-Jun N-terminal Kinase Isoforms 1 and 2
L200CBD	Odourous 200 µL Limonene and Intracranial Cannabidiol
L2KCBD	Odourous 2000 µL Limonene and Intracranial Cannabidiol
L200VEH/L200V	Odourous 200 µL Limonene and Intracranial Phosphate-Buffered Saline
L2KVEH/L2KV	Odourous 2000 µL Limonene and Intracranial Phosphate-Buffered Saline
LDB	Light-Dark Box Test
LIM	Limonene
MAPK	Mitogen-Activated Protein Kinase
MCL	Mesocorticolimbic pathway
MRN	Median Raphe Nucleus
mTOR	Mammalian Target of Rapamycin
NAc	Nucleus Accumbens
NAcc	Nucleus Accumbens Core Region
NAcSh/NASh	Nucleus Accumbens Shell Region

NAD299	(3*R*)-3-(Dicyclobutylamino)-8-fluoro-3,4-dihydro-2*H*-1-benzopyran-5-carboxamide hydrochloride; 5-HT_{1A} antagonist
NMDA	N-methyl-D-aspartate
NMDAR	N-methyl-D-aspartate Receptor
OF	Open Field
PFC	Prefrontal Cortex
PI3K	Phosphoinositide 3-Kinase
PKA	Protein Kinase A
PTSD	Post-Traumatic Stress Disorder
S1/S2/S3	Stage 1, Stage 2, Stage 3 (in the context of a behavioural assay)
SAD	Social Anxiety Disorder
SEM	Standard Error of the Mean
SI	Social Interaction
SMI	Social Motivation Index
SRI	Social Recognition Index
SSRI	Selective Serotonin Reuptake Inhibitor
SNRI	Serotonin and Norepinephrine Reuptake Inhibitors
Δ9-THC/THC	Δ^9-tetrahydrocannabinol
TNF-α	Tumor Necrosis Factor-Alpha
TAN	Tonically Active Interneurons
VCBD	Odourous Vehicle (air) and Intracranial Cannabidiol
VVEH/VV	Odourous Vehicle (air) and Intracranial Phosphate-Buffered Saline
VST	Ventral Striatum
VTA	Ventral Tegmental Area

WHO	World Health Organization

1. Introduction

1.1 Properties of *Cannabis sativa*

Cannabis sativa, also referred to as marijuana or simply as cannabis, is the most popular drug consumed with approximately 147 million people using it worldwide (*WHO|Cannabis*, n.d.). This figure equates to 2.5% of the global population – higher than the prevalence rate of 0.2% for cocaine and 0.2% for opiates (*WHO|Cannabis*, n.d.). With the legalization of marijuana in several American states and federally in Canada in October 2018, there has been growing concern with regards to how recreational or medicinal use can impact human health. While evidence suggests that cannabis consumption can have detrimental effects on learning, memory, and motor function, several other studies have alluded to the potential therapeutic benefits of various cannabis constituents (Niesink & van Laar, 2013; Renard et al., 2017; Volkow et al., 2014). This heterogeneity of cannabis-induced psychological effects reflects the complex molecular interactions between its various phytochemicals and their biological substrates.

The cannabis plant contains over 500 phytochemicals of which 104 have been identified as cannabinoids (components that interact specifically with the cannabinoid (CB) receptors of the brain) (Pertwee, 2014). Two of these cannabinoids, Δ^9-tetrahydrocannabinol (Δ9-THC or THC) and cannabidiol (CBD), have garnered particular scientific interest due to their relatively high concentrations in the plant and their distinctive psychological effects. Ingestion of THC, the main psychoactive component of cannabis, has been shown to lead to impairments in learning and memory, with chronic exposure resulting in psychosis and schizophrenia (Lafaye, 2017; Renard et al., 2017). In contrast, evidence suggests that CBD may have a neuroprotective effect, potentially antagonizing the negative consequences of THC

(Niesink & van Laar, 2013). Specifically, pre-clinical and clinical assays have alluded to the ability of CBD to alleviate symptoms of stress, anxiety, and depression (Crippa et al., 2018; Millar et al., 2019). For instance, a study by Crippa and colleagues (2011) found that oral administration of cannabidiol resulted in a significant reduction in subjective levels of anxiety in individuals with social anxiety disorder (SAD), with subsequent changes in activity in the limbic and paralimbic systems of the brain.

In addition to cannabinoids, the aromatic compounds known as terpenes, have been shown to possess psychoactive properties (Russo, 2011). Terpenes (organic hydrocarbons) are most abundantly found in essential oils and to varying degrees in different strains of cannabis. Over 200 of the 20,000 terpenes described have been identified in cannabis with the most common being limonene, β-myrcene, α-pinene, linalool, and β-caryophyllene (Russo, 2011). While terpenes constitute less than 1% of most cannabis assays, concentrations as low as 0.05% have been deemed to be pharmacologically significant (Başer & Buchbauer, 2016; Russo, 2011). Moreover, emerging preclinical evidence has alluded to the potential anticonvulsant, antidepressant, and anxiolytic effects of specific monoterpenes (Carvalho-Freitas & Costa, 2002; Elisabetsky et al., 1995; Komiya et al., 2006). For instance, inhalation of limonene by mice results in a reduction in anxiety-related behaviour (Lima et al., 2013). While several reports demonstrate the potential clinical significance of terpenes, studies characterizing their pharmacological profile remain scarce.

In addition to the isolated effects of cannabis-derived compounds, various accounts demonstrate synergistic efficacy between these substances (Russo, 2011). The phenomenon, dubbed the ‘Entourage Effect’, suggests that multiple components within cannabis may interact, thereby producing a stronger influence on the body than each component in isolation. For example, Carlini et al. (1974) found that

administration of crude cannabis extracts produced effects two to four times greater on pulse rate and psychological disturbance than their pure THC counterparts. Such findings alluding to cannabis synergism are of particular interest for the development of therapeutic agents for various mental health disorders. Specifically, the demonstrated anxiolytic effects of certain terpenes, such as limonene, and the phytocannabinoid, CBD, combined with the rationale of the Entourage Effect, suggest that specific terpene-phytocannabinoid formulations may work together to more effectively reduce symptoms of anxiety. Consequently, we utilized a rodent model to examine whether specific combinations of CBD and the monoterpene, d-limonene, could more effectively alleviate anxiety-related behaviours. Moreover, we sought to characterize the associated molecular changes in the brain induced by these drug formulations.

1.2 Overview of Anxiety

1.2.1 Defining Anxiety and Anxiety Disorders

The feeling of anxiety is often composed of three parts: 1) worried thoughts, 2) specific bodily sensations, such as increased heart rate or respiration, and 3) particular actions, such as running away or freezing (Craske & Stein, 2016). Anxiety is a normal reaction to potentially threatening or stressful life events and healthy individuals manage their anxiety by employing adaptive coping mechanisms. These strategies are often divided into two categories: emotion-based behaviours wherein, the individual seeks to regulate their emotions that arise as a consequence of the stressor, or problem-based behaviours that directly resolve the stressor (Biggs et al., 2017). Occasionally, however, anxiety may persist in the absence of an external stressor, or symptoms may be unusually severe or frequent. When symptoms of

anxiety begin to interfere with one or more activities of daily living, the individual may be diagnosed with an anxiety disorder.

The nine types of anxiety disorders characterized by the American Psychiatric Association in the Diagnostic and Statistical Manual of Mental Disorders, 5th Edition (DSM-V) are generalized anxiety disorder (GAD), social anxiety disorder (SAD) or social phobia, separation anxiety disorder, selective mutism, specific phobias, panic disorder, agoraphobia, substance/medication-induced anxiety disorder, and anxiety disorder due to another medical condition, such as post-traumatic stress disorder (PTSD). For many individuals diagnosed with one or more of these conditions, management of anxiety requires therapeutic intervention in the form of psychotherapy and/or medication.

1.2.2 Prevalence of Anxiety Disorders

Anxiety disorders are the most prevalent type of neuropsychiatric illness. In 2017, the proportion of the global population with an anxiety disorder was estimated to be 3.8% (equivalent to 284 million people) – higher than depression (3.4%), alcohol abuse (1.4%), and eating disorders (0.2%) (Global Burden of Disease, 2017). Moreover, the lifetime prevalence of having an anxiety disorder, based on large population-based surveys was 33.7%, with estimates of rates as high as 70% in people with chronic health conditions (Bandelow & Michaelis, 2015; Remes et al., 2016). While these findings signify the already high occurrence of the disease, more recent evidence suggests that these figures can increase during socioeconomic and political upheavals. During the COVID-19 pandemic of 2020, several studies reported an increase in anxiety amongst the general population, with one meta-analysis reporting a proportion as high as 31.9% in a sample of 63,439 individuals (Luo et al., 2020;

Salari et al., 2020). Not surprisingly, economic uncertainty influences population mental health.

Anxiety disorders are highly treatable and when left untreated have significant personal and societal costs such as frequent medical visits, decreased work productivity, unemployment, and impaired social relationships (Simpson et al., 2010). Moreover, untreated anxiety is a risk factor for the development of other anxiety and mood disorders, as well as substance abuse (Remes et al., 2016), consequently, making treatment more difficult and contributing to low remission rates, poor prognosis, and risk of suicide (Nutt et al., 2007; Simpson et al., 2010). Given the pervasiveness of the illness and the associated consequences when left untreated, it is imperative that effective treatment exists for those struggling with an anxiety disorder.

1.2.3 Current Treatment for Anxiety

Treatment for anxiety is broadly categorized into two types: psychotherapy and anti-anxiety medication. Psychotherapy-based interventions include cognitive-behavioural therapy (CBT), psychodynamic therapy, and behavioural therapy. CBT is the most widely utilized of the psychotherapies and is highly effective for various types of anxiety disorders (Hoffman & Smits, 2008). However, a recent meta-analysis by van Dis and colleagues (2020) suggests that CBT's efficacy may be short-lived for some forms of anxiety. Over 69 randomized clinical trials including 4118 patients, skills and insights acquired through CBT were maintained 12-months post-treatment but failed to result in improved outcomes after 12-months for panic disorder (with or without agoraphobia) (van Dis et al., 2020). Given the chronic nature of anxiety-related disorders, the long-term efficacy of therapeutic intervention is critical for successfully managing the illness.

In addition to psychotherapy, anxiety is frequently treated using pharmacotherapeutic agents. First-line anxiolytics include benzodiazepines, selective serotonin reuptake inhibitors (SSRIs), and serotonin and norepinephrine reuptake inhibitors (SNRIs), which modulate GABAergic, serotonergic, and serotonergic and norepinephrine brain pathways, respectively. In comparison to CBT, these drugs demonstrate similar treatment efficacy in terms of long-term symptom management and relapse rates, while being more convenient than recurrent weekly sessions of psychotherapy (Bandelow et al., 2018). Regardless of their remedial effects, however, anxiolytic drugs are associated with considerable side-effects, dependence, and when terminated, severe symptoms of withdrawal (Bandelow et al., 2018; Mackinnon & Parker, 1982; Owen & Tyrer, 1983).

A review by Ravindran and Stein (2010) found that SSRI usage frequently resulted in symptoms of nausea, dizziness, jitteriness, sleep difficulties, and gastrointestinal disturbances – akin to the symptoms associated with anxiety. Due to the severity of these adverse reactions, patients often terminated their treatment prematurely before the medication had enough time to reach maximum efficacy (Ravindran & Stein, 2010). These findings were further corroborated by a more recent study, wherein, 318 children and adolescents reported their adherence to antidepressants for anxiety 3-12 years after initial treatment (Kagan et al., 2020). Researchers found that 40.6% of the 318 patients discontinued their medication with reports of primary concerns of perceived ineffectiveness (31.8%) and side-effects (25.5%) (Kagan et al., 2020). When side-effects are managed and treatment can be continued long-term, these drugs can lead to cognitive impairments and increased risk of developing dementia (Barker et al., 2004; Chan et al., 2017; Ravindran & Stein, 2010). Moreover, when drug doses are reduced or terminated, associated symptoms of

withdrawal include, perceptual disturbances, epileptic seizures, weight loss, insomnia, and autonomic symptoms, often leading to the reliance on higher drug doses (Owen & Tyrer, 1983).

In summary, while current interventions can be successful in relieving anxiety they are replete with short and long-term problems. Thus, there is an urgent need for the development of safer, novel pharmacotherapies that not only possess fewer side-effects and better long-term outcomes, but also encourage patient adherence which is critical for the treatment of chronic anxiety.

1.3 Serotonergic (5-HT) System and Anxiety

Multiple treatment studies, genetic research, and neuroimaging data have implicated dysregulated serotoninergic (5-HTergic) neurotransmission as a primary contributor in mood and anxiety disorders (Durant et al., 2010; Jans et al., 2007; Ravindran & Stein, 2010). That said, the existing literature is contradictory on whether anxiety is provoked in states of 5-HT excess or deficiency (Albert et al., 2014; Durant et al., 2010). Some assays employing 5-HT agonists report anxiogenic symptoms (Charney et al., 1987), while others demonstrate anxiolysis (Crippa et al., 2011). This discrepancy is a result of the differential binding of these agonists to specific 5-HT receptors (5-HTR). For example, the anxiogenic agent utilized by Charney et al. (1987), *m-chlorophenylpiperazine* (m-CPP), is a 5-HT_{1C}R agonist, while the anti-anxiety agent employed by Crippa et al. (2011), cannabidiol, is a well-established 5-HT_{1A}R agonist. Thus, the 5-HT receptor subtype modulated by potential therapeutic agents for anxiety is an important aspect to consider.

5-HT receptors are broadly categorized into five families based on their mechanism of action: 5-HT_1, 5-HT_2, 5-HT_3, 5-HT_5, and the family of 5-HT_4, 5-HT_6, and 5-HT_7 (Siegel, 1999). Within these families, receptors may be further subdivided

into A, B, C, D, E, and F subtypes (such as 5-HT_{1A}, 5-HT_{1D}, 5-HT_{2A}, etc.) (Siegel, 1999). Among them, the 5-HT_{1A} receptor in particular has been implicated in the etiology of anxiety disorders.

Specifically, 5-HT_{1A} heteroreceptors are reduced in patients with social anxiety disorder, and in the cortical regions of patients with panic disorder (Lanzenberger et al., 2007; Neumeister et al., 2004). Additionally, homozygous and heterozygous 5-HT_{1A}R knockout (KO) mice display significantly increased levels of anxiety, indicating that a partial receptor deficit is sufficient to elicit the phenotype (Akimova et al., 2009). Generally, extensive evidence suggests that alterations in 5-HT_{1A}R expression is common in several affective and anxiety-related disorders, suggesting that it may be a general marker of psychopathology.

1.3.1 Mechanism of Action of 5-HT_{1A} Receptors

5-HT_{1A}R is a major inhibitory G-protein coupled receptor (GPCR) subtype that exerts its effects through Gi/Go proteins, thereby, modulating several intracellular signalling pathways such as, adenylyl cyclase (specifically inhibition) (Barnes & Sharp, 1999), mitogen-activated protein kinases (MAPK), and Akt (Polter & Li, 2010). 5-HT_{1A} receptors are classified into two populations within the central nervous system (CNS): autoreceptors and heteroreceptors.

5-HT_{1A} autoreceptors can be found on the soma and dendrites of the 5-HT producing neurons in the dorsal and median raphe nuclei (DRN and MRN, respectively) of the brainstem, where their activity results in hyperpolarization and reduced firing of the cell (Hjorth & Sharp, 1991; Verge et al., 1985). Thus, activation of these autoreceptors creates a negative feedback loop that decreases the release of extracellular 5-HT into projection areas (Hjorth & Sharp, 1991; Verge et al., 1985) **(Figure 1)**.

5-HT$_{1A}$ heteroreceptors innervated by 5-HTergic neurons are expressed on postsynaptic excitatory pyramidal neurons (Azmitia et al., 1996; Palchaudhuri & Flügge, 2005) and GABAergic interneurons (Santana et al., 2004) mainly in the prefrontal cortex (PFC) and regions of the limbic system such as the hippocampus (HP), nucleus accumbens (NAc), and amygdala (AMG) (Azmitia & Segal, 1978; Beck et al., 1992; Li et al., 2006; Pompeiano et al., 1992). Similar to 5-HT$_{1A}$ autoreceptors, stimulation of heteroreceptors results in nerve cell hyperpolarization (Dong et al., 1998; Sprouse & Aghajanian, 1988). Common anxiolytics, such as SSRIs and SNRIs, work by preventing the re-uptake of released 5-HT, enabling activation of postsynaptic 5-HT$_{1A}$ heteroreceptors (Palchaudhuri & Flügge, 2005; Santana et al., 2004). Thus, hyperpolarization of target neurons leads to the subsequent reduction of fear and anxiety-related symptoms (Palchaudhuri & Flügge, 2005; Santana et al., 2004).

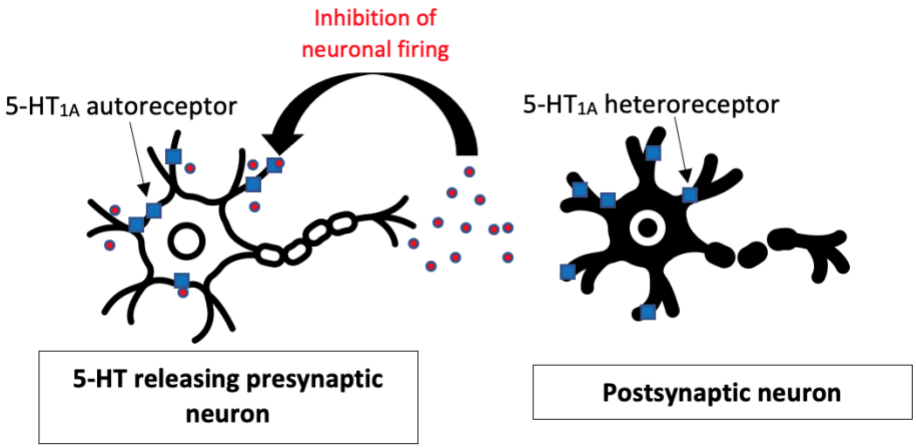

Figure 1: Depiction of a Serotonergic (5-HT) Neuron Negative Feedback via 5-HT$_{1A}$ Autoreceptors. 5-HT$_{1A}$ activation inhibits neuronal firing and 5-HT release. Blue squares represent 5-HT$_{1A}$ receptors; Red circles are 5-HT.

1.4 The Mesocorticolimbic (MCL) Pathway

The mesocorticolimbic system, often referred to as the 'reward pathway', represents the brain's major dopaminergic (DA) system and has been implicated in numerous mood and anxiety disorders (Alex & Pehek, 2007). Subdivided into the mesocortical and mesolimbic pathways, they both contain dopaminergic (A10) neurons that originate primarily from the ventral tegmental area (VTA) located in the midbrain (Alex & Pehek, 2007) (**Figure 2**). The mesocortical pathway projects to the prefrontal cortex (PFC), which regulates complex cognitive processes (Alex & Pehek, 2007). Conversely, the mesolimbic pathway projects to the nucleus accumbens (NAc) and olfactory tubercle (referred together as the ventral striatum of the basal ganglia), mediating endogenous and exogenous drug-induced reward responses (Ekhtiari & Paulus, 2016; Robbins & Everitt, 1996; Schultz, 2016); this path is also associated with complex circuits involving the amygdala, hippocampus, and the bed nucleus of the stria terminalis (BNST), which modulate emotion, memory, and autonomic/neuroendocrine/behavioral responses, respectively (Ekhtiari & Paulus, 2016). Notably, the MCL pathway receives prominent serotonergic innervations from the MRN and DRN and thus, 5-HT_{1A}R agonists have been shown to modulate 5-HT release and DA neuronal activity within the MCL system (Chen & Reith, 2002).

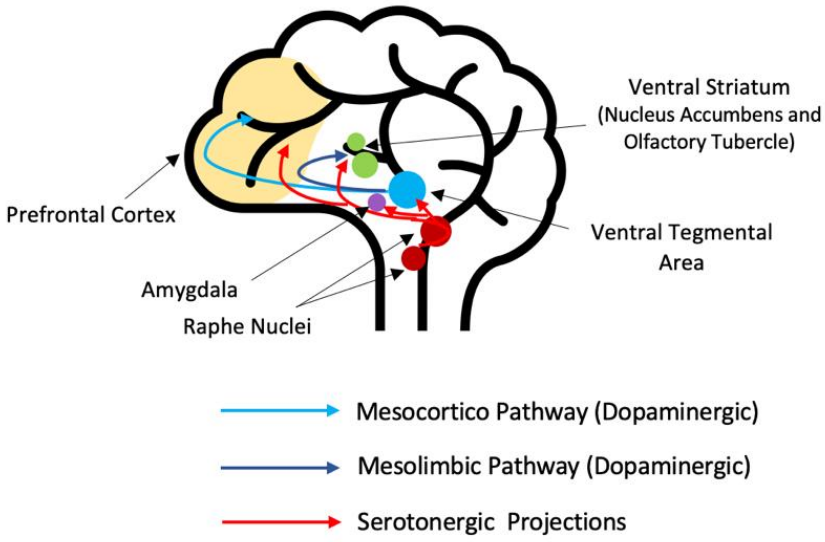

Figure 2: Depiction of the Mesocorticolimbic (MCL) System Differentiating the Cortico and Limbic Pathways. Dopaminergic projections are represented in blue; Serotonergic projections from the raphe nuclei to the VTA, VST, PFC, and AMG are shown in red.

1.4.1 Nucleus Accumbens (NAc)

The nucleus accumbens consists of two anatomical components: the core (NAcc) and the shell (NAcSh/NASh). The main output neurons of both divisions are the medium spiny neurons (MSN) – a special type of GABAergic inhibitory cell containing multiple dopamine receptors (Salgado & Kaplitt, 2015). The activity of these efferent projections is modulated by glutamatergic afferents arising from the prefrontal cortex, hippocampus, and amygdala, by dopaminergic afferents from the ventral tegmental area, by serotonergic afferents from the raphe nucleus, and by noradrenergic afferents from the locus coeruleus (Shirayama & Chaki, 2006).

The NAc is central in modulating social motivation (i.e., the need to obtain social rewards and avoid social punishment) – a behaviour often disrupted in mood/anxiety disorders (American Psychiatric Association, 2013). Utilizing event-related functional magnetic resonance imaging (fMRI), Kohls et al. (2013) observed that the anticipation of social reward (i.e., approval) and the anticipation of avoidable

social punishment (i.e., disapproval) resulted in the activation of the nucleus accumbens. Additionally, an fMRI study conducted by Levita et al. (2012) found that the NAc was associated with active and passive avoidance. Specifically, the NAc displayed increased activation during active avoidance (i.e., pressing of a button that stopped the presentation of an aversive image), and increased deactivation during passive avoidance (i.e., withholding a button press that prevented the aversive image from appearing); critically, the degree of these activity patterns was correlated with individual levels of anxiety (Levita et al., 2012).

Given the extensive evidence associating the nucleus accumbens with anxious phenotypes, the NAc has garnered research interest as a stereotaxic target for therapeutic agents. For instance, the application of three different antipsychotic drugs has been shown to increase Fos expression (a marker of metabolically active neurons) within the NASh, highlighting its role as a site of antipsychotic action (Deutch et al., 1992). Moreover, a study by Norris et al. (2016) alluded to the therapeutic effects of intra-NASh cannabidiol in ameliorating anxiety-related behaviour. Specifically, microinfusions of CBD blocked the formation of fear-related memory (via 5-HT_{1A} receptor activation) (for more details see *Properties and Mechanisms of Action of Cannabidiol***)**. Consequently, CBD within the NASh represents a potential remedy in alleviating behaviours associated with anxiety.

1.4.2 Functional Associations between the NAc, VTA, PFC, and BLA

Nucleus Accumbens and Ventral Tegmental Area (NAc-VTA)

The NAc-VTA circuit has been implicated in various anxiety-related behaviours such as aversion (Danjo et al., 2014) and social dysfunction (van der Kooij et al., 2018). While the VTA sends dopaminergic projections to the NAc (Han et al., 2017), the NAc in turn reciprocally modulates VTA DAergic and non-DAergic

neuronal activity via MSN GABAergic efferents (Kalivas et al., 1993; Nauta et al., 1978; Norris et al., 2016)(**Figure 3**). Notably, this NAc→VTA connection is modulated via 5-HT_{1A}R signalling and regulates fear-related behaviour associated with pre-clinical assays of anxiety (Norris et al., 2016) (for a detailed description see *Properties and Mechanisms of Action of Cannabidiol).* Consequently, these effects of the NAc on the VTA indirectly alter the activity of the PFC via the mesocortical pathway (Alex & Pehek, 2007), and equally, PFC→VTA connectivity modulates NAc dopamine release (Karreman & Moghaddam, 2002).

Nucleus Accumbens and Prefrontal Cortex (NAc-PFC)

In addition to indirect regulation, the PFC executes top-down control of the NAc via direct glutamatergic excitatory input (Brady, 2004; Sesack & Pickel, 1992) and GABAergic input (Lee et al., 2014; Torregrossa et al., 2008)(**Figure 3**). Dysfunction within the PFC-NAc circuitry is associated with abnormal cognitive behaviours observed in schizophrenia (Meyer-Lindenberg et al., 2002; Pantelis, 1997), and glutamatergic PFC→NAc projections have been linked to drug-seeking behaviours (Bossert et al., 2012; Park et al., 2002). Furthermore, the PFC→NAc GABAergic transmission is shown to provoke real-time avoidance behaviour upon optogenetic stimulation, suggesting that this path transmits aversive signals (Lee et al., 2014), having implications for disorders such as agoraphobia and panic disorder.

Basolateral Amygdala (BLA)

Evidence suggests that the amygdala is functionally connected to the MCL circuitry (Nazari-Serenjeh & Rezayof, 2013; Reznikov et al., 2018). The two subregions of the human amygdala include: 1) the basolateral amygdala (BLA) and 2) the cortico-medial amygdala; the cortico-medial amygdala is linked to agonistic behaviour related to fear and the basolateral amygdala is known for encoding the

threat value of a stimulus (Etkin et al., 2004; Luiten et al., 1985; Mai et al., 2016). Notably, the BLA-VTA mutually interact to form memories associated with avoidance learning (Nazari-Serenjeh & Rezayof, 2013) and conditioned fear (de Oliveira et al., 2011), and similarly, deep brain stimulation within the PFC attenuates fear and anxiety-related symptoms by reducing BLA firing in pre-clinical assays of PTSD (Reznikov et al., 2018). It should be noted that while extensive literature has alluded to the top-down regulation of PFC-BLA relations, the BLA in turn project glutamatergically to the PFC, modulating emotional responses within this circuit (Cheriyan et al., 2016; McGarry & Carter, 2016). Finally, the BLA-NAc circuit suppresses punished reward-seeking responses, and thus, dysfunction within this path is proposed to have implications for compulsive behaviours related to OCD (Piantadosi et al., 2017).

In summary, these findings illustrate the integrative and systematic nature in which the NAc, VTA, PFC, and BLA function, and the distinctive and analogous effects they have on anxiety-related behaviours. Critically, this physiological connectivity and interactive affective processing highlight the importance of considering collective neural activity in the development of pharmacological agents that are specific and minimally disruptive to the functioning of associated brain regions.

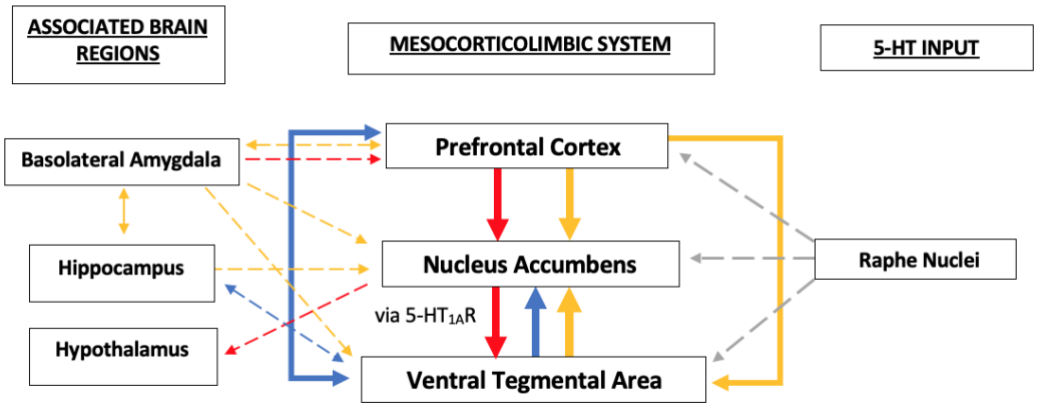

Figure 3: Functional Connections between the MCL System and Associated Brain Regions. Arrow legend: Blue = Dopaminergic, Red = GABAergic, Yellow = Glutamatergic, Grey = Serotoninergic.

1.4.3 Targeting the MCL System in the Treatment of Anxiety

Given the robust evidence demonstrating the role of the mesocorticolimbic system in emotion and cognition from pre-clinical assays of anxiety, this system is frequently a region of interest in clinical interventions. Sturm et al. (2003) demonstrated that the application of deep brain stimulation (DBS) to the NASh in patients with severe anxiety and OCD (who were unresponsive to psycho- and pharmacotherapy) was correlated with a significant reduction in anxiety symptoms. These results were corroborated by a subsequent study by Denys and colleagues (2010) validating the effective use of intra-NAc DBS in patients with OCD. Aside from being a focus for therapy, the NAc represents a potential biomarker for predicting treatment outcomes as greater pre-treatment NAc volume is linked to a greater reduction in anxiety symptoms upon treatment with CBT and SSRIs (Burkhouse et al., 2020). Moreover, similar changes in structural volume have been demonstrated in the PFC, with decreased PFC volumes positively correlating with

worry scores in persons with generalized anxiety disorder (GAD) (Mohlman et al., 2009).

As a chief component of the cortical limb of the MCL pathway and with its top-down inhibition of the amygdala, the PFC plays a significant role in modulating cognitive states and fear-responses related to anxiety. While adolescents with GAD display increased activation of the PFC in response to angry faces relative to control subjects, increased PFC activation is associated with less severe anxiety, implying that it may serve a compensatory role by enabling those with GAD to regulate their responses to anxiety-provoking stimuli, possibly through the PFC's functional connections with the amygdala (Monk et al., 2006). This assertion is supported by Ironside et al. (2019) whereby, transcranial direct current stimulation (tDCS) of the PFC in subjects with high trait anxiety increased attentional control, while simultaneously reducing amygdala threat reactivity.

The activity of the principal dopaminergic input of the MCL system, the ventral tegmental area, is likewise connected to clinical anxiety. Patients with GAD display heightened VTA-mesocorticolimbic coupling and attenuated VTA-hippocampal coupling in response to generalized stimuli during fear generalization tasks (Cha et al., 2014) supporting the contention that the treatment of anxiety necessitates the consideration of related brain regions at the systems-level as opposed to isolated structures. As a note, the long-term memory consolidating structure, the hippocampus, possesses circuit-level interactions with the VTA, NAc, and PFC (Godsil et al., 2013; Kahn & Shohamy, 2013) with dysfunctional associations having implications for the onset of anxiety symptoms (Marusak et al., 2017). Finally, pharmacological targeting of the VTA with specific natural medicines has been shown to ameliorate symptoms of GAD potentially via VTA dopaminergic

associations within the MCL system (Herrera-Arellano et al., 2012; Prieto-Gómez et al., 2003).

1.5 Receptor Targets and Downstream Molecular Signalling of Anxiety

As mentioned previously, atypical serotonergic conduction specifically with regards to 5-HT_{1A}R is associated with the onset, maintenance, and treatment of anxious phenotypes (see *Serotonergic (5-HT) System and Anxiety*). Additionally, increasing data have revealed the anxiogenic effects of reduced GABA transmission (Goddard et al., 2001), specifically as it pertains to key brain structures of the MCL system. Reduced GABA levels within the PFC and GABAR antagonists within the VTA have been observed to increase anxiety (Frye & Paris, 2009; Ghosal et al., 2017), and in a similar vein, the injection of GABAR agonists into the NASh is linked to anxiolytic behaviours (Lopes et al., 2012). Notably, the functional connections between the NAc and VTA (specifically, the GABAergic NAc→VTA projections) signify that GABA alterations in one region can have implications for the other (Norris et al., 2016). Ultimately, these studies highlight the importance of inhibitory control within the MCL system in modulating anxiety and the clinical relevance of GABA receptors, as exhibited by the conventional anxiolytic benzodiazepine.

Both 5-HTR and GABAR are associated with anxiety-related downstream signalling pathways with notable protein biomarkers being: ERK, JNK, Akt, and GSK3 (Ailing et al., 2008; Crofton et al., 2017; Hollos et al., 2018; Matsuda et al., 2019) (**Figure 4**). While the activity of these proteins results in phenotypically similar anxiety-associated symptoms, their effects on downstream targets exhibit distinctive mechanisms of action.

1.5.1 ERK

The extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2, respectively) are members of the mitogen-activated protein kinase (MAPK) superfamily of signalling cascades that mediate cellular processes such as proliferation, differentiation, and apoptosis (Mebratu & Tesfaigzi, 2009). In addition to their physiological effects, phosphorylated ERK (p-ERK) within the PFC is linked to high levels of anxiety (Ailing et al., 2008), and inhibited 5-HT_{1A}R has been found to increase activation of the ERK pathway (via increased p-ERK) within the hippocampi of PTSD mice thereby, increasing anxiety symptoms (Xiang et al., 2017). Moreover, compounds in cannabis are able to modulate this cascade. In particular, intra-hippocampal infusions of THC increases salience attribution in fear conditioning assays, which are reversed upon the co-application of CBD due to the downregulation of p-ERK1/2; additionally, pharmacological reactivation of pERK1/2 blocks these behavioural effects by CBD (Hudson et al., 2019). Thus, the antipsychotic effects of cannabidiol appear to be modulated by the ERK1/2 pathway, specifically within the context of brain regions associated with the MCL system.

1.5.2 JNK

Also, members of the MAPK family, c-Jun N-terminal kinases 1, 2, and 3 (JNK1, JNK2, and JNK3, respectively) are involved in cellular processes and activated by stressful stimuli similar to ERK proteins (Johnson & Nakamura, 2007). Consequently, given that oxidative stress can induce anxiety and depression (Hassan et al., 2014), it is unsurprising that the JNK1 isoform has been implicated in these mood disorders (Hollos et al., 2018). Specifically, JNK1 knockout mice display increased hippocampal neurogenesis and subsequent alleviation of anxiety and depression, with JNK inhibition being sufficient in yielding these effects (Mohammad

et al., 2018). Thus, JNK has been causally implicated in the neurogenesis hypothesis of anxiety, which postulates that the generation of new neurons within the hippocampus throughout adulthood suppresses anxious behaviour (Revest et al., 2009). Furthermore, while 5-HT_{1A}R-mediated ERK phosphorylation leads to the initiation of anti-apoptotic pathways, 5-HT_{1A}R stimulation of JNK in the same cell line results in pro-apoptosis, suggesting that the ultimate consequence of 5-HT_{1A}R activity on MAPK proteins is dependent on their respective role within the tissue (Masson et al., 2012).

1.5.3 Akt

Protein kinase b (Akt) is a member of the PI3K-Akt signal transduction pathway, which is activated by the phosphorylation of its serine-473 and threonine-308 residues; this phosphorylates downstream targets, including the protein glycogen synthase kinase 3 (GSK3), involved in cell survival and growth (Manning & Cantley, 2007). Notably, this phosphorylation is related to 5-HT homeostasis, as mice with inhibited phosphorylation of Akt (p-Akt) at Ser473 display elevated cortical expression of 5-HT_{1A}R (Saunders et al., 2014). Moreover, the isoform Akt2 is involved in mood stabilization and fear memory with Akt knockout mice displaying significantly increased anxiety in pre-clinical behavioral assays such as the light-dark box and open field test (Leibrock et al., 2013). Finally, varying genotypes of the Akt2 gene have been associated with human personality traits related to anxiety and depression, highlighting its pharmacological significance in treating mood disorders (Engeli et al., 2014).

1.5.4 GSK3

Glycogen synthase kinase 3 (GSK3) is a serine/threonine protein kinase primarily phosphorylated and inactivated by Akt as a downstream target of the PI3K-Akt pathway (Cross et al., 1995). Existing as two isoforms, GSK3α and GSK3β, it participates in cell signalling and transport, as well as in the etiology of mood disorders (Jope, 2011). In mice, silencing GSK3β within the NASh decreases the intrinsic excitability of tonically active interneurons (TANs), consequently leading to a reduction in anxiety-like behaviour (Crofton et al., 2017). Indeed, several studies have validated that GSK3β modulates behaviours linked to aberrant 5-HT transmission and that 5-HT_{1A}R agonists increase phosphorylated GSK3β within the cerebral cortex, hippocampus, and striatum (Latapy et al., 2012; Polter & Li, 2010). Finally, several classes of 5-HT modulating drugs, such as SSRIs, exert their actions by inhibiting GSK3, reinforcing the significance of targeting this protein in neuropsychiatric disease (Polter & Li, 2011).

It should be noted that the aforementioned proteins, of which anxiety-related modulatory roles are mentioned in isolation, work in a concerted manner through direct and indirect interactions with each other. Consequently, their ultimate behavioural outcomes, based on activation or inactivation, is dependent on the brain region targeted, as well as the complex interplay between these molecules.

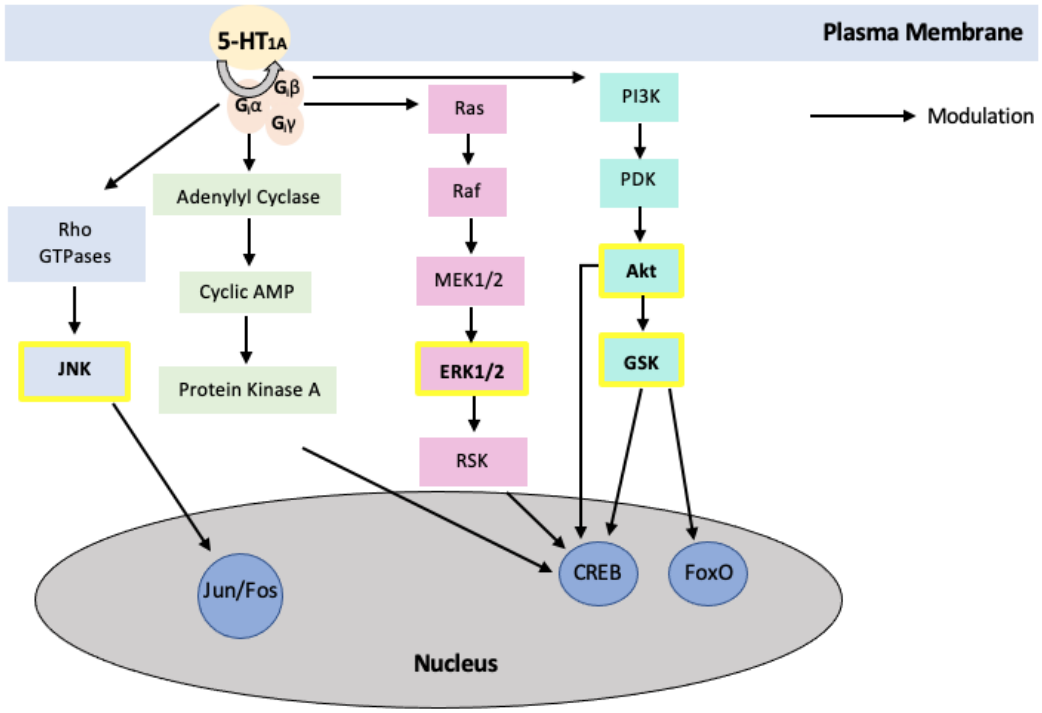

Figure 4: 5-HT$_{1A}$ Receptor-Mediated Downstream Molecular Targets. Proteins discussed in this thesis are highlighted. Jun/Fos, CREB, and FoxO represent transcription factors. JNK = c-Jun N-terminal kinase, ERK = extracellular signal-regulated kinase, Akt = protein kinase b, GSK = glycogen synthase kinase.

1.6 The Endocannabinoid System and Mental Health

Despite thousands of years of cannabis use for recreational and medicinal purposes, interest in cannabinoid receptor pharmacology did not begin until the isolation of THC and the discovery of its psychoactive properties in 1964 (Maroon & Bost, 2018; Russo, 2011). Subsequent research in the early 1990s unveiled the specific membrane receptors of THC, thus leading to the identification of the endogenous signalling system – the endocannabinoid system (ECS) (Maroon & Bost, 2018). Consequently, the endogenous cannabinoids (cannabis-like substances) derived from arachidonic acid, *N*-arachidonoylethanolamine (anandamide; AEA) and 2-arachidonoylglycerol (2-AG) were identified (Maroon & Bost, 2018).

The two major endogenous cannabinoid receptors of the ECS system are the CB_1 and CB_2 G protein-coupled receptors (Pertwee, 2008). Binding of phytocannabinoids and endogenous cannabinoids to these receptors within the mammalian brain has been found to modulate effects on emotion, motor control, and cognition (Rodrigues de Fonseca et al., 2005). Additionally, within the central and peripheral nervous systems, they play a crucial role in regulating the autonomic nervous system, immunity, and microcirculation (Rodrigues de Fonseca et al., 2005).

Notably, the antagonistic effects of CBD and THC are owing to their differential receptor binding. While THC is a partial agonist at the CB_1 and CB_2 receptors, CBD acts as a negative allosteric modulator of the CB_1 receptor and a weak indirect antagonist of the CB_2 receptor (Pertwee, 2008). Critically, by targeting these receptors, their exogenous and endogenous ligands, and endocannabinoid degradative enzymes, recent pharmacological advances have successfully manipulated the ECS system to treat pain, neurological disease, and psychiatric and emotional disorders, like anxiety and drug addiction (Maroon & Bost, 2018).

1.6.1 Cannabis-Derived Treatment

While there is evidence of cannabis-derived treatment dating back to 2700 B.C. and reports of pharmaceutical use in the 19th century, their definitive induction into Western medicine is a more recent phenomenon (Zuardi, 2006). The first cannabis-derived medication, Sativex, was approved and launched in the United Kingdom on June 21st, 2010, with 29 other countries, including Canada, following suit shortly after; this 1:1 THC-CBD oral spray is used for the treatment of pain and spasticity caused by multiple sclerosis (Freeman et al., 2019). On June 25th, 2018, the US Food and Drug Administration (FDA) approved Epidiolex, an oral CBD solution, for the treatment of seizures associated with two severe forms of childhood epilepsy –

Lennox-Gastaut syndrome and Dravet syndrome (Mead, 2019). Additionally, some countries, including the US, Netherlands, and Germany, have now licensed the use of two synthetic-THC products, Dronabinol and Nabilone, both of which serve as a weight loss treatment for patients with AIDS and an anti-emetic for patients receiving chemotherapy (Freeman et al., 2019). Although whole-plant extracts have yet to be approved by the FDA, growing evidence of the therapeutic effects of cannabis has prompted the World Health Organization to propose that cannabis should be rescheduled for medical use within international law (Mayor, 2019). Specifically, in the case of CBD, its excellent safety profile and tolerance even at high doses have highlighted its clinical value in treating mood and anxiety disorders (Taylor et al., 2018).

1.6.2 Properties and Mechanisms of Action of the Phytocannabinoid Cannabidiol

Cannabidiol (CBD) comprises about 40% of most *Cannabis sativa* extracts, making it the second most abundant component after THC (Grlic, 1976). CBD interacts with a host of different receptors; critically, its anxiolytic effects have been associated with the vanilloid receptor TRPV1 (Iannotti et al., 2014), the serotonin receptor 5-HT_{1A} (Norris et al., 2016), and the endocannabinoid receptor CB_1 (Pertwee, 2008).

Activation of the TRPV1 channel is linked to the etiology of psychiatric disorders such as anxiety and fear-associated responses (Chahl, 2011). For instance, compared to wild-type mice, TRPV1 knockout mice display less anxiety-related symptoms as measured by the light-dark box test, the elevated plus maze, and the fear conditioning paradigm (Marsch et al., 2007; Santos et al., 2008). Moreover, CBD has been shown to dose-dependently activate and subsequently desensitize TRPV1

channels (Iannotti et al., 2014); thus, the reported anxiolytic effects of CBD may be related to its direct interactions with this receptor.

Conversely, evidence suggests that CBD may modulate these effects indirectly through the endocannabinoid anandamide (AEA). AEA is an agonist at the TRPV1 channel (Ross, 2003) and the CB_1 receptor (of which CBD is not a primary ligand) (Dasilva et al., 2014). Studies demonstrate that CBD increases levels of AEA by inhibiting its hydrolytic enzyme, fatty acid amide hydrolase (FAAH) (Leweke et al., 2012), and by inhibiting AEA uptake via its presumed AEA transporter (Bisogno et al., 2001). Since increased AEA is negatively associated with psychotic symptoms, the antipsychotic effects of CBD have been attributed to its effects on AEA levels. Notably, the administration of CBD in patients with acute schizophrenia results in the reduction of psychotic symptoms comparable to the potent antipsychotic drug amisulpride, while bearing less negative side-effects (Leweke et al., 2012).

While the exact biochemical interactions of CBD are still debated, extensive behavioural and molecular assays have robustly linked it as an agonist at the 5-HT_{1A} receptor (de Gregorio et al., 2019; Norris et al., 2016; Resstel et al., 2009; Russo et al., 2005). The study by Norris et al. (2016) (mentioned earlier under *Nucleus Accumbens*) demonstrating that intra-NASh CBD can block the formation of conditioned freezing behaviors, unveiled a novel circuit between the NASh and the ventral tegmental area via NASh 5-HT_{1A}R transmission. Specifically, CBD decreased DAergic neuronal activity in the VTA, while simultaneously increasing VTA GABAergic neuronal activity, with the reversal of these neuronal effects and restoration of associative fear memory formation upon administration of NAD299 (a 5-HT_{1A}R antagonist) (Norris et al., 2016).

Furthermore, the ability for CBD to facilitate GABAergic activity and modulate anxiety has been associated with its interactions with the GABA receptor subtype, $GABA_A$, while exhibiting no interactions at $GABA_BR$ (Bakas et al., 2017; Straiker et al., 2018). Specifically, CBD acts as a positive allosteric modulator at the $GABA_A$ receptor (Bakas et al., 2017; Onaivi et al., 1990), which is similar to the actions of the anxiolytic drug, benzodiazepine (Roy-Byrne, 2005). Given that $GABA_A$ activation inhibits GABA interneurons within the ventral striatum and subsequently increases NAc firing (Mallet, 2005), CBD may possess a $GABA_AR$-NAc-mediated route in inhibiting VTA dopaminergic transmission within the MCL system, thereby, modulating anxiety symptoms.

In addition to its reported regulation of multiple anxiety-related receptor targets, oral ingestion of CBD demonstrates an excellent safety profile with adverse reactions being mild or moderate in severity after two daily doses of 1500 mg (Taylor et al., 2018). Notably, a single oral dose of 400 mg of CBD is enough to significantly decrease subjective levels of anxiety in patients with GAD (Crippa et al., 2011), while a 600 mg dose is effectual in patients with SAD (Bergamaschi et al., 2011). Consequently, given its relatively superior tolerability and effective modulation of 5-HT and GABA, CBD represents an excellent candidate as an alternative or supplementary agent to conventional anxiolytics.

1.6.3 Properties and Mechanisms of Action of the Monoterpene Limonene

For centuries, essential oils have been utilized for the treatment of infection, inflammation, and mood disorders (Ali et al., 2015). In particular, there are extensive reports from traditional and folk medicine on the anxiolytic effects of Citrus essential oils (such as that found in *Citrus aurantium L.*) with the emergence of more recent empirical evidence supporting its therapeutic use in pre-clinical and clinical settings

(Lehrner et al., 2000; Leite et al., 2008; Lima et al., 2013; Rombolà et al., 2017). For instance, numerous rodent *in-vivo* studies have demonstrated that lemon essential oil can reduce levels of stress, anxiety, and depression (d'Alessio et al., 2014; de Almeida et al., 2012; Komiya et al., 2006; Lima et al., 2013). In humans, drops of lemon essential oil scattered in a dental office lobby were shown to reduce anxiety of awaiting patients (Lehrner et al., 2000), while oral administration reduced preoperative anxiety levels of patients scheduled for minor elective surgery (Akhlaghi et al., 2011).

The anxiolytic effects of essential oils are largely attributed to the aromatic compounds known as terpenes. The major chemical component (constituting up to 96.24%) of Citrus essential oil is the monoterpene hydrocarbon limonene (Leite et al., 2008); in cannabis extracts, limonene represents 16% of the fresh bud oil (Ross & ElSohly, 1996). Moreover, emerging pre-clinical data has alluded to the pharmacologically similar effects of limonene and cannabidiol and their analogous modulation of activity within the MCL system.

Komiya et al. (2006) reported that inhalation of lemon essential oil by mice decreased measures of anxiety in the pre-clinical assay, the elevated plus maze (EPM). Moreover, pre-treatment with the anxiolytic buspirone (a 5-HT_{1A} agonist) and the antipsychotic haloperidol (a D2, D3, D4 antagonist) potentiated the anxiolytic effects of inhaled lemon oil, while flumazenil (a $GABA_AR$ antagonist) blocked these effects (Komiya et al., 2006). Considered together these findings provide support for the assertion that lemon oil modulates DAergic activity via interactions with the 5-HTnergic and/or GABA receptor complex (Komiya et al., 2006). One possible mechanism of action is that components of lemon oil, such as limonene, activate raphe nuclei serotonergic transmission to the VTA and associated regions of the MCL

system, which subsequently suppresses DAergic neuronal activity (Komiya et al., 2006). These results of limonene regulation of the 5-HT, dopamine, and GABA systems are corroborated by other studies (Fukumoto et al., 2006; Lima et al., 2013; Yun, 2014; Zhou et al., 2009) with one study by Yun (2014) demonstrating limonene-associated reversal of methamphetamine-induced dopamine release specifically in the nucleus accumbens.

Furthermore, in addition to its reported effects on stress and mood, limonene represents an attractive candidate in drug therapy due to its potential non-invasive, olfactory administration and low toxicity. The LD_{50} values for d-limonene in male and female rats (in g/kg body weight), respectively, are 4.4 and 5.1 (oral), 3.6 and 4.5 (intraperitoneal), > 20 and > 20 (subcutaneous), and 0.12 and 0.11 (intravenous), categorizing limonene as 'practically non-toxic' according to the Hodge and Sterner toxicity scale (Tsuji et al., 1975). While extrapolation of these doses to humans should be conducted with caution, evidence from a Phase I clinical trial suggests that limonene toxicity, upon oral administration, is limited to gastrointestinal symptoms (such as irritation, nausea, and diarrhea), and are associated with exceptionally high doses in the range of 6.5–12 g/m^2 body surface area per day (Vigushin et al., 1998).

Given its relatively safe pharmacological profile and described anxiolytic efficacy, limonene represents an appealing alternative to current anxiolytic pharmacotherapies. This is further supported by its reported interactions with well-established anxiety-related targets, such as $5\text{-}HT_{1A}R$ (Costa et al., 2013). Moreover, the similar mechanistic profile that limonene shares with CBD makes it a suitable co-drug for the examination of synergistic efficacy as it relates to the Entourage Effect.

1.7 Synergy and the Entourage Effect (EE)

Synergy is the enhanced potency of combined pharmacological agents that is greater than their presumed additive effects (Greco et al., 1996). These co-operative interactions often allow for the use of lower doses of each drug, thereby, reducing the likelihood for adverse reactions. In line with this synergism principle, the Entourage Effect refers to the proposed mechanism demonstrating the enhanced psychoactive potency of combined phytocannabinoid components relative to their isolated administrations (Ben-Shabat et al., 1998; Russo, 2011). Originally identified amongst endogenous cannabinoids, recent developments have extended this effect to exogenous cannabinoids, such as THC and CBD, terpenes, and flavonoids (Ferber et al., 2020a; Russo, 2011). Critically, studies specifically demonstrating the augmented therapeutic efficacy of whole-plant or combinatorial extracts of cannabis have significant implications for the treatment of brain and anxiety disorders.

In an *in-vitro* rodent model of epilepsy, comparison of a standardized cannabis extract (SCE) with a matched concentration of pure THC, yielded the SCE as a stronger and more rapidly-acting anticonvulsant (Wilkinson et al., 2003). Moreover, Ryan et al. (2006) found that evoked calcium responses within hippocampal neurons and glia were increased with combinatorial administrations of CBD and THC relative to pure CBD and THC counterparts and that the response sizes and maximal responder rates were heightened by CBD-THC mixtures containing additional phytocannabinoids rather than a pure 1:1 formula of CBD:THC. Consequently, these findings provide evidence of synergy between THC and CBD, as well as potential interactions with other cannabis constituents (Ryan et al., 2006).

Four possible mechanisms of synergy have been postulated by Wagner and Ulrich-Merzenich (2009) which include i) effects at multiple target receptors, ii)

improved pharmacokinetics such as, enhanced bioavailability, iii) interactions affecting bacterial resistance, and iv) tempering of adverse events, as in the case of CBD-inhibition of THC-induced psychotropic effects (Hudson et al., 2019; Niesink & van Laar, 2013). However, while studies have demonstrated synergistic efficacy between CBD and THC, as well as within constituents of lemon essential oil (Costa et al., 2013), there is currently no study that has assessed possible Entourage Effects between Limonene-CBD formulations (Ferber et al., 2020b). Given the previously described similarities between CBD and limonene in their modulation of receptor targets, neuronal communication, and consequent changes in anxiety-associated behaviours, Limonene-CBD formulations offer a promising avenue in EE-potentiated treatment of anxiety disorders. Consequently, this thesis examined the potential synergistic and anxiolytic efficacy of combined sub-threshold doses of Limonene-CBD formulations relative to pure administrations of these phytocannabinoids.

1.8 Hypothesis and Research Aims

General Hypothesis: This study hypothesized that combining sub-threshold cannabidiol and d-limonene would lead to synergistic efficacy (based on the concept of the cannabis Entourage Effect, discussed previously), thus, resulting in greater anxiolytic efficacy in both behavioural and molecular assays compared to the administration of either compound in isolation.

The following research aims were conducted to test this hypothesis:

Aim 1: Characterize the anxiolytic efficacy of Limonene-CBD formulations relative to limonene and CBD alone by the administration of compounds through inhalation and intracranially, respectively, and by subsequent assessment of established rodent anxiety-related phenotypes in behavioural assays.

Aim 2: Examine possible 5-HT_{1A} receptor-mediated anxiolysis by co-application of the antagonist (NAD299) with Limonene-CBD formulations and assess for changes in behaviour in the same assays conducted in *Aim 1.*

Aim 3: Determine whether Limonene-CBD formulations alter molecular pathways associated with anxiety and 5-HT_{1A}R signalling by examining changes in associated biomarkers (such as ERK1/2 and Akt).

2. Methods

2.1 Animal Housing

Adult male Sprague Dawley rats (300-400 grams) were housed in pairs upon arrival from the Charles River Laboratories, Quebec, Canada until surgery. The following conditions were maintained: 12-hour light/dark cycle, food (rodent chow) and water ad libitum, and constant temperature and humidity. Cages were plexiglass rectangular boxes filled with approximately two inches of corn bedding containing approved objects for environmental enrichment (paper nesting and wood chewing blocks). Beginning one day after arrival, animals were handled every day for at least one week to acclimate them to handling procedures. After one week, animals were ready for surgical cannulation. All experimental procedures were performed in accordance with the regulations of the Canadian Council on Animal Care (CCAC) and the University of Western Ontario Animal Care Committee (AUS protocol 2018-053). Animals were monitored by both Animal Care and Veterinary Services (ACVS) and laboratory technicians in the designated animal care facility at Western University.

2.2 Stereotaxic Surgery

Rats were anesthetized with a 2:1 mixture of ketamine (80 mg/kg; 26 Vetoquinol) and xylazine (6 mg/kg; Bayer) via intraperitoneal injection. Appropriate anesthetic depth was ensured by the absence of reflexive movement in response to a toe pinch and lack of whisker twitch in response to light stroking. After confirmation of anesthesia, subjects were treated with a subcutaneous injection of meloxicam (1mg/kg) to prevent pain and inflammation; additionally, a second dose was administered 24-hours post-surgery. Rats were positioned in the stereotaxic apparatus. Subjects' body temperature was measured immediately before, during, and after

surgery and maintained around 36ºC - 37ºC using a warm heat pad positioned below a urine pad placed under the rat.

An incision was made to expose the skull and eight-millimeter stainless steel guide cannulas (22 G; Plastics1) were implanted into the NASh bilaterally using the following stereotaxic coordinates: 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 based on the Rat Brain Atlas by Paxinos and Watson (2005). Cannulas were secured to the skull using four miniature screws and dental acrylic cement. Dust caps were fitted to the cannulas to prevent obstruction by debris. Following at least one week of recovery, rats were tested in behavioural paradigms.

2.3 Drug Preparation and Administration

2.3.1. d-limonene

D-limonene (96.9%; MP Biomedicals) was administered through inhalation according to the following protocol (Harada et al., 2018).

One Day Before Test Day:

To habituate the subjects to the cage where they would receive the odourous exposure to d-limonene, rats were placed in the cage for 30 minutes one day before the first test day. In this set-up, subjects were exposed to 200 µL of distilled water.

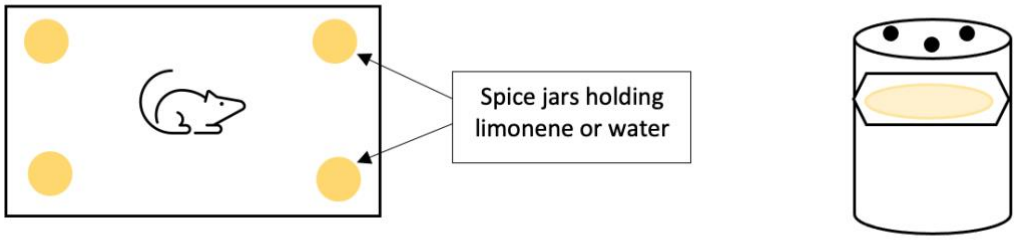

Figure 5: Schematic of d-limonene Exposure. Left: Top-down view schematic of plexiglass-cage depicting the position of the four spice jars. **Right:** Schematic of spice jar containing a weigh boat at the jar mouth. Weigh boats were filled with limonene or water depending on treatment designation.

The limonene exposure apparatus was a transparent, rectangular plexiglass cage with a filter top (which prevented odours from entering or leaving the cage) and four spice jars in each of the four corners secured to the floor using Velcro adhesive. Small weigh boats containing distilled water (200 or 2000 µL; V) or d-limonene (200 µL or 2000 µL; L200 or L2K, respectively), were placed on the mouth of each spice jar. The jars were then secured with lids which contained three holes that allowed the odours to diffuse into the cage. On test day, rats were exposed to this treatment for 30 minutes just before intracranial CBD infusion.

Test Day:

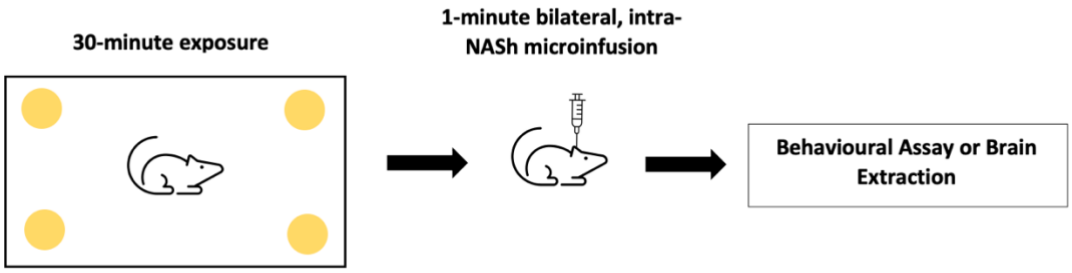

Figure 6: Timeline of Drug Administration Prior to Behavioural Test or Brain Extraction. For brain extractions, animals were administered Euthanyl three minutes after intracranial CBD infusion and brains were collected immediately after confirmed euthanasia.

2.3.2 Vehicle, Cannabidiol (CBD), and NAD299 Microinfusions

Vehicle (V) solutions comprised of dimethyl sulfoxide (DMSO; Sigma-Aldrich), cremophor (Sigma-Aldrich), and 0.9% saline (pH 7.4) in a 1:1:18 ratio. CBD-only (Tocris) and NAD299 hydrochloride-only (Tocris; 5-HT_{1A}R antagonist) solutions were created by dissolving the respective drug in DMSO and diluted to their final concentrations (final DMSO 5% in saline containing 5% cremophor). Target CBD concentrations were either 1 ng/0.5 µL (CBD(1)) or 5 ng/0.5 µL (CBD(5)) (sub-threshold doses; Norris et al., (2016)), while NAD299-only solutions were 100 ng/0.5 µL (effective dose in attenuating CBD anxiolytic effects; Norris et al., (2016)). CBD-NAD299 solutions contained 5 ng of CBD and 100 ng of NAD299 in 0.5 µL of vehicle solution (CBD(5)NAD).

Intracranial microinfusions were administered into the NASh (volume of 0.5 mL per hemisphere) using microinjectors attached to a Hamilton syringe over a 1-minute period. Microinjectors were kept in place for an additional one minute following infusion to ensure that all of the solution had diffused out of the injector tip. After intracranial infusion, subjects were immediately placed into the designated behavioural test.

2.4 Behavioural Assays

2.4.1 Open Field (OF)

The open field test was conducted primarily as a measure of general locomotor activity, wherein, subjects that displayed significantly abnormal locomotor activity were excluded from further analysis. The apparatus consisted of a transparent plexiglass box with approximately two inches of wood chip bedding placed on the floor for comfort. A grid system of laser interference detection (San Diego Instruments) assessed various motor movements such as total distance travelled (in cm) and the total time spent in the center and periphery of the chamber (in seconds). Rats were placed in this chamber to freely explore for 30 minutes. Data was analyzed only for the first 5 minutes of the test, however, as most subjects tended to stop exploration after this time.

2.4.2 Elevated Plus Maze (EPM)

The elevated plus maze task measures open-space anxiety exhibited by rats. The test apparatus was a black acrylic maze consisting of four arms (10 x 50 cm), extending from a 10 x 10 cm base platform elevated 50 cm above the floor. The two ‘closed arms’ were shielded with 40 cm high walls, while the two opposing ‘open arms’ were unshielded except for a 1 cm high ledge which prevented subjects from falling off the platform during exploration. Relevant measures of this test were: total time spent in the open arms and the total number of arm transitions. Subjects were placed diagonally on the center platform facing no particular arm and allowed to explore the maze for 10 minutes. Exploration behavior was recorded and analyzed offline (Behaview software; www.pmbogusz.net).

2.4.3 Three-Chamber Social Interaction (SI)

This test is a measure of the degree of the rodent's social motivation and social recognition memory and consists of three-stages (Loureiro et al., 2015). The test apparatus consisted of three compartments each separated by a removable gate.

One Day Before Test Day:

To acclimate the subjects to the chamber a habituation phase was conducted one day before test day. The subject was placed in the center compartment with both gates closed and allowed to explore the center for 5 minutes. After this, the two gates were opened and the rat was allowed to explore the whole chamber for 8 minutes.

Test Day:

i) Stage One (S1): Habituation

The next day, the subject was placed in the center compartment for 5 minutes with both gates closed.

ii) Stage Two (S2): Social Motivation

After this 5-minute session, an unfamiliar male rat (matched in age and size to the subject) was placed inside a small wire cage and placed in one compartment, while a similar empty cage was placed in the other compartment. Both gates were then lifted and the test subject was allowed to explore both cages for 8 minutes. The following data was analyzed: time spent sniffing the 'stranger' rat and time spent sniffing the empty cage. Throughout the testing protocol, the placement of the empty cage and stranger rat were counterbalanced between the left and right compartments of the test apparatus.

iii) Stage Three (S3): Social Recognition

Immediately after the second stage, a novel and unfamiliar male rat (matched in age and size to the subject) was introduced into the empty cage. The test subject

was allowed to explore both cages for 8 minutes. The following data was analyzed: time spent sniffing the 'familiar' rat (from S2) and time spent sniffing the 'novel' rat (from S3). Subjects' recorded interaction times with the cages during the task were analyzed offline (Behaview software; www.pmbogusz.net).

2.4.4 Light-Dark Box (LDB)

The light-dark box test measures bright-space anxiety exhibited by rats, based on their instinctive tendency to avoid brightly lit environments. The test apparatus was a non-transparent plexiglass box (50 x 25 x 37 cm) consisting of two equally sized compartments. The 'light compartment' was white, uncovered, and illuminated by a lamp placed approximately 125 cm above the floor of the compartment. The 'dark compartment' was black and lidded. The two compartments were separated by a 10 x 10 cm open doorway, which allowed subjects to easily traverse them.

Subjects were placed in the light compartment with their back facing the open doorway and observed for a total of 10 minutes. The following data was analyzed: total time spent in the light, total time spent in the dark, risk assessment (total time spent by the subject placing its nose or its forepaws into the light compartment from the dark compartment), as well as the total number of compartmental transitions during the test session. A transition into a compartment was considered only when all four feet of the rat was placed in the respective compartment. Test sessions were recorded and analyzed offline (Behaview software; www.pmbogusz.net).

2.4.5 Contextual Fear Conditioning (CFC)

The contextual fear conditioning paradigm is a test of associative learning and memory created between an aversive footshock stimulus and a given environment. While it can be used to measure both memory acquisition and memory recall, this

thesis examined memory acquisition only. The test apparatus, adapted from Norris et al. (2016) consisted of a lidless, tall chamber containing a metallic grid floor connected to a shocker. The task consisted of two stages conducted over two days.

i) Stage One: Conditioning Phase

Day 1 of the protocol involved pairing a specific context to an aversive footshock stimulus. Immediately after subjects received treatment, they were placed inside a black-and-white striped walled chamber (the context) and administered 10 supra-threshold footshocks. The shock was 0.8 mA, 1 second in duration and, given at randomized intervals over a 25-minute session.

ii) Stage Two: Testing Phase

The next day, within 24 hours, rat subjects were placed in this same chamber and their subsequent freezing behaviour was measured. Recorded data was then analyzed offline (Behaview software; www.pmbogusz.net)

2.4.6 Experimental Timeline of Behavioural Assays

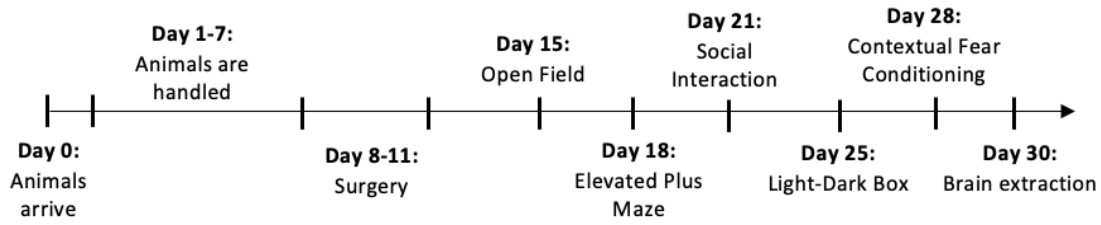

Figure 7: Testing Timeline of Behavioural Assays (not to scale)**.**

2.4.7 Experimental Groups in Behavioural Assays

Table 1: Summary of Experimental Cohorts and Treatment Designations Per Cohort

Research Aim	Experimental Cohort	Treatment Groups
Aim 1: Characterizing anxiolysis and synergy	Baseline Cohort (9 potential groups)	VV, VCBD(1) and/or VCBD(5), L200V, L2KV, L200CBD(1) and/or L200CBD(5), L2KCBD(1) and/or L2KCBD(5)
Aim 2: Examining 5-$HT_{1A}R$ modulation	NAD299 Challenge (8 potential groups)	VV, VNAD, L200CBD(1) and/or L200CBD(5), L2KCBD(1) and/or L2KCBD(5), L200CBD(5)NAD, L2KCBD(5)NAD

2.5 Molecular Analyses

2.5.1. Tissue Extraction

After completion of all behavioural tests, rodent brains were collected for Western Blot analysis. Prior to euthanasia, rats were subjected to their respective treatment (odour and drug combination) from behavioural testing. Euthanasia was an intraperitoneal injection of sodium pentobarbital (Euthanyl$_{TM}$; 240 mg/kg) administered 3 minutes after intracranial infusion. Animals were decapitated and extracted brains were frozen at -80^{o}C and sliced within three weeks. To account for potential minor variations in extracting methods, brains from each cohort of animals were extracted on the same day.

Using a cryostat, coronal sections (99 μm) of the PFC, NASh, and BLA were collected and mounted on glass slides. Bilateral tissue samples were extracted from

these brain regions. NASh 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.

2.5.2 Western Blots

The Western Blot protocol was adopted from Lyons et al. (2013). Protein samples were denatured in Laemmli buffer and diluted to ensure an equal concentration amongst all samples. Each well was loaded with 25 μg of protein sample and subjected to 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 ERK1/2 (p-ERK; rabbit; 1:1000; Cell Signalling Technology), total ERK1/2 (t-ERK; mouse; 1:2000; Cell Signalling Technology), phosphorylated Akt Ser473 (p-Akt Ser473; rabbit; 1:1000; Cell Signalling Technology), and total Akt (t-Akt; mouse; 1:1000; Cell Signalling Technology).

Membranes were subsequently 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.

2.6 Histology

Over four cohorts, a total of 145 animals underwent surgery for intra-cranial cannulation. Following tissue extraction, slides containing the NASh region were stained using cresyl violet dye as detailed by Loureiro et al. (2015). Stained slides with visible cannula tips under a confocal microscope were photographed and assessed for appropriate cannula placement using the Rat Brain Atlas by Paxinos and Watson (2005). Due to improper cannulation, 11 animals were excluded from analysis from the following groups: VV [2], VCBD [3], L200V [2], L200CBD [1], and L2KCBD [3].

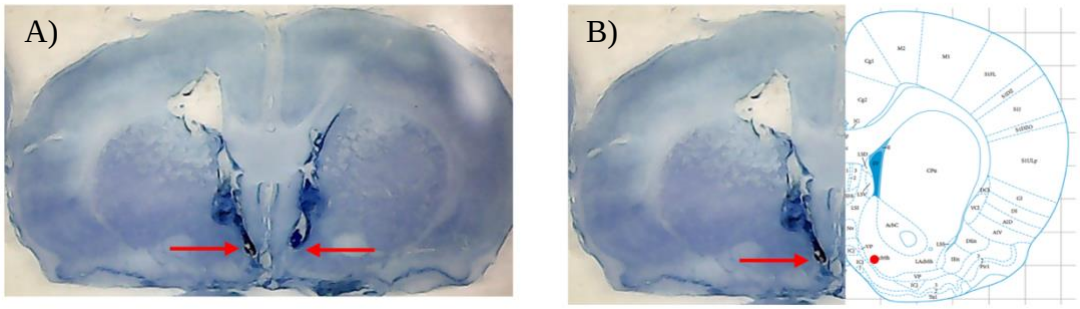

Figure 8: Histological Analysis of the Intra-Shell Region of the Mesolimbic Nucleus Accumbens (NASh) Microinjection Sites. A) Microphotograph of a representative slide depicting bilateral injector placements within the NASh **B)** Slide presented alongside an overlay of the relevant depiction in the Rat Brain Atlas by Paxinos and Watson (2005). Red arrows point towards the injector tips.

2.7 Statistical Analysis

All statistical analyses were performed using GraphPad Prism (Version 8). Behavioural and molecular data were analyzed for Gaussian distribution using the Kolmogorov-Smirnov test. Data was then analyzed using a one-way ANOVA (or Kruskal-Wallis analysis) or t-test (or Mann-Whitney U-Test). The *post-hoc* analysis utilized was Fisher's LSD. Statistical significance was determined at $p < 0.05$ and the

confidence interval was 95%. Statistical outliers were identified using the Grubbs' Test and removed.

3. Results

3.1 Histology

Histological analysis confirmed injector placements were correctly localized to the nucleus accumbens shell (NASh) according to the anatomical boundaries specified in the Atlas by Paxinos and Watson (2005) (**Figure 8**). Only these animals were analyzed for further study.

3.2 Baseline Cohort Behavioural Results

3.2.1 Open Field (OF)

The open field test was conducted to assess if treatment groups affected general locomotor activity. For this test, the number of animals in each group (excluding histological outliers mentioned in *2.6 Histology*) were: VV [9], VCBD(5) [9], L200V [10], L2KV [12], L200CBD(5) [11], and L2KCBD(5) [9].

General Locomotion

There were no significant differences in the distance travelled between any of the treatment groups based on a one-way ANOVA analysis ($F_{(5,52)} = 0.472$, $p = .795$; **Figure 9B**), suggesting that treatment did not affect general locomotion. Thus, the results from the OF test conclusively eliminated variable locomotor activity as a confounding variable in all subsequent behavioural assays. Statistical outliers were identified and removed from the following groups: L200V [1] and L200CBD(5) [1].

Center Time

The time spent in the center of the chamber was analyzed as a measure of rodent open-space anxiety. There were no significant differences in the time spent in the center of the chamber between any of the treatment groups based on the Kruskal-Wallis test ($\chi^2_{(5)} = 5.584$, $p = .345$; **Figure 9C**). However, there appeared to be a trend of increasing time spent in the center with increasing volume of limonene and for Limonene-CBD combination groups. Statistical outliers were identified and removed from the following group: L2KV [1].

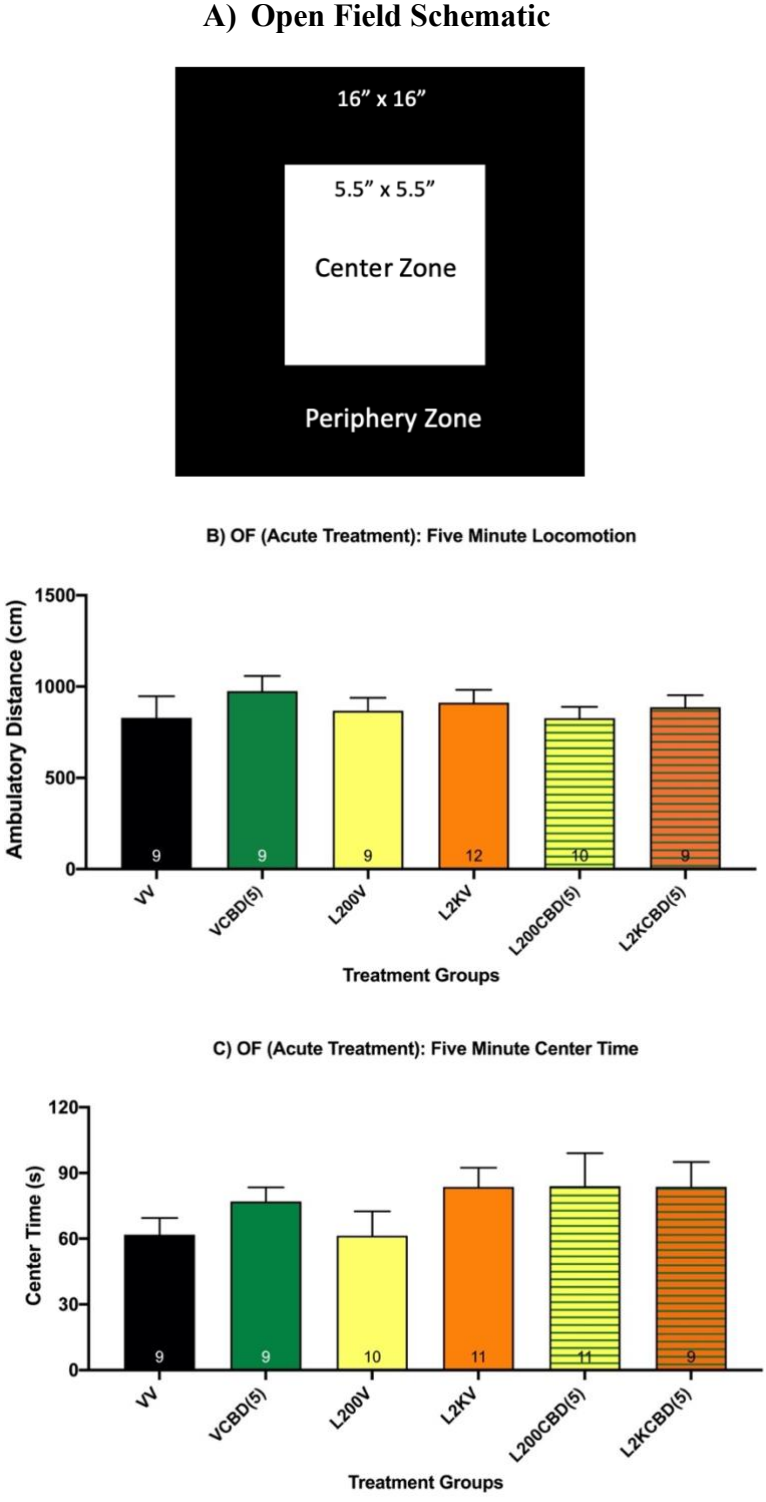

Figure 9: Open Field (Baseline Cohort). A) A top-down view schematic of the OF apparatus **B)** Distance travelled in the first five minutes of the test. No significant differences were observed between treatment groups; one-way ANOVA **C)** Time spent in the center of the apparatus in the first five minutes of the test. No significant differences were observed between treatment groups; K-S test. CBD doses were 5 ng/0.5 µL; limonene doses were 200 µL (L200) or 2000 µL (L2K). Plotted values are the mean ± SEM; the sample size of each group is represented on the bottom of their respective bar; $p < 0.05$.

3.2.2 Elevated Plus Maze (EPM)

The elevated plus maze test was used to assess rodents' inherent aversion to open spaces. Subjects that spend more time in the open arms of the maze are deemed to be less anxious. Two parameters from this test were analyzed: time in open arms and the total number of open arm entries. For this test, the number of animals in each group (excluding histological outliers mentioned in *2.6 Histology*) were: VV [9], VCBD(1) [10], VCBD(5) [10], L200V [8], L2KV [14], L200CBD(1) [8], L200CBD(5) [13], L2KCBD(1) [8], and L2KCBD(5) [9]. Animals that fell off the maze and therefore, did not complete the 10-minute assay were excluded from analysis. These were from: VV [1], VCBD(5) [2], and L200CBD(5) [2].

Time in Open Arms

In comparison to the VV group, L2KV and all Limonene-CBD combination groups (i.e., L200CBD(1), L200CBD(5), L2KCBD(1), and L2KCBD(5)) spent significantly more time in the open arms of the maze; $p = .041$, $p = .014$, $p = .004$, $p = .011$, and $p = .014$, respectively. In comparison to the VCBD(1) group, all Limonene-CBD combination groups (i.e., L200CBD(1), L200CBD(5), L2KCBD(1), and L2KCBD(5)) spent significantly more time in the open arms of the maze; $p = .027$, $p = .008$, $p = .021$, and $p = .028$, respectively.

In comparison to the VCBD(5) group, all Limonene-CBD combination groups (i.e., L200CBD(1), L200CBD(5), L2KCBD(1), and L2KCBD(5)) spent significantly more time in the open arms of the maze; $p = .026$, $p = .009$, $p = .020$, and $p = .026$, respectively.

In comparison to the lowest dose of limonene, L200V, all Limonene-CBD combination groups (i.e., L200CBD(1), L200CBD(5), L2KCBD(1), and L2KCBD(5)) spent significantly more time in the open arms of the maze; $p = .024$, $p = .008$, $p =$

.018, and $p = .024$, respectively. **Figure 10B** summarizes these findings; one-way ANOVA analysis ($F_{(8, 75)} = 3.173$, $p = .004$).

Total Number of Open Arm Entries

In comparison to the VV group, L2KV and all Limonene-CBD combination groups except the L200CBD(1) group made significantly more open arm entries. Individual p values between VV and L2KV, L200CBD(5), L2KCBD(1), and L2KCBD(5) were $p = .026$, $p = .011$, $p = .015$, and $p = .006$, respectively.

Compared to the VCBD(1) group, L200CBD(5), L2KCBD(1), and L2KCBD(5) made significantly more open arm entries; $p = .037$, $p = .047$, $p = .019$, respectively.

Finally, compared to the VCBD(5) group, the L2KCBD(5) group displayed significantly more open arm entries; $p = .039$. **Figure 10C** summarizes these findings; one-way ANOVA analysis ($F_{(8, 74)} = 2.120$, $p = .044$). Statistical outliers were identified and removed from the following group: L2KV [1].

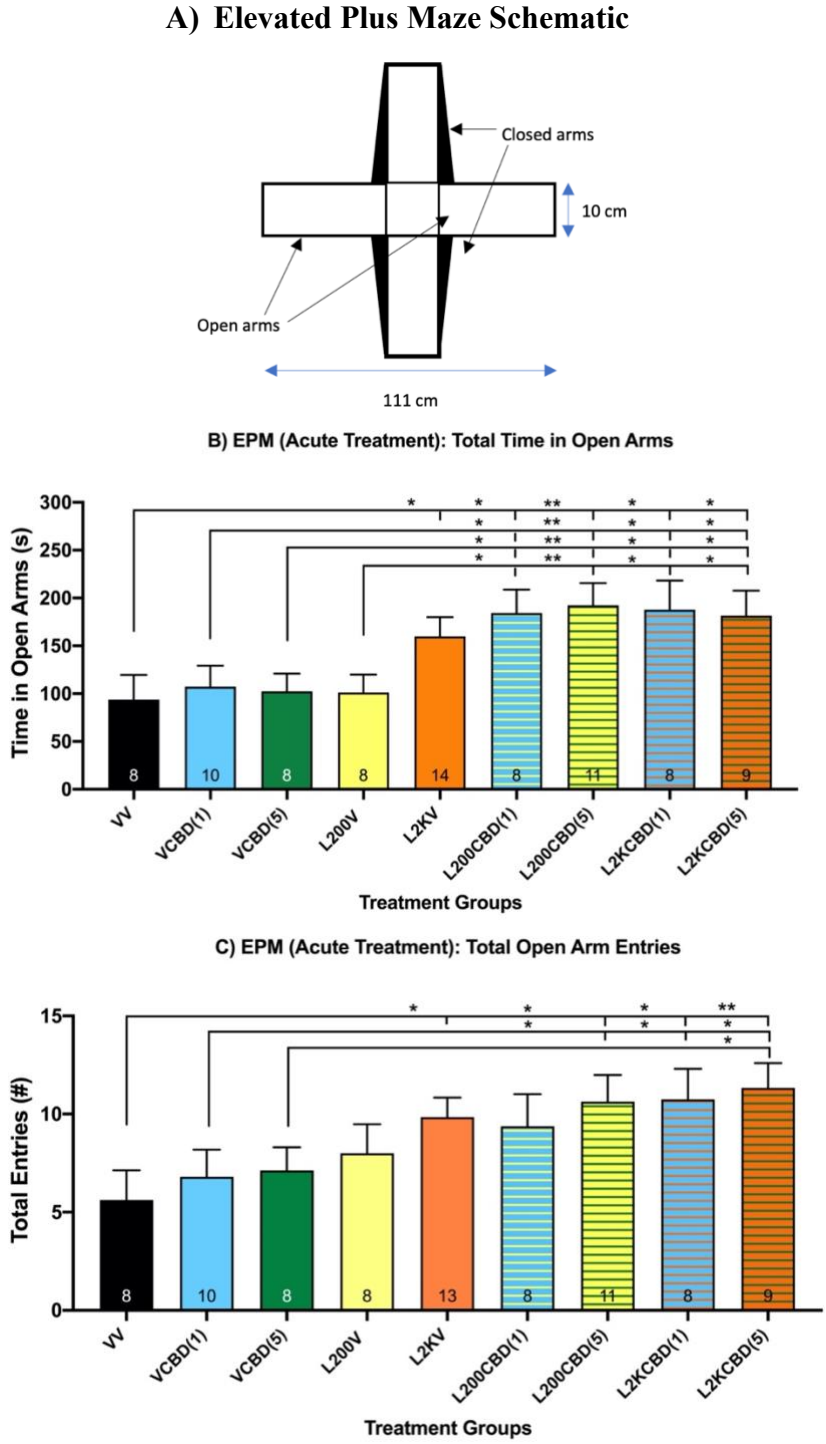

Figure 10: Elevated Plus Maze (Baseline Cohort). A) A top-down view schematic of the EPM apparatus **B)** The total time spent in the open arms of the maze by treatment groups. The group receiving the highest dose of limonene (L2KV) and all Limonene-CBD combination groups spent significantly more time in the open arms compared to the VV group. Compared to the CBD only groups (VCBD(1) and VCBD(5)) and the group receiving the lowest dose of limonene (L200V), all Limonene-CBD combination groups spent significantly more time in the open arms **C)** The total number of open arm entries by treatment groups during the 10-minute test. The group receiving the highest dose of limonene (L2KV) and all Limonene-CBD combination groups (except L200CBD(1)) made significantly more entries than the VV group. Compared to the VCBD(1) group, all Limonene-CBD combination groups (except L200CBD(1)) made significantly more entries. Additionally, the groups receiving the highest dose of CBD and limonene (L2KCBD(5)) made significantly more entries than the VCBD(5) group. Single annotation = $p \leq 0.05$, double annotation = $p \leq .01$, triple annotation = $p \leq .001$. CBD doses were 1 ng/0.5 µL (CBD(1)) or 5 ng/0.5 µL (CBD(5)); limonene doses were 200 µL (L200) or 2000 µL (L2K). Plotted values are the mean ± SEM; the sample size of each group is represented on the bottom of their respective bar.

3.2.3 Three-Chamber Social Interaction (SI)

The three-chamber social interaction test was conducted as a pre-clinical model of social anxiety. Rodents normally prefer to spend time with a conspecific than alone and also tend to prefer a novel conspecific than a familiar one. Thus, the assay consists of two measurements: general sociability and preference for social novelty. These are quantified as 'social motivation index' and 'social recognition index', respectively. For this test, the number of animals in each group (excluding histological outliers mentioned in *2.6 Histology*) were: VV [9], VCBD(5) [9], L200V [10], L2KV [11], L200CBD(5) [10], and L2KCBD(5) [10].

Social Motivation Index (SMI)

The social motivation index is the normalized value of the time spent sniffing the cage with the rat divided by the total time spent sniffing in Stage Two.

There were no significant differences in the SMI values between any of the treatment groups based on a one-way ANOVA analysis ($F_{(5,51)} = 0.789$, $p = .563$; **Figure 11B**). Statistical outliers were identified and removed from the following group: L2KV [2].

Social Recognition Index (SRI)

The social recognition index is the normalized value of the time spent sniffing the cage with the novel rat divided by the total time spent sniffing in Stage Three. There were no significant differences in the SRI values between any of the treatment groups based on a one-way ANOVA analysis ($F_{(5,54)} = 0.065$, $p = .997$; **Figure 11C**).

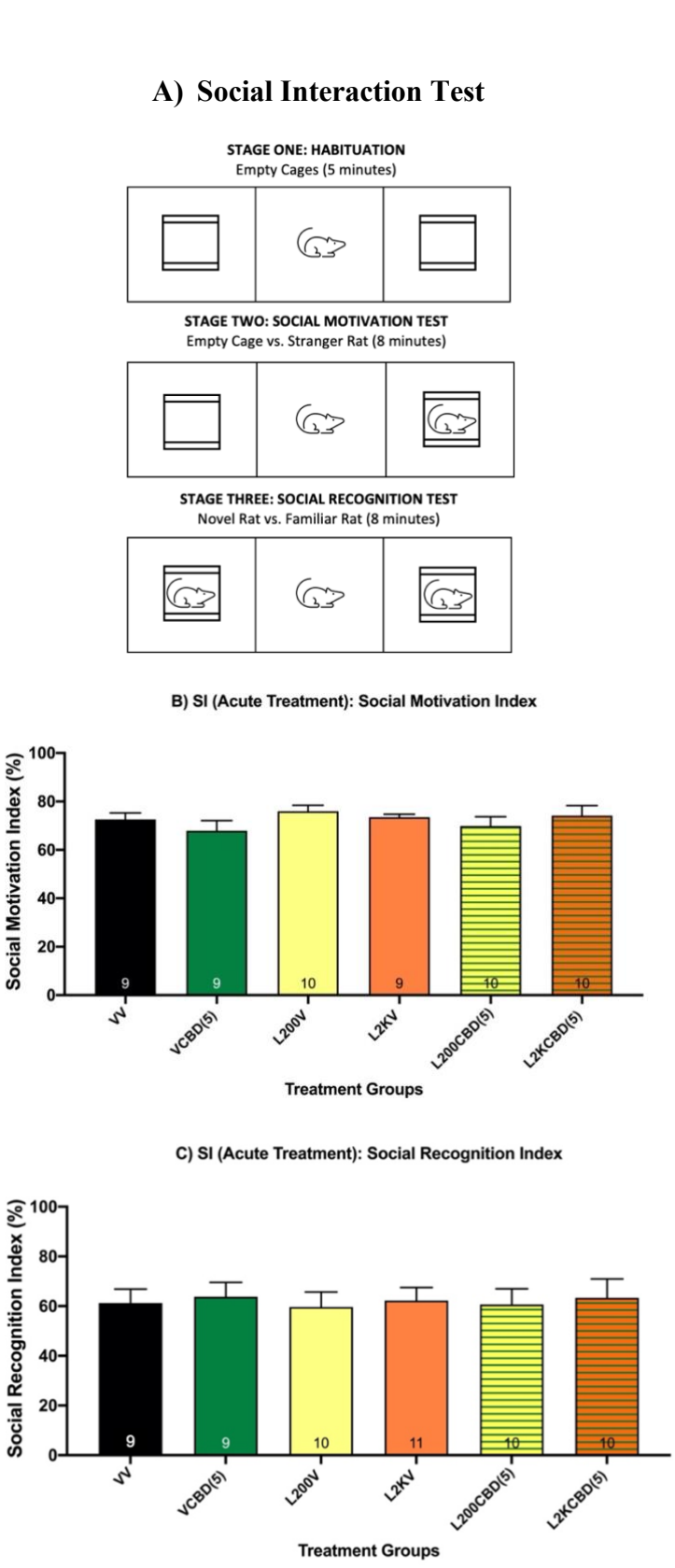

Figure 11: Social Interaction (Baseline Cohort). A) A front-view schematic of the SI apparatus **B)** The social motivation indices measured in Stage Two of the test. No significant differences were observed between treatment groups; one-way ANOVA **C)** The social recognition indices measured in Stage Three of the test. No significant differences were observed between treatment groups; one-way ANOVA. CBD doses were 5 ng/0.5 µL (CBD(5)); limonene doses were 200 µL (L200) or 2000 µL (L2K). Plotted values are the mean ± SEM; the sample size of each group is represented on the bottom of their respective bar; $p < 0.05$.

3.2.4 Light-Dark Box (LDB)

The light-dark box test was used to assess rodents' inherent aversion for brightly-lit spaces. Subjects that spend more time in the light compartment are deemed to be less anxious. Three parameters from this test were analyzed: time in light, time in dark, and risk assessment. For this test, the number of animals in each group (excluding histological outliers mentioned in *2.6 Histology*) were: VV [9], VCBD(5) [8], L200V [8], L2KV [9], L200CBD(5) [9], and L2KCBD(5) [9].

Time in Light

Both the L200CBD(5) and L2KCBD(5) groups spent significantly more time in the light compartment of the box compared to VV ($p = .007$ and $p = .001$, respectively) and VCBD(5) ($p = 0.005$ and $p < .001$, respectively) based on a one-way ANOVA analysis ($F_{(5, 46)} = 4.217$, $p = 0.003$; **Figure 12A**).

Time in Dark

Compared to the VV treatment group, L200V, L2KV, L200CBD(5), and L2KCBD(5) spent significantly more time in the dark compartment of the box ($p = .016$, $p = .002$, $p < .001$, and $p < .001$, respectively). Additionally, compared to the VCBD(5) group significant differences were found with L200V, L2KV, L200CBD(5), and L2KCBD(5) ($p = .018$, $p = .003$, $p < .001$, and $p < .001$, respectively); one-way ANOVA analysis ($F_{(5, 45)} = 6.814$, $p < .0001$; **Figure 12B**). It should be clarified that time in dark is not simply the opposite of time in light as instances of risk assessment behaviour (see below) are not included in this analysis. Statistical outliers were identified and removed from the following group: L200CBD(5) [1].

Risk Assessment

The risk assessment measure included (time the subject spent in the light) + (time the subject spent with its head or forepaws in the light while the body remained in the dark). Compared to the VV treatment group, L200V, L2KV, L200CBD(5), and L2KCBD(5) spent significantly more time in risk assessment behaviours ($p = .016$, $p = .002$, $p < .0001$, $p < .0001$, respectively). Compared to the VCBD(5) group, L200V, L2KV, L200CBD(5), and L2KCBD(5) displayed significantly more risk assessment ($p = .018$, $p = .003$, $p < .0001$, $p < .0001$, respectively); one-way ANOVA analysis ($F_{(5, 45)} = 6.814$, $p < .0001$; **Figure 12C**). Statistical outliers were identified and removed from the following group: L200CBD(5) [1].

Total Number of Transitions

Analysis of the total number of transitions made between the light and dark compartments revealed a significant difference between the VV group and L2KV ($p = .013$), L200CBD(5) ($p = .007$), and L2KCBD(5) ($p = .002$) and between the VCBD(5) group and L2KCBD(5) ($p = .022$) according to the Kruskal-Wallis test ($\chi^2_{(5)} = 14.52$, $p = .013$; **Figure 12D**).

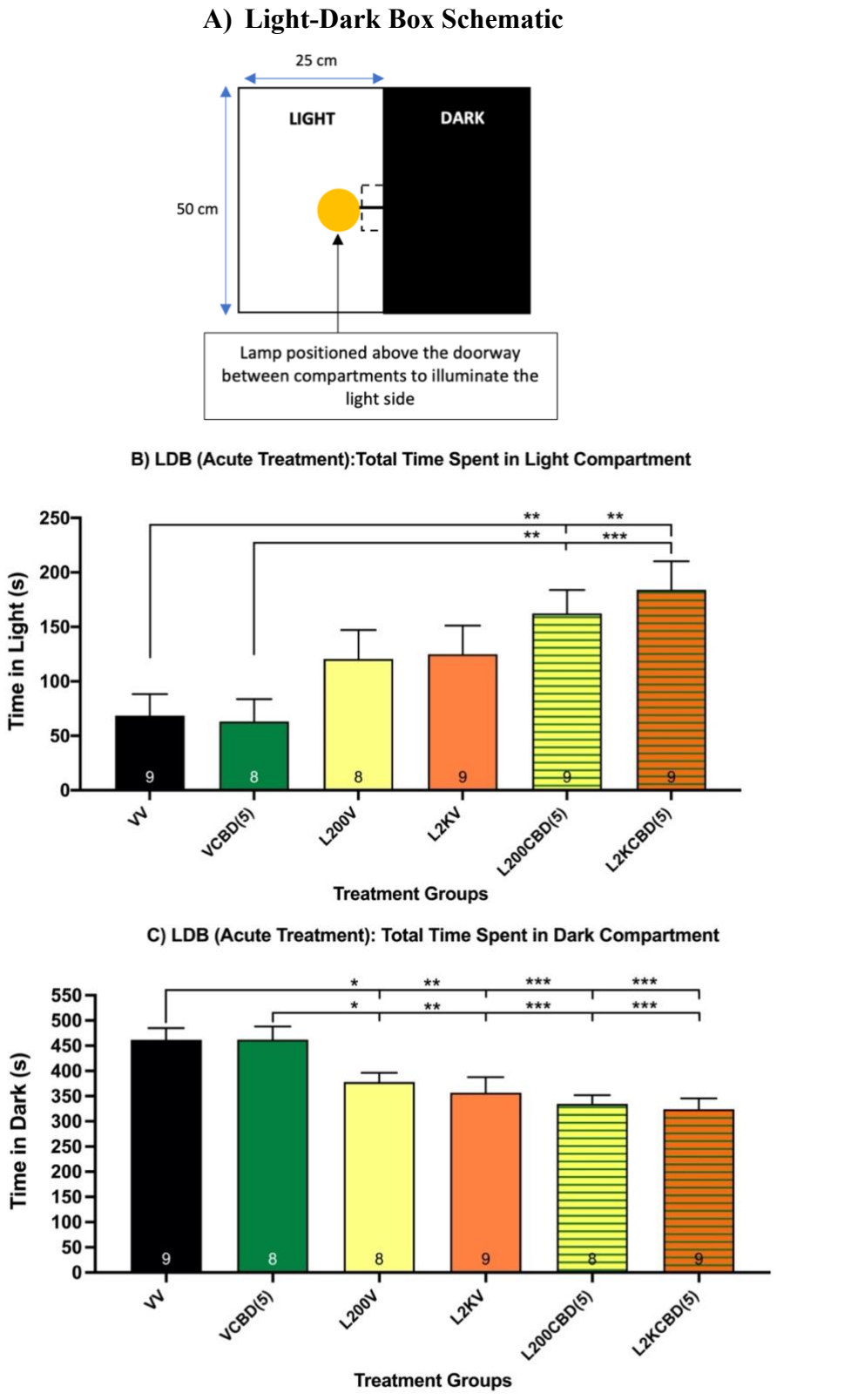

Figure 12: Light Dark Box (Baseline Cohort). A) A top-down view schematic of the LDB apparatus **B)** The total time spent in the light compartment of the box by treatment groups. Limonene-CBD combination groups spent significantly more time in the light compared to VV and VCBD(5) groups **C)** The total time spent in the dark compartment of the box by treatment groups. Limonene only and Limonene-CBD combination groups spent significantly less time in the dark compared to VV and VCBD(5) groups. Single annotation = $p \leq 0.05$, double annotation = $p \leq .01$, triple annotation = $p \leq .001$. CBD doses were 5 ng/0.5 µL; limonene doses were 200 µL (L200) or 2000 µL (L2K). Plotted values are the mean ± SEM; the sample size of each group is represented above their respective error bar.

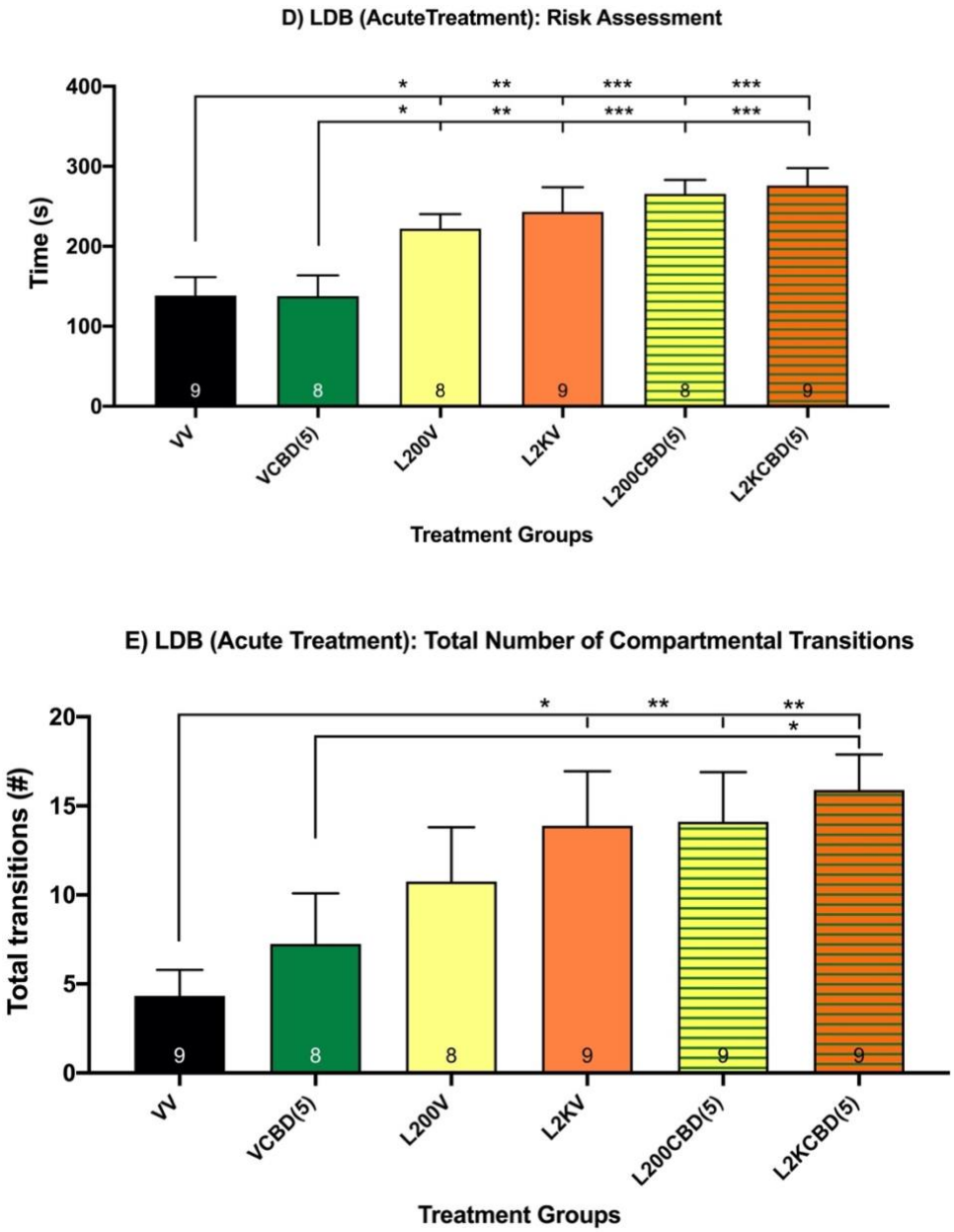

Figure 13 (continued): Light Dark Box (Baseline Cohort). D) The total time spent in risk assessment behaviours. Limonene only and Limonene-CBD combination groups spent significantly more time in these behaviours compared to VV and VCBD(5) groups **E)** The total number of transitions made between the light and dark compartments during the 10-minute test. L2KV and Limonene-CBD groups made significantly more transitions compared to the VV group. Additionally, the L2KCBD(5) group made significantly more transitions compared to the VCBD(5) group. Single annotation = $p \leq 0.05$, double annotation = $p \leq .01$, triple annotation = $p \leq .001$. CBD doses were 5 ng/0.5 µL; limonene doses were 200 µL (L200) or 2000 µL (L2K). Plotted values are the mean ± SEM; the sample size of each group is represented above their respective error bar.

3.2.5 Contextual Fear Conditioning (CFC)

The contextual fear conditioning paradigm assesses rodents' associative memory between an aversive foot-shock stimulus and the environment. Specifically, this study assessed rodents' fear-related memory acquisition. The parameter measured was freezing behaviour in response to the foot-shock-associated context. For this test, the number of animals in each group (excluding histological outliers mentioned in *2.6 Histology*) were: VV [9], VCBD(1) [10], VCBD(5) [9], L200V [9], L2KV [8], L200CBD(1) [9], L200CBD(5) [9], and L2KCBD(1) [8].

Freezing Behaviour

All treatment groups (except for VCBD(1)) displayed significantly less time freezing compared to the VV group. Individuals *p* values respectively, for VCBD(5), L200V, L2KV, L200CBD(1), L200CBD(5), and L2KCBD(1) were: .037, .008, .020, .005, .017, and .002; Kruskal-Wallis test ($\chi^2_{(7)} = 13.45$, $p = .061$; **Figure 13B**). Additionally, based on the Welch's t-test the L2KCBD(1) group spent significantly less time freezing compared to the VCBD(1) group; ($t_{(16)} = 2.245$, $p = .045$; **Figure 13B**).

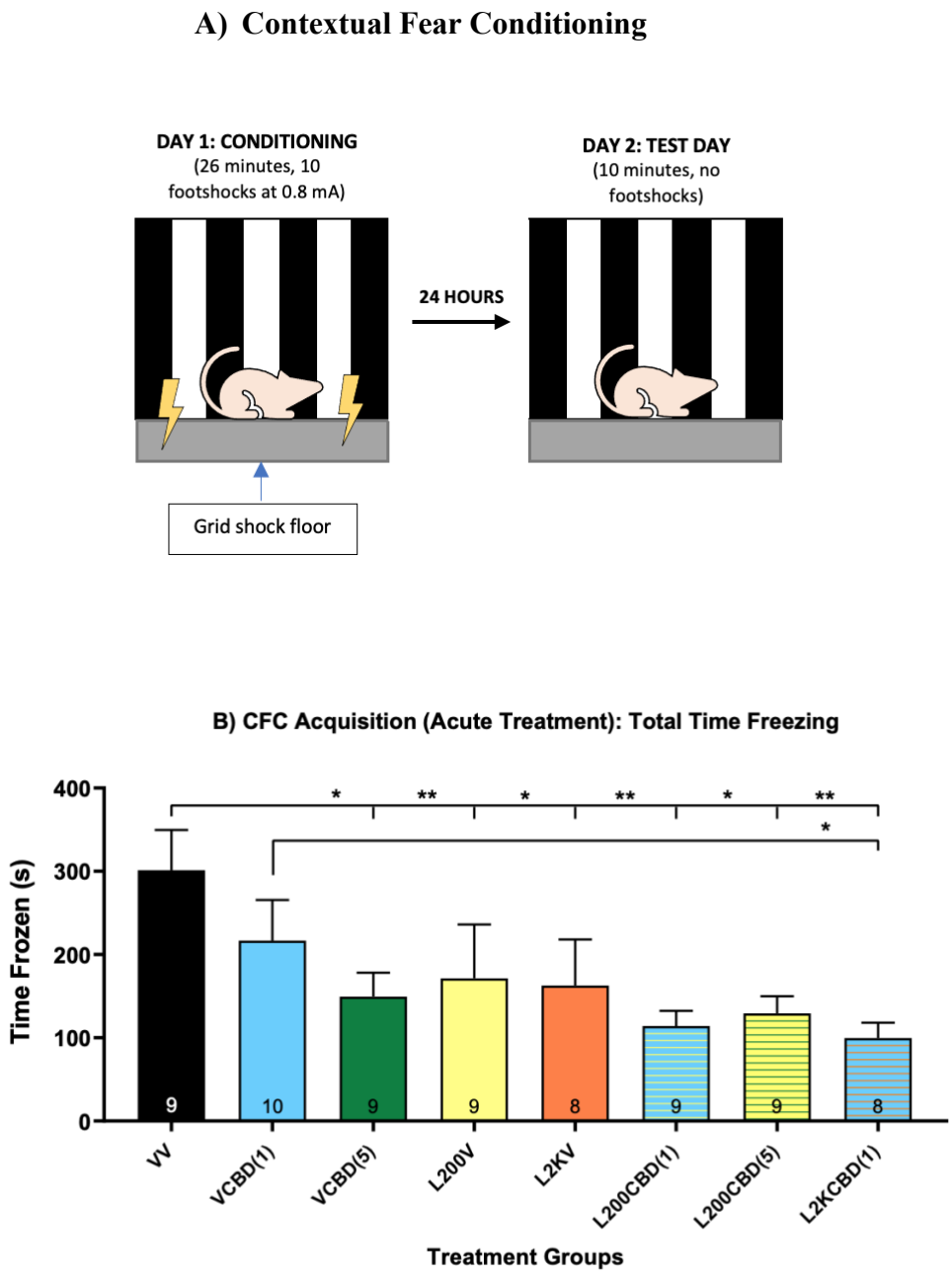

Figure 14: Contextual Fear Conditioning (Baseline Cohort). A) A schematic of the CFC paradigm **B)** Total time spent freezing in response to the foot-shock associated environment on Day 2. Compared to the VV group, all groups (except for VCBD(1)) spent significantly less time freezing. Compared to the VCBD(1) group, the L2KCBD(1) group spent significantly less time freezing. Single annotation = $p \leq 0.05$, double annotation = $p \leq .01$, triple annotation = $p \leq .001$. CBD doses were 1 ng/0.5 µL (CBD(1)) or 5 ng/0.5 µL (CBD(5)); limonene doses were 200 µL (L200) or 2000 µL (L2K). Plotted values are the mean ± SEM; the sample size of each group is represented above their respective error bar.

3.3 NAD299 Challenge Cohort Behavioural Results

3.3.1 Open Field (OF)

The open field (OF) test was conducted to assess if treatment groups affected general locomotor activity. For this test, the number of animals in each group (excluding histological outliers mentioned in *2.6 Histology*) were: VV [9], L200CBD(5) [11], L2KCBD(5) [9], L200CBD(5)NAD [11], L2KCBD(5)NAD [11], and VNAD [11].

General Locomotion

There were no significant differences in the distance travelled between any of the treatment groups based on a one-way ANOVA analysis ($F_{(4,45)} = 0.369$, $p = .829$; **Figure 14A [left]**), suggesting that treatment did not affect general locomotion. Thus, the results from the OF test conclusively eliminated differing locomotor activity as a confounding variable in all subsequent behavioural assays in the NAD299 challenge experiments. Additionally, the Welch's t-test with VV and VNAD confirmed that locomotor ability did not differ significantly between these groups ($t_{(18)} = 1.032$, $p = .318$; **Figure 14A [right]**). Statistical outliers were identified and removed from the following group: L200CBD(5) [1].

Center Time

The time spent in the center of the chamber was analyzed as a measure of rodent open-space anxiety. There were no significant differences in the time spent in the center of the chamber between any of the treatment groups based on the Kruskal-Wallis test ($\chi^2_{(4)} = 2.855$, p = .582; **Figure 14B [left]**). Notably, however, the time spent in the center of the chamber was highest for the Limonene-CBD combination groups with a trend in the reversal of this effect in the groups receiving NAD299.

Additionally, there was no significant difference in the time spent in the center between the VV and VNAD groups based on the Mann-Whitney test ($U = 33$, $p = .356$; **Figure 14B [right]**). Statistical outliers were identified and removed from the following groups: L200CBD(5)NAD [1] and VNAD [1].

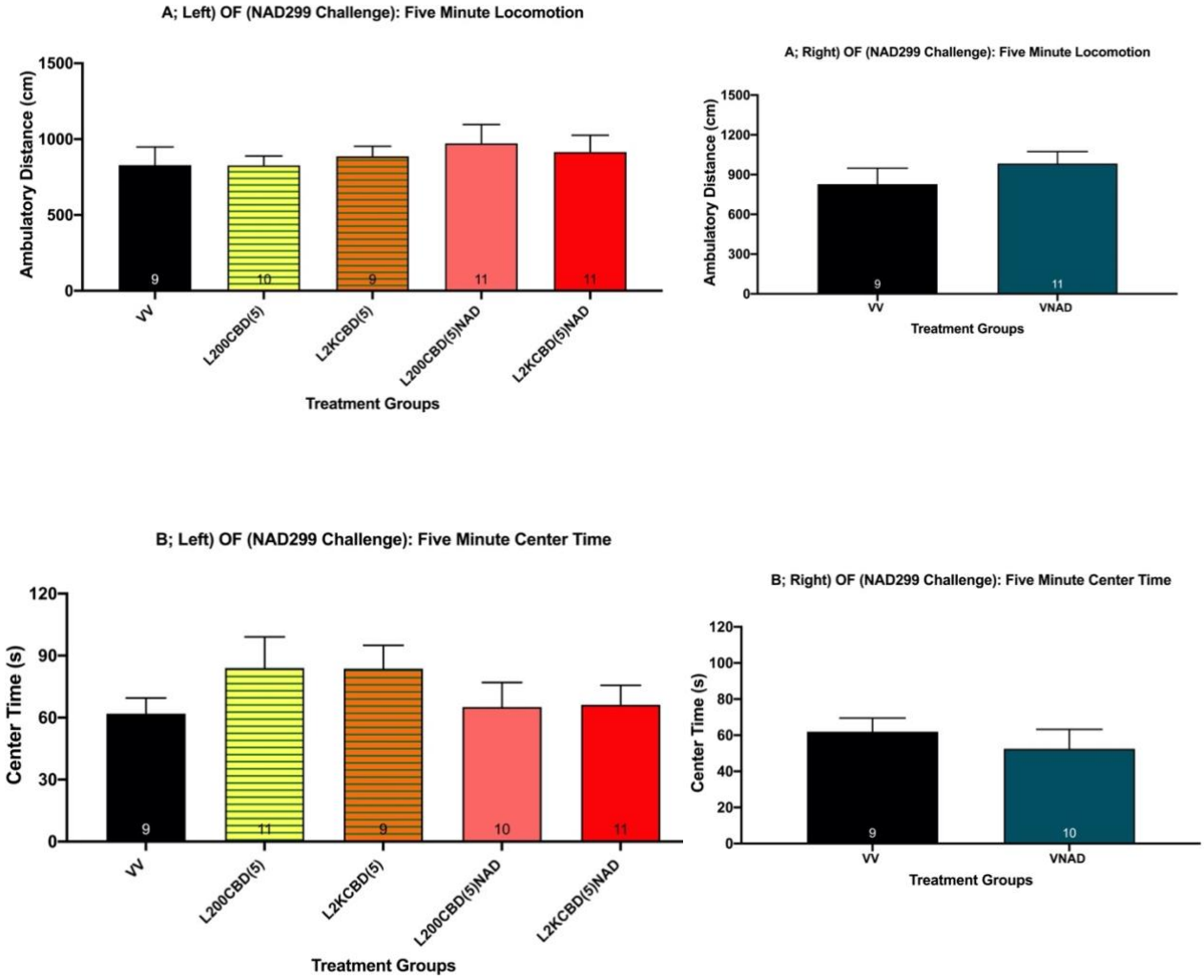

Figure 15: Open Field (NAD299 Challenge Cohort). A) Left: The distance travelled in the first five minutes of the test. No significant differences were observed between treatment groups; one-way ANOVA **A) Right:** The distance travelled between VV and VNAD groups. No significant differences were observed between groups; Welch's t-test **B) Left:** Time spent in the center of the apparatus in the first five minutes of the test. No significant differences were observed between groups; K-S test **B) Right:** Time spent in the center between VV and VNAD groups. No significant differences were observed between groups; Mann-Whitey U-test. CBD doses were 5 ng/0.5 µL (CBD(5)); limonene doses were 200 µL (L200) or 2000 µL (L2K); NAD299 doses were 100 ng/0.5 µL. Plotted values are the mean ± SEM; the sample size of each group is represented on the bottom of their respective bar; $p < 0.05$.

3.3.2 Elevated Plus Maze (EPM)

The elevated plus maze test was used to assess rodents' inherent aversion to open spaces. Subjects that spend more time in the open arms of the maze are deemed to be less anxious. Two parameters from this test were analyzed: time in open arms and the total number of open arm entries. For this test, the number of animals in each group (excluding histological outliers mentioned in *2.6 Histology*) were: VV [9], L200CBD(1) [8], L200CBD(5) [13], L2KCBD(1) [8], L2KCBD(5) [9], L200CBD(5)NAD [8], L2KCBD(5)NAD [8], and VNAD [8]. Animals that fell off the maze and therefore, did not complete the 10-minute assay were excluded from analysis. These were from: VV [1] and L200CBD(5) [2].

Time in Open Arms

In comparison to the VV group, all Limonene-CBD combination groups (i.e., L200CBD(1), L200CBD(5), L2KCBD(1), and L2KCBD(5)) spent significantly more time in the open arms of the maze; $p = .016$, $p = .005$, $p = .012$, and $p = .016$, respectively. Additionally, there appeared to be a reversal of this affect with NAD299 as displayed by the significant difference between L200CBD(1) and L200CBD(5)NAD ($p = .012$), L200CBD(1) and L2KCBD(5)NAD ($p = .035$), L200CBD(5) and L200CBD(5)NAD ($p = .004$), L200CBD(5) and L2KCBD(5)NAD ($p = .013$), L2KCBD(1) and L200CBD(5)NAD ($p = .009$), L2KCBD(1) and L2KCBD(5)NAD ($p = .028$), L2KCBD(5) and L200CBD(5)NAD ($p = .012$), and L2KCBD(5) and L2KCBD(5)NAD ($p = .036$). **Figure 15A [left]** illustrates these results; one-way ANOVA analysis ($F_{(6, 53)} = 3.774$, $p = .003$). The Welch's t-test revealed no significant difference between the VV and VNAD groups ($t_{(14)} = 1.151$, $p = .271$; **Figure 15A [right]**).

Total Number of Open Arm Entries

In comparison to the VV group, L200CBD(5), L2KCBD(1), and L2KCBD(5) groups made significantly more open arm entries (p = .018, p = .025, and p = .011, respectively). Additionally, there was a significant reversal of this effect with NAD299 with decreased open arm entries in the L200CBD(5)NAD group compared to the L2KCBD(5) group (p = .038). **Figure 15B [left]** summarizes these findings; one-way ANOVA analysis ($F_{(6, 53)}$ = 2.238, p = .054). The Welch's t-test revealed no significant difference between the VV and VNAD groups ($t_{(14)}$ = .674, p = .512; **Figure 15B [right]**).

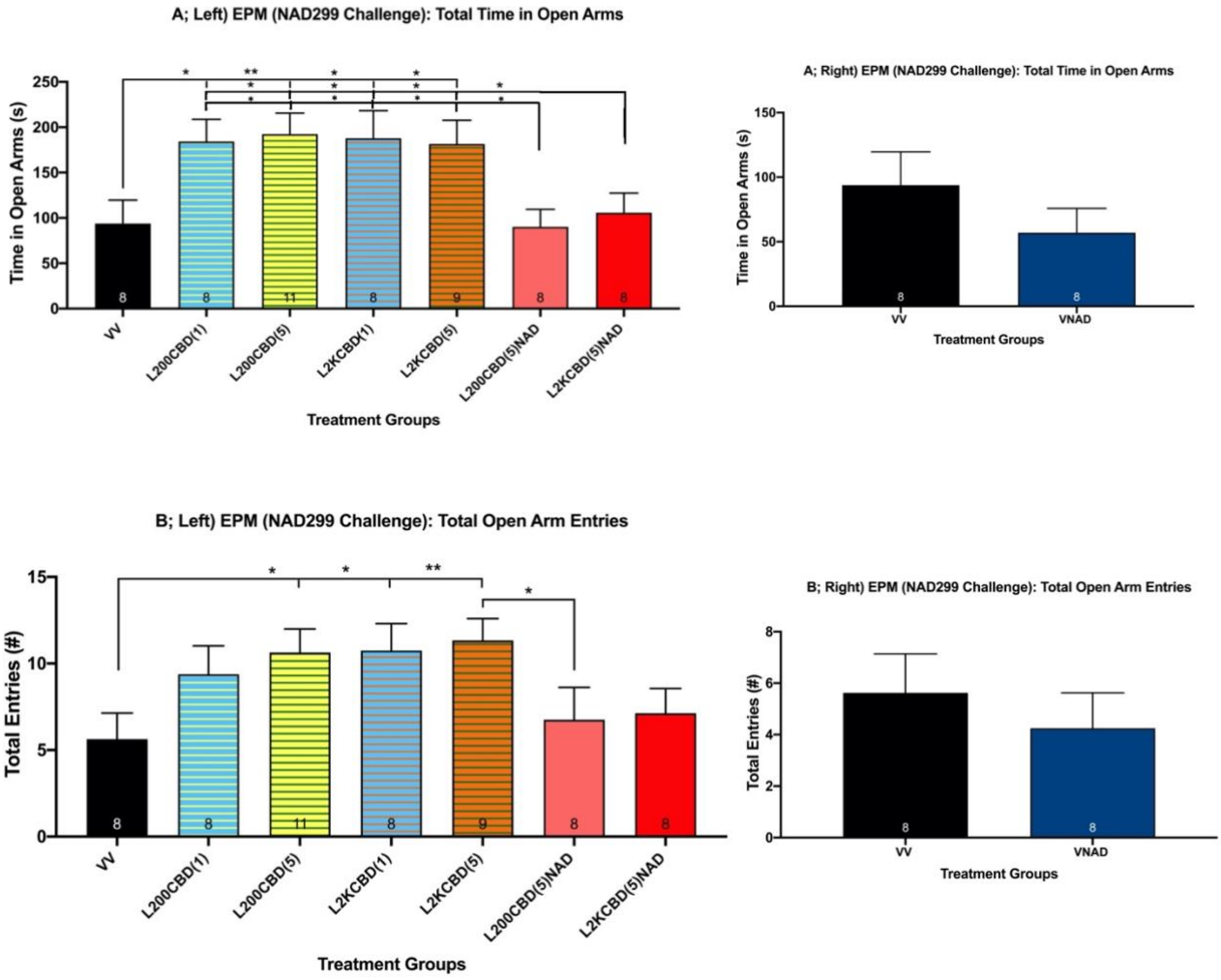

Figure 16: Elevated Plus Maze (NAD299 Challenge Cohort). **A) Left:** Total time spent in the open arms of the maze by treatment groups. Compared to the VV group, all Limonene-CBD combination groups spent significantly more time in the open arms. There was a reversal of this effect illustrated by the groups receiving NAD299 spending significantly less time in the open arms compared to the Limonene-CBD combination groups **A) Right:** Graph displaying no significant differences in the total time spent in the open arms of the maze between VV and VNAD groups **B) Left:** Total number of open arm entries by treatment groups during the 10-minute test. Compared to the VV group, all Limonene-CBD combination groups (except L200CBD(1)) made significantly more entries. Additionally, the L200CBD(5)NAD group made significantly fewer entries than the L2KCBD(5) group **B) Right:** Graph displaying no significant difference in the total number of open arm entries between VV and VNAD groups. Single annotation = $p \leq 0.05$, double annotation = $p \leq .01$, triple annotation = $p \leq .001$. CBD doses were 1 ng/0.5 µL (CBD(1)) or 5 ng/0.5 µL (CBD(5)); limonene doses were 200 µL (L200) or 2000 µL (L2K); NAD299 doses were 100 ng/0.5 µL. Plotted values are the mean ± SEM; the sample size of each group is represented on the bottom of their respective bar.

3.3.3 Three Chamber Social Interaction (SI)

The three-chamber social interaction test was conducted as a pre-clinical model of social anxiety. Rodents normally prefer to spend time with a conspecific than alone and also tend to prefer a novel conspecific than a familiar one. Thus, the assay consists of two measurements: general sociability and preference for social novelty. These are quantified as 'social motivation index' and 'social recognition index', respectively. For this test, the number of animals in each group (excluding histological outliers mentioned in *2.6 Histology*) were: VV [9], L200CBD(5) [10], L2KCBD(5) [10], L200CBD(5)NAD [9], L2KCBD(5)NAD [9], and VNAD [10].

Social Motivation Index (SMI)

The social motivation index is the normalized value of the time spent sniffing the cage with the rat divided by the total time spent sniffing in Stage Two. There were no significant differences in the SMI values between any of the treatment groups based on a one-way ANOVA analysis ($F_{(4,42)} = 0.290$, $p = .883$; **Figure 16A [left]**). Additionally, the Welch's t-test with VV and VNAD confirmed that social motivation indices did not differ significantly between these groups ($t_{(17)} = 1.753$, $p = .098$; **Figure 16A [right]**).

Social Recognition Index (SRI)

The social recognition index is the normalized value of the time spent sniffing the cage with the novel rat divided by the total time spent sniffing in Stage Three. There were no significant differences in the SRI values between any of the treatment groups based on a one-way ANOVA analysis ($F_{(4,43)} = 0.329$, $p = .857$; **Figure 16B [left]**). Additionally, the Welch's t-test with VV and VNAD confirmed social recognition indices did not differ significantly between these groups ($t_{(18)} = 1.217$, $p = .0239$; **Figure 16B [right]**).

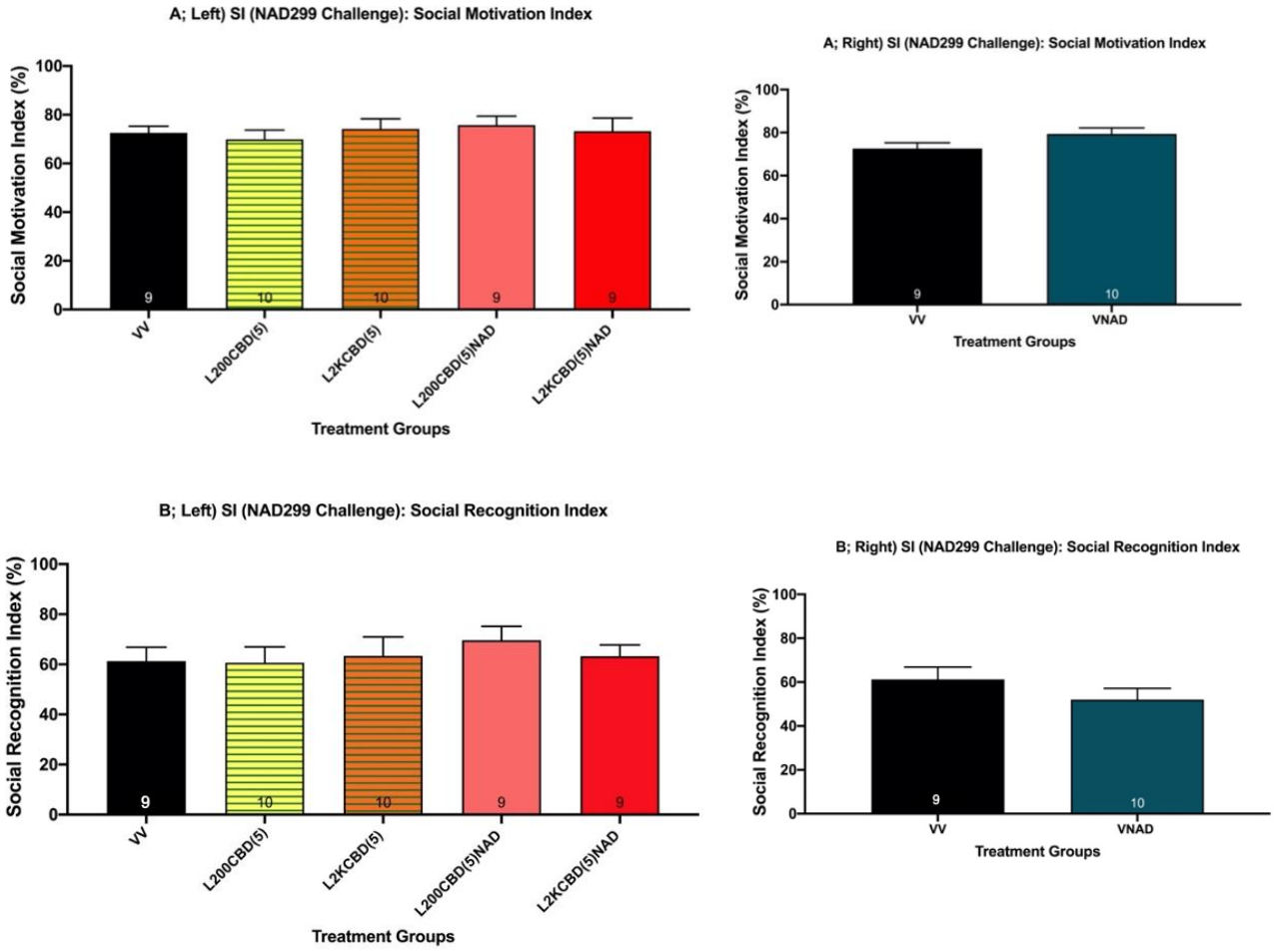

Figure 17: Social Interaction (NAD299 Challenge Cohort). A) Left: Social motivation indices measured in Stage Two of the test. No significant differences were observed between treatment groups; one-way ANOVA **A) Right:** No significant difference in social motivation indices between VV and VNAD; Welch's t-test **B) Left:** Social recognition indices measured in Stage Three of the test. No significant differences were observed between treatment groups; one-way ANOVA **B) Right:** No significant difference in social recognition indices between VV and VNAD; Welch's t-test. CBD doses were 5 ng/0.5 µL (CBD(5)); limonene doses were 200 µL (L200) or 2000 µL (L2K); NAD299 doses were 100 ng/0.5 µL. Plotted values are the mean ± SEM; the sample size of each group is represented on the bottom of their respective bar; $p < 0.05$.

3.3.4 Light-Dark Box (LDB)

The light-dark box test was used to assess rodents' inherent anxiety for brightly-lit spaces. Three parameters from this test were analyzed: time in light, time in dark, and risk assessment. For this test, the number of animals in each group (excluding histological outliers mentioned in *2.6 Histology*) were: VV [9], L200CBD(5) [9], L2KCBD(5) [9], L200CBD(5)NAD [12], L2KCBD(5)NAD [12], and VNAD [11].

Time in Light

Both the L200CBD(5) and L2KCBD(5) groups spent significantly more time in the light compartment of the box compared to VV ($p = .014$ and $p = .003$, respectively; one-way ANOVA analysis ($F_{(4, 46)} = 2.917$, $p = .031$); **Figure 17A [left]**). The Mann-Whitney test revealed no significant difference between the VV and VNAD groups ($U = 48$, $p = .941$; **Figure 17A [right]**). Statistical outliers were identified and removed from the following group: L2KCBD(5)NAD [1].

Time in Dark

Compared to the VV treatment group, L200CBD(5), and L2KCBD(5) spent significantly more time in the dark compartment of the box ($p = .005$ and $p = .002$, respectively). Additionally, there was a significant reversal of this effect with NAD299 as observed by the significant difference between L200CBD(5) and L200CBD(5)NAD ($p = .048$) and L2KCBD(5) and L200CBD(5)NAD ($p = .022$); one-way ANOVA analysis ($F_{(4, 44)} = 3.864$, $p = .009$; **Figure 17B [left]**). The Mann-Whitney test revealed no significant difference between the VV and VNAD groups ($U = 44$, $p = .710$; **Figure 17B [right]**). It should be clarified that time in dark is not simply the opposite of time in light as instances of risk assessment behaviour (see below) are not included in this analysis. Statistical outliers were identified and removed from the following groups: L200CBD(5) [1] and L2KCBD(5)NAD [1].

Risk Assessment

The risk assessment measure included (time the subject spent in the light) + (time the subject spent with its head or forepaws in the light while the body remained in the dark). Compared to the VV treatment group, L200CBD(5), L2KCBD(5), and L2KCBD(5)NAD spent significantly more time in risk assessment behaviours (p = .009, p = .004, p < .019, respectively). Compared to the L2KCBD(5) group, the L2KCBD(5)NAD displayed significantly less risk assessment (p = .037); one-way ANOVA analysis ($F_{(4, 45)} = 3.378$, $p = .017$; **Figure 17C [left]**). The Mann-Whitney test revealed no significant difference between the VV and VNAD groups ($U = 44$, $p = .710$; **Figure 17C [right]**). Statistical outliers were identified and removed from the following group: L200CBD(5) [1].

Total Number of Transitions

Analysis of the total number of transitions made between the light and dark compartments revealed a significant difference between VV and L200CBD(5) ($p = .004$), VV and L2KCBD(5) ($p = .001$), and L2KCBD(5) and L2KCBD(5)NAD groups ($p = .030$); Kruskal-Wallis test ($\chi^2_{(4)} = 14.09$, $p = .007$; **Figure 17D [left]**). The Mann-Whitney test revealed no significant difference between the VV and VNAD groups ($U = 47.5$, $p = .901$; **Figure 17D [right]**.

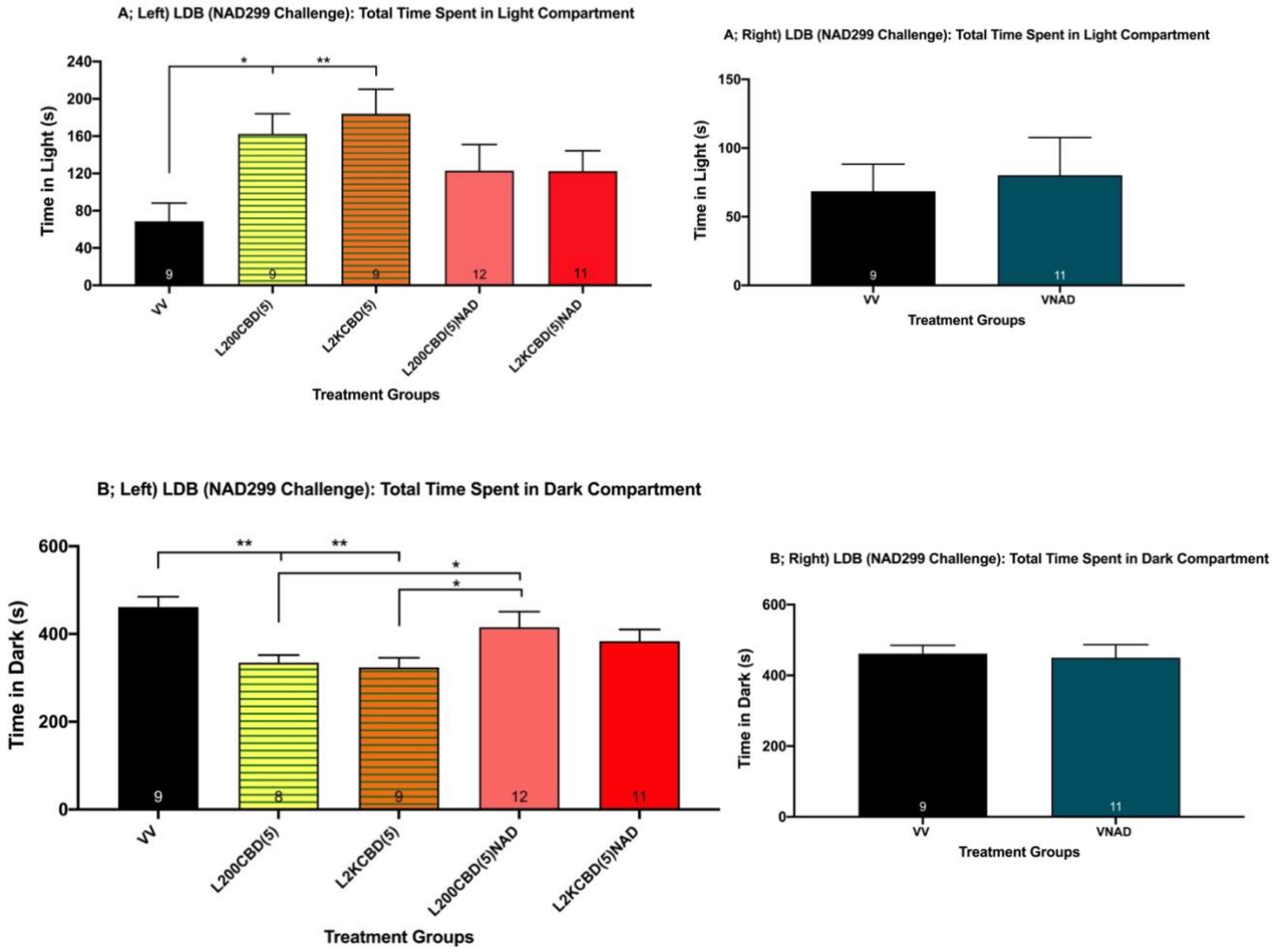

Figure 18: Light Dark Box (NAD299 Challenge Cohort). **A) Left:** Total time spent in the light compartment of the box by treatment groups. Limonene-CBD combination groups spent significantly more time in the light compared to the VV group **A) Right:** Graph displaying no significant differences in the total time spent in the light compartment of the box between VV and VNAD groups **B) Left:** Total time spent in the dark compartment of the box by treatment groups. Limonene-CBD combination groups spent significantly less time in the dark compared to the VV group. Additionally, the L200CBD(5)NAD group displayed significantly more time in the dark compared to both the Limonene-CBD combination groups **B) Right:** Graph displaying no significant differences in the total time spent in the light compartment of the box between VV and VNAD groups**.** Single annotation = $p \leq 0.05$, double annotation = $p \leq .01$, triple annotation = $p \leq .001$. CBD doses were 5 ng/0.5 µL; limonene doses were 200 µL (L200) or 2000 µL (L2K); NAD299 doses were 100 ng/0.5 µL. Plotted values are the mean ± SEM; the sample size of each group is represented on the bottom of their respective bar.

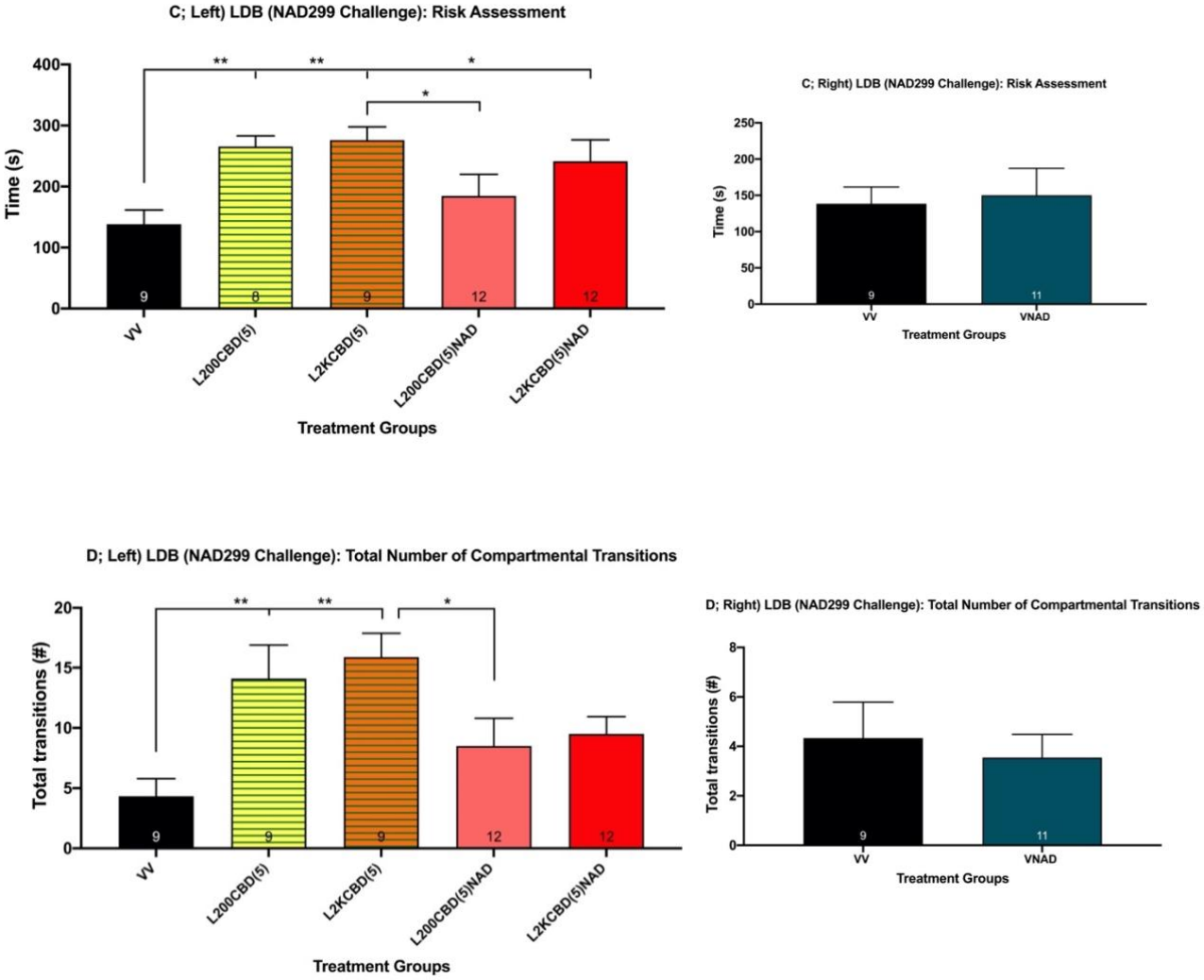

Figure 19 (continued): Light Dark Box (NAD299 Challenge Cohort). **C) Left:** Graph displaying significant differences in risk assessment between VV and Limonene:CBD combination groups and the L2KCBD(5)NAD group, and between the L2KCBD(5) and L200CBD(5)NAD groups **C) Right:** Graph displaying no significant differences in risk assessment between VV and VNAD groups **D) Left:** Total number of transitions made between the light and dark compartments during the 10-minute test. Limonene-CBD groups made significantly more transitions compared to the VV group. The L200CBD(5)NAD group displayed significantly less transitions than the L2KCBD(5) group **D) Right:** Graph displaying no significant differences in the total number of transitions made between VV and VNAD groups. Single annotation = $p \leq 0.05$, double annotation = $p \leq .01$, triple annotation = $p \leq .001$. CBD doses were 5 ng/0.5 µL; limonene doses were 200 µL (L200) or 2000 µL (L2K); NAD299 doses were 100 ng/0.5 µL. Plotted values are the mean ± SEM; the sample size of each group is represented on the bottom of their respective bar.

3.3.5 Contextual Fear Conditioning (CFC)

The contextual fear conditioning paradigm assesses rodents' associative memory between an aversive foot-shock stimulus and the environment. Specifically, this study assessed rodents' fear-related memory acquisition. The parameter measured was freezing behaviour in response to the foot-shock-associated context. For this test, the number of animals in each group (excluding histological outliers mentioned in *2.6 Histology*) were: VV [9], L200CBD(1) [9], L200CBD(5) [9], L2KCBD(1) [8], L200CBD(5)NAD [10], L2KCBD(5)NAD [10], and VNAD [9].

Freezing Behaviour

All treatment groups displayed significantly less time freezing compared to the VV group. Individuals *p* values respectively, for L200CBD(1), L200CBD(5), L2KCBD(1), L200CBD(5)NAD, and L2KCBD(5)NAD were: .004, .015, .002, .005, and .014. Notably, groups receiving NAD299 displayed a trend of greater freezing time compared to the Limonene-CBD combination groups. These findings are illustrated in **Figure 18A [left]**; Kruskal-Wallis test ($\chi^2_{(5)} = 13.22$, $p = .021$). The VV and VNAD groups displayed no significant difference in freezing time; Mann-Whitney test ($U = 23$, $p = .136$; **Figure 18A [right]**).

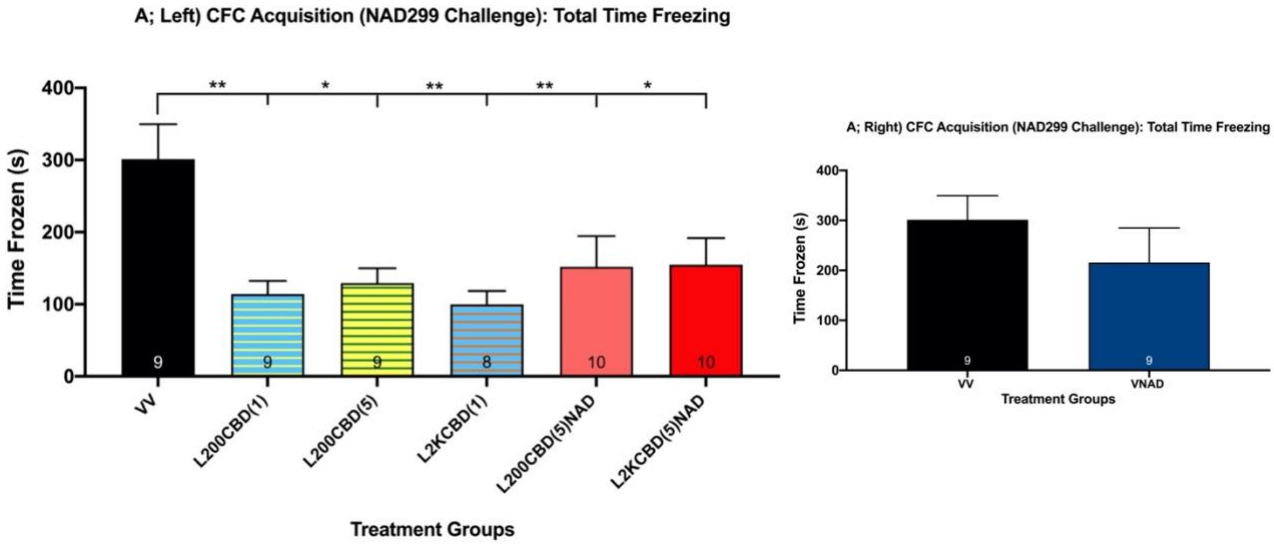

Figure 20: Contextual Fear Conditioning (NAD299 Challenge Cohort). A) Left: Total time spent freezing in response to the foot-shock associated environment on Day 2. Compared to the VV group, all groups spent significantly less time freezing. **A) Right:** VV and VNAD groups did not differ significantly in freezing time. Single annotation = $p \leq 0.05$, double annotation = $p \leq .01$, triple annotation = $p \leq .001$. CBD doses were 1 ng/0.5 µL (CBD(1)) or 5 ng/0.5 µL (CBD(5)); limonene doses were 200 µL (L200) or 2000 µL (L2K); NAD299 doses were 100 ng/0.5 µL. Plotted values are the mean ± SEM; the sample size of each group is represented on the bottom of their respective bar.

3.4 Molecular Assays

Downstream molecular biomarkers associated with anxiety and the 5-HT_{1A} system were quantified to elucidate potential mechanisms of action of the formulations utilized in the behavioural assays. Three brain regions were analyzed: the PFC, NASh, and BLA. One-way ANOVA analyses were conducted with protein expression levels normalized to the control group (VV) and represented as a percentage out of 100.

3.4.1 Mitogen-Activated Protein Kinase Pathway (ERK 1/2)

Prefrontal Cortex

With regards to the ERK1 isoform in the PFC, p-ERK1 levels were significantly lower in the L2KCBD(5) group in comparison to the VV and VCBD(5) groups ($F_{(3, 12)} = 5.482$, $p = .013$; $p = .006$ and $p = .005$, respectively; **Figure 19A**). T-ERK1 expression was significantly higher in the VCBD(5) and L2KCBD(5) groups compared to VV ($F_{(3, 12)} = 2.216$, $p = .139$; $p = .044$ and $p = .048$, respectively; **Figure 19B**). The ratio of p/T ERK1 levels were significantly lower in the L2KCBD(5) group and the L2KCBD(5)NAD group compared to the VV group ($F_{(3, 12)} = 9.810$, $p = .002$; $p < .001$ and $p = .006$, respectively; **Figure 19C**) and additionally, L2KCBD(5) displayed significantly lower p/T ERK1 levels relative to the VCBD(5) group ($p = .005$; **Figure 19C**).

Comparing the expression of the ERK2 isoform demonstrated that p-ERK2 levels were significantly lower in the L2KCBD(5) group compared to the VV and VCBD(5) groups ($\chi^2_{(3)} = 10.55$, $p = .003$; $p = .004$ and $p = .009$, respectively; **Figure 19A**). There were no significant differences in the T-ERK2 levels between treatment groups ($F_{(3, 12)} = 1.432$, $p = .282$; **Figure 19B**). p/T ERK2 levels were significantly

lower in L2KCBD(5) group compared to the VV and VCBD(5) groups ($F_{(3, 12)}$ = 10.19, p = .001; p < .001 and p = .002 respectively; **Figure 19C**) and in the L2KCBD(5)NAD group compared to the VV and VCBD(5) groups (p = .004 and p = .028, respectively; **Figure 19C)**. Each treatment group contained tissue samples from 4 animals.

Nucleus Accumbens Shell

Within the NASh, no significant differences were found in any measures of the ERK1 isoform (p-ERK1: $F_{(3, 16)}$ = 1.590, p = .231 [**Figure 19D**]; T-ERK1: $F_{(3, 16)}$ = 0.127, p = .943 [**Figure 19E**]; p/T-ERK1: $F_{(3, 15)}$ = 1.264, p = .322 [**Figure 19F**]).

ERK2 isoform expression levels were significantly lower for p-ERK2 in the VCBD(5), L2KCBD(5), and L2KCBD(5)NAD groups compared to the VV group ($F_{(3, 15)}$ = 5.093, p = .013; p = .022, p = 002, and p = .041, respectively; **Figure 19D**). No significant differences were found in the T-ERK2 levels between groups ($F_{(3, 15)}$ = 0.107, p = .955; **Figure 19E**), while p/T ERK2 was significantly lower only in the L2KCBD(5) group relative to the VV group ($F_{(3, 15)}$ = 2.185, p = .132; p = .024; **Figure 19F)**. Each treatment group contained tissue samples from 5 animals. Statistical outliers were identified using the Grubbs' Test and removed from the following groups: one L2KCBD(5) sample from p-ERK2 and T-ERK2 and one VCBD(5) sample from p/T-ERK1.

Basolateral Amygdala

Quantification in the BLA revealed no significant differences in any measures of the ERK1 isoform (p-ERK1: $F_{(3, 10)}$ = 1.030, p = .420 [**Figure 19G**]; T-ERK1: $F_{(3, 10)}$ = 0.579, p = .642 [**Figure 19H**]; p/T-ERK1: $F_{(3, 10)}$ = 0.847, p = .499 [**Figure 19I**]).

Similarly, p-ERK2 levels did not display significant differences between groups ($F_{(3, 10)}$ = 1.080, p = .401; **Figure 19G**) while T-ERK2 was only significantly

lower in the L2KCBD(5)NAD group compared to the VV group ($F_{(3, 10)} = 1.698$, $p = .230$; $p = .048$; **Figure 19H**). Finally, there were no differences in the p/T ERK2 levels ($F_{(3, 10)} = 1.609$, $p = .249$; **Figure 19I**). Each treatment group contained tissue samples from 4 animals. Statistical outliers were identified using the Grubbs' Test and removed from the following groups: one L2KCBD(5) sample from p-ERK1/2 and T-ERK1/2 and one L2KCBD(5)NAD sample from p-ERK1/2 and T-ERK1/2.

Mitogen-Activated Protein Kinase Pathway (ERK 1/2)

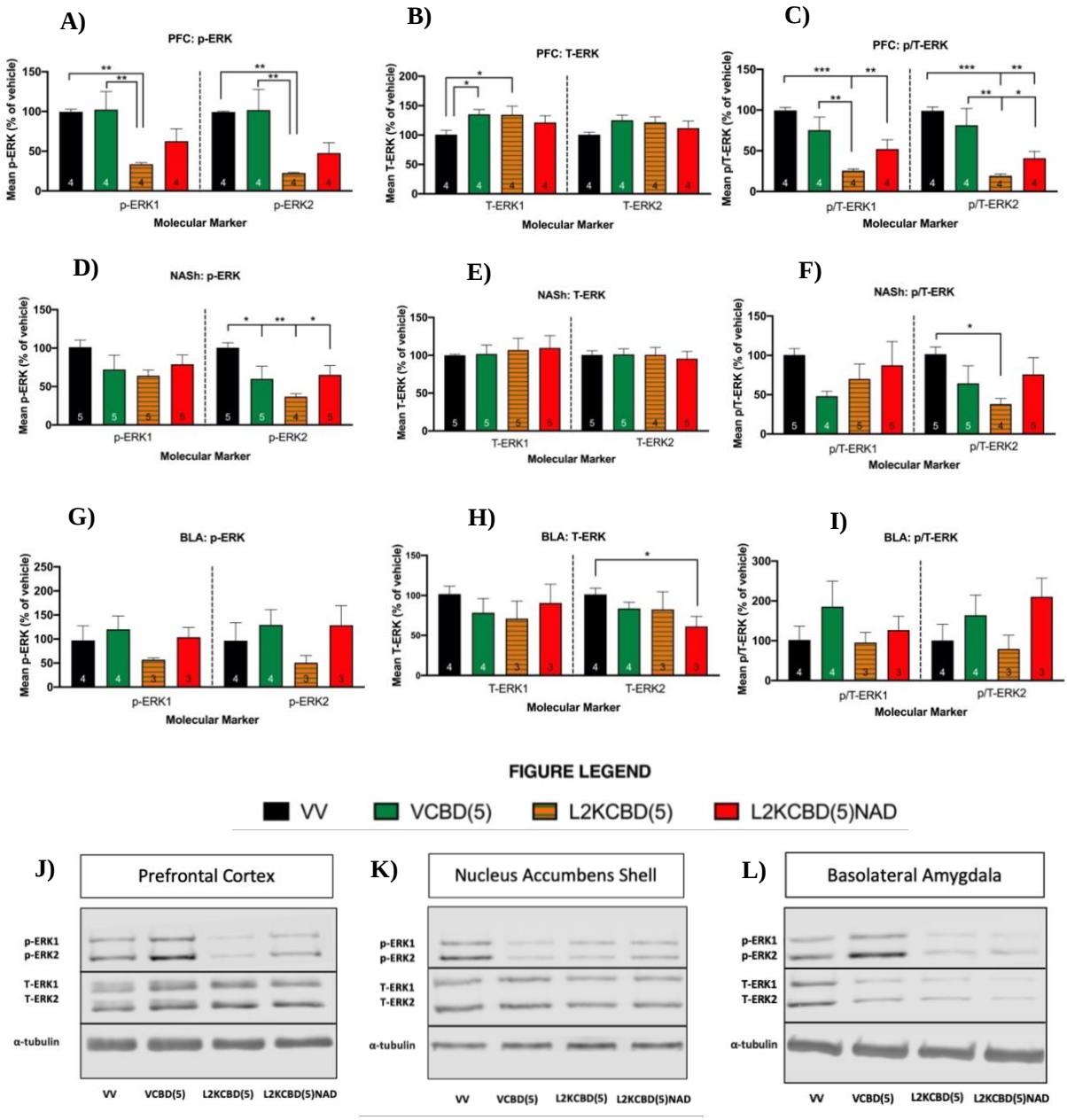

Figure 21: ERK Expression Levels Within the PFC, NASh, and BLA. **PFC**: **A)** p-ERK1 and 2 were significantly lower in the L2KCBD(5) group compared to VV and VCBD(5) **B)** T-ERK1 was significantly higher in the VCBD(5) and L2KCBD(5) groups compared to VV **C)** p/T-ERK1 and 2 were significantly lower in the L2KCBD(5) group compared to VV and VCBD(5). The L2KCBD(5)NAD group displayed significantly lower p/T-ERK1 and 2 compared to VV and significantly lower p/T-ERK2 compared to VCBD(5). **NASh: D)** p-ERK2 was significantly lower in the VCBD(5), L2KCBD(5), and L2KCBD(5)NAD groups compared to VV **E)** No significant differences were observed in the T-ERK1 and 2 levels between treatment groups **F)** p/T- ERK2 was significantly lower in the L2KCBD(5) group compared to VV. **BLA: G)** No significant differences were found between treatment groups in p-ERK1 and 2 expression **H)** T-ERK2 was significantly lower in the L2KCBD(5)NAD group compared to VV **I)** No significant differences were observed in the p/T-ERK1 and 2 levels between treatment groups. **J, K, L)** Representative Western blots of **J)** PFC, **K)** NASh, and **L)** BLA showing p-ERK1-2, T-ERK1-2, and α-tubulin expression. Separate one-way ANOVA analyses were conducted as indicated by the dotted vertical lines. Single annotation = $p \leq 0.05$, double annotation = $p \leq .01$, triple annotation = $p \leq .001$. All CBD doses were 5 ng/0.5 µL; limonene doses were 2000 µL (L2K); the NAD299 dose was 100 ng/0.5 µL. Plotted values are the mean ± SEM; the sample size of each group is represented on the bottom of their respective error bar.

3.4.2 PI3K-Akt Signal Transduction Pathway (Akt Ser473)

Prefrontal Cortex

No significant differences were found between treatment groups in any quantifications of Akt Ser473 within the PFC (p-AktSer473: $F_{(3, 12)} = 0.132$, $p = .940$ [**Figure 20A**]; T-Akt Ser473: $F_{(3, 12)} = 0.060$, $p = .980$ [**Figure 20B**]; p/T-Akt Ser473 $F_{(3, 12)} = 0.068$, $p = .976$ [**Figure 20C**]). Each treatment group contained tissue samples from 4 animals.

Nucleus Accumbens Shell

No significant differences were found in the total Akt levels in the NASh (T-Akt Ser473: $F_{(3, 15)} = 0.235$, $p = .870$; **Figure 20E**). However, p-Akt Ser 473 was significantly lower in the L2KCBD(5) group compared to the VV, VCBD(5), and L2KCBD(5)NAD groups ($F_{(3, 15)} = 4.782$, $p = .016$; $p = .004$, $p = .049$, and $p = .006$, respectively; **Figure 20D**). Moreover, p/T Akt Ser473 was significantly lower in the L2KCBD(5) group compared VV ($F_{(3, 15)} = 1.760$, $p = .198$; $p = .045$; **Figure 20F**). Each treatment group contained tissue samples from 5 animals. Statistical outliers were identified using the Grubbs' Test and removed from the following groups: one L2KCBD(5) sample from p-Akt Ser473 and T-Akt Ser473.

Basolateral Amygdala

No significant differences were observed between treatment groups within the BLA in T-Akt Ser 473 levels ($F_{(3, 12)} = 0.810$, $p = .513$; **Figure 20H**) and in P/T-Akt Ser 473 levels levels ($F_{(3, 12)} = 1.890$, $p = .185$; **Figure 20I**). However, p-Akt Ser 473 was significantly lower in the L2KCBD(5) group compared to the VV group ($F_{(3, 12)} = 2.203$, $p = .141$; $p = .038$; **Figure 20G**). Each treatment group contained tissue samples from 4 animals.

PI3K-Akt Signal Transduction Pathway (Akt Ser 473)

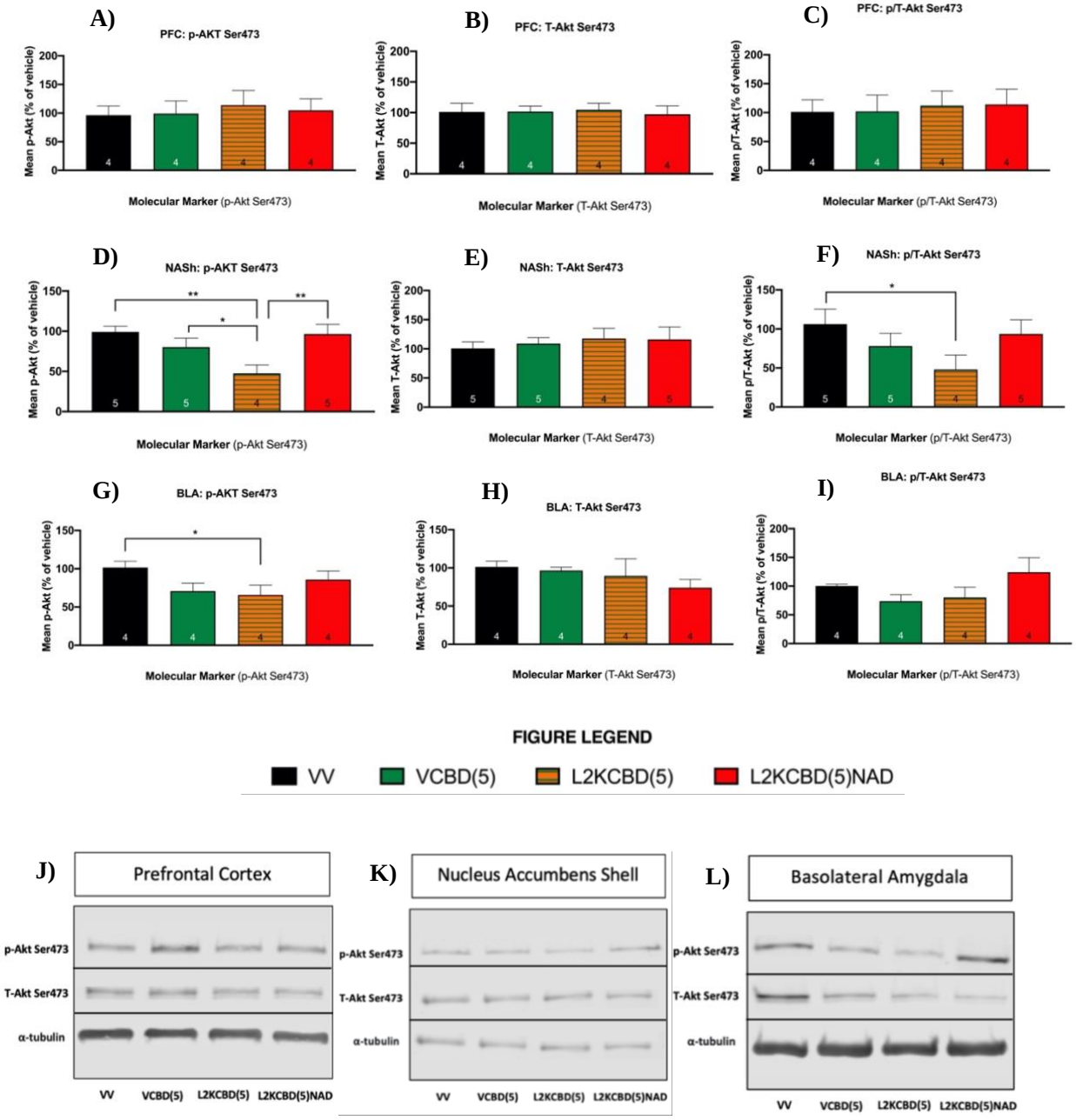

Figure 22: Akt Ser 473 Expression Levels Within the PFC, NASh, and BLA. PFC: No significant differences were found in the **A)** p-Akt Ser473, **B)** T-Akt Ser 473, and **C)** p/T-Akt Ser 473 levels within the PFC. **NASh: D)** L2KCBD(5) displayed significantly lower p-Akt Ser 473 compared to VV, VCBD(5), and L2KCBD(5)NAD groups **E)** No significant differences were found in the T-Akt Ser 473 levels between treatment groups **F)** The L2KCBD(5) group exhibited significantly lower p/T-Akt Ser 473 levels compared to VV **BLA: G)** p-Akt Ser 473 was significantly lower in the L2KCBD(5) groups compared to VV **H)** No significant differences were found in the T-Akt SER 473 levels between treatment groups **I)** No significant differences were found in the p/T-Akt Ser 473 levels between treatment groups**. J, K, L)** Representative Western blots of **J)** PFC, **K)** NASh, and **L)** BLA showing p-Akt Ser 473, T-Akt Ser 473, and α-tubulin expression. One-way ANOVA analysis. Single annotation = $p \leq 0.05$, double annotation = $p \leq .01$, triple annotation = $p \leq .001$. All CBD doses were 5 ng/0.5 µL; limonene doses were 2000 µL (L2K); the NAD299 dose was 100 ng/0.5 µL. Plotted values are the mean ± SEM; the sample size of each group is represented above their respective error bar.

4. Discussion

Previous evidence has alluded to the synergistic potential of cannabis-derived compounds in modulating mental and physical states (Russo, 2011). This phenomenon generally referred to as the 'Entourage Effect' (EE), has not yet been explored with regards to combinatorial administrations of cannabidiol (CBD) and d-limonene. By utilizing a rodent model, this thesis demonstrated for the first time the synergistic potential and greater anxiolytic efficacy of Limonene-CBD formulations relative to isolated administrations of CBD or limonene, specifically within the nucleus accumbens shell (NASh) brain region (*Aim 1)*. Additionally, by utilizing a molecular antagonist (NAD299) and via analysis of specific molecular markers, this study elucidated the potential 5-HT_{1A}-mediated mechanism of these Limonene-CBD formulations (*Aim 2)*, as well as changes in specific downstream signalling molecules associated with this activation (*Aim 3)*.

Overall, the greater reduction of anxiety-related symptoms in behavioural assays in the Limonene-CBD groups relative to isolated groups of CBD or limonene supported the proposed hypothesis of EE-potentiated anxiolysis in Limonene-CBD formulations. Moreover, Western blots demonstrating the differential protein expression levels exhibited by isolated CBD relative to the Limonene-CBD combinatorial group allude to a distinctive mechanism in the modulation of neuronal activity that likely underlies this synergism.

4.1 Baseline Cohort: Limonene-CBD Demonstrate EE-Potentiated Anxiolysis

Table 2: Summary of Baseline Cohort Behavioural Results

Behavioural Test	Limonene-CBD groups relative to isolated CBD or Limonene groups	Conclusion
Open-Field	No difference in locomotion No difference in center time	-
Elevated-Plus Maze	Increase in time spent in open arms and total number of open arm entries	EE-potentiated anxiolysis
Social Interaction	No differences in social motivation index or social recognition index	-
Light-Dark Box	Increase in time spent in light compartment and total number of compartmental transitions Decrease in time spent in dark compartment	EE-potentiated anxiolysis
Contextual Fear Conditioning (Memory Acquisition)	Decrease in freezing time	EE-potentiated anxiolysis

The CBD doses of 1 ng/0.5 µL and 5 ng/0.5 µL utilized in this cohort were deemed to be sub-threshold doses in modulating anxiety-related behaviour based on previous literature on intra-NASh microinfusions in rats (Norris et al., 2016). An exception was in the contextual fear conditioning test, where 5 ng/0.5 µL of CBD proved to be an effective dose. The volumes of limonene, 200 µL and 2000 µL, were established from a study by Harada et al. (2018) assessing the anxiolytic effects of a similar monoterpene, linalool. Consequently, for this thesis, significant reduction in anxiety-related measures in the Limonene-CBD groups relative to the CBD or limonene alone group suggested synergistic effects and thereby, EE-potentiated anxiolysis.

The open-field (OF) test was conducted primarily to assess for potential locomotor variations evoked by the drug assays. Analysis of the first five minutes of the test yielded no significant differences between the treatment groups in the total distance travelled consistent with previous findings on CBD (Kasten et al., 2019; Long et al., 2010) and with inhaled limonene (Satou et al., 2012). Critically, this outcome suggested that changes in anxiety-related behaviours observed in other experimental assays are unlikely to be mediated by alterations in motor functionality and can be more confidently associated with modulation within the limbic system.

The secondary measure of center time in the first five minutes of the OF test yielded no significant differences between treatment groups consistent with previous findings for intraperitoneally administered CBD (Kasten et al., 2019). Although we observed a trend of increased center time in the Limonene-CBD groups, the similar levels of anxiolysis found in the isolated CBD and limonene groups suggest that Limonene-CBD synergy was not established in this test. Conversely, it is plausible that 5 ng/0.5 µL of CBD and 2000 µL limonene are suprathreshold doses in this assay, and thus utilizing a dose of 1 ng/0.5 µL of CBD may help elucidate potential synergistic effects.

It should be noted that criticisms exist for the use of the OF test as a reliable measure of anxiety as it has been shown to be insensitive to some conventional anxiolytics, such as triazolobenzodiazepines and anti-depressants (Prut & Belzung, 2003). Moreover, findings such as the anxiogenic drug amphetamine showing an increase in center exploration (Einat, 2006), suggests that there exists some ambiguity on how center time should be interpreted in this paradigm. Consequently for this study, the OF test cannot conclusively eliminate the ability of Limonene-CBD formulations in modulating open-space anxiety.

The most robust evidence of open-space anxiolysis and synergism between cannabidiol and limonene was found in the elevated-plus maze (EPM) test, wherein, all combinations of Limonene-CBD (L200CBD(1), L200CBD(5), L2KCBD(1), and L2KCBD(5)) resulted in significantly more time spent in the open arms of the maze relative to the isolated administration of CBD at both experimental doses (VCBD(1) and VCBD(5)) and relative to the isolated administration of limonene at the lowest dose (L200V). Moreover, subjects in three of the combinatorial treatment groups (L200CBD(5), L2KCBD(1), and L2KCBD(5)) made significantly more open arm entries relative to those in VCBD(1); additionally, the L2KCBD(5) group made significantly more open arm entries relative to VCBD(5). These findings are substantiated by prior literature linking limonene to increased values on these parameters (Gurgel do Vale et al., 2002; Komiya et al., 2006; Lima et al., 2013), as well as that of cannabidiol (Guimarães et al., 1990).

With regards to the three-chamber social interaction (SI) test, we found no differences in the social motivation index and the social recognition index values between treatment groups suggesting a lack of EE-potentiated improvement in social anxiety and social cognition by the Limonene-CBD formulations. Our SI results are consistent with previous SI findings demonstrating that isolated CBD does not affect social interaction (Long et al., 2010; Malone et al., 2009; Szkudlarek et al., 2019; van Ree et al., 1984). While there are currently no studies assessing the effects of limonene on this test, evidence using inhaled orange essential oil (OEO) (containing 96.24% limonene) suggests that it may increase active social interaction in this paradigm (Leite et al., 2008). However, given that OEO contains many components in addition to limonene, namely: 0.53% alpha-pinene, 0.27% sabinene, 2.24% myrcene, 0.44% linalool, and 0.25% decanal, its reported effects on social interaction could

also be a result of synergy and thus, explain why we did not find similar effects in isolated limonene groups (Leite et al., 2008).

Despite the findings of this thesis, the possibility that CBD and limonene could be effective agents for alleviating social anxiety should not be dismissed. Research suggests that CBD administration can reverse the social deficits associated with genetically-altered mouse models of autism (Kaplan et al., 2017) and in THC-induced social withdrawal (Malone et al., 2009). Moreover, while acute CBD may not significantly improve social interaction, chronic administration can (Osborne et al., 2017). Thus, while Limonene-CBD formulations did not significantly improve social interaction in our study, this does not suggest that they will be ineffective in those already suffering from social anxiety, especially with chronic treatment. Further studies wherein, Limonene-CBD formulations are administered over an extended period or in rodent models of social deficit could clarify these effects.

Within the light-dark box (LDB) test, combinatorial groups (L200CBD(5) and L2KCBD(5)) spent significantly greater time in the light compartment and significantly less time in the dark compartment in comparison to the VCBD(5) group. Moreover, the L2KCBD(5) group made significantly more compartmental transitions relative to the VCBD(5) group. These results imply that CBD and limonene synergistically alleviate bright-space anxiety in rodents, as expected from previous reports using effective doses of CBD (Long et al., 2010) and inhaled limonene (Satou et al., 2012).

Interestingly, the limonene groups (L200V and L2KV) also spent significantly less time in the dark compared to the VCBD(5) group but did not display a consequent increase in light time. This discrepancy is a result of the limonene groups spending significantly more time in risk assessment behaviours (placing nose or

forepaws into the light compartment from the dark compartment) as opposed to wholly entering the light area. Taken together then, these findings offer further support that combining CBD with limonene potentiates limonene's anxiolytic efficacy.

Finally, the contextual-fear conditioning (CFC) test, found that all treatment groups (except VCBD(1)) displayed a significant reduction in freezing time relative to the VV group. Consequently, for this paradigm, 1 ng/0.5 μL of CBD represents a sub-threshold dose (Norris et al., 2016), while 5 ng/0.5 μL is an effective dose as verified by the significant extinguishment of freezing behaviour within the VCBD(5) group. Additionally, the significant reduction in freezing exhibited by the L2KCBD(1) group relative to the VCBD(1) group supports the proposition that CBD and limonene synergistically attenuate the formation of fear-related memory, and thereby, may be an effective therapeutic agent in preventing the onset of anxiety-related symptoms, such as in post-traumatic stress disorder. While there are presently no studies examining the effect of limonene on contextual fear conditioning, a study by d'Alessio et al. (2014) found that orally administered d-limonene reversed the onset of freezing behaviour induced by a stress paradigm. Moreover, the CFC findings of this study are in agreement with previous work using this paradigm and cannabidiol (Fogaça et al., 2014; Gomes et al., 2012; Lemos et al., 2010; Resstel et al., 2009) with some studies utilizing specific receptor antagonists proposing that these effects are modulated through 5-HT_{1A}-mediated neurotransmission (Fogaça et al., 2014; Gomes et al., 2012).

4.2 NAD299 Challenge Cohort: Limonene-CBD EE-Potentiation is 5-HT$_{1A}$-Dependent

Table 3: Summary of NAD299 Challenge Behavioural Results

Behavioural Test	Limonene-CBD-NAD vs. Limonene-CBD	Conclusion
Open-Field	No difference in locomotion No difference in center time	-
Elevated-Plus Maze	Decrease in time spent in open arms and total number of open arm entries	5-HT$_{1A}$-dependent anxiolysis
Social Interaction	No differences in social motivation index or social recognition index	-
Light-Dark Box	Decrease in time spent in light compartment and total number of compartmental transitions Increase in time spent in dark compartment	5-HT$_{1A}$-dependent anxiolysis
Contextual Fear Conditioning (Memory Acquisition)	No difference in freezing time	-

The CBD doses of 1 ng/0.5 µL and 5 ng/0.5 µL utilized in this cohort were deemed to be sub-threshold doses in modulating anxiety-related behaviour based on previous literature on intra-NASh microinfusions in rats (Norris et al., 2016). An exception was in the contextual fear conditioning test, where 5 ng/0.5 µL of CBD proved to be an effective dose. The volumes of limonene, 200 µL and 2000 µL, were established from a study by Harada et al. (2018) assessing the anxiolytic effects of a similar monoterpene, linalool. The utilized antagonist, NAD299, is highly specific in attenuating 5-HT$_{1A}$ receptor-induced anxiolysis and individual administration of NAD299 did not by itself modify any of the anxiety-related parameters assessed in

this study similar to previous literature findings (Johansson et al., 1997; Norris et al., 2016; Szkudlarek et al., 2019). Consequently, for this thesis, a reduction in anxiety-related behaviours in the Limonene-CBD-NAD groups relative to the Limonene-CBD groups suggested a 5-HT_{1A} receptor-mediated anxiolytic effect.

Discussion of NAD299 Challenge Behavioural Results

In the open field task, the absence of difference in ambulatory distance between the treatment groups verified that NAD299 does not alter locomotor ability. Consequently, deviations in anxiety-related measures in subsequent assays within this cohort were confidently attributed to changes within the limbic system, as opposed to variability in motor function. Although there was a trend of increased center time in the Limonene-CBD groups that was reversed by the addition of NAD299 (**Figure 14B [left]**), our results from the Baseline Cohort showing similar levels of anxiolysis in the isolated CBD and limonene groups relative to Limonene-CBD groups suggest that Limonene-CBD synergy was not established in the OF test (**Figure 9C**). That said, the perceived trend in the reversal of this effect by NAD299 suggests that any potential mechanism of anxiolysis is likely mediated by 5-HT_{1A}R. As mentioned previously, however, there is some uncertainty on how center time should be interpreted (Einat, 2006; Prut & Belzung, 2003), thus, conclusions about open-space anxiety from this assay should be made cautiously.

In contrast, the elevated plus maze (EPM) is a well-established test of open-space anxiety. We found a significant EE-potentiated increase in the total time spent in the open arms for all Limonene-CBD groups that was significantly reduced upon co-administration of NAD299 in both combinatorial doses (L200CBD(5)NAD and L2KCBD(5)NAD). Furthermore, there was a reversal of the EE-potentiated increase in the total number of open arm entries with a significant reduction displayed by the

L200CBD(5)NAD group compared to the L200CBD(5) group. Taken together, these results strongly propose that open-space anxiolysis by Limonene-CBD formulations are mediated by 5-HT_{1A}R consistent with the proposed 5-HT_{1A} mechanism in previous research on the EPM assay using effectual doses of CBD (de Gregorio et al., 2019; Szkudlarek et al., 2019) and limonene (Komiya et al., 2006).

The lack of significant differences in the social motivation and social recognition indices between the Limonene-CBD formulations and the Limonene-CBD-NAD groups in the three-chamber social interaction (SI) test suggests that at least within the context of this study, CBD, limonene, and NAD do not modulate measures of rodent social anxiety (for a more detailed discussion of this test see *Discussion of Baseline Cohort Behavioural Results*).

In regards to the light dark box (LDB) test, the observed decrease in the time spent in the light, the decrease in the total number of compartmental transitions, and the subsequent increase in the time spent in the dark amongst the Limonene-CBD-NAD groups relative to the Limonene-CBD groups, signify that the EE-potentiated anxiolytic effects of CBD and limonene towards bright spaces are 5-HT_{1A}R mediated. This conclusion is further corroborated by findings by Costa et al. (2013) showing that the essential oil (EO) extracted from *Citrus aurantium* (containing 98.66% limonene) similarly modifies these same LDB parameters with effects being mediated by the 5-HT_{1A} receptor. Notably, however, these researchers found that the administration of pure limonene at its prevalent dose within the EO did not modify these LDB behaviors, implying that the anxiolytic effects of the EO are a result of synergism between limonene and other EO phytochemicals (Costa et al., 2013). These findings, combined with the results from this thesis, strongly imply that Limonene-

CBD formulations are acting synergistically at the 5-HT_{1A} receptor to exhibit their anxiolytic effects within the LDB test.

In the last assay, the contextual fear conditioning (CFC) paradigm, there was no significant reversal of the anxiolytic effect observed in the Limonene-CBD formulations upon co-application of NAD299. This is in contrast to the findings by Norris and colleagues (2016) demonstrating 5-HT_{1A}-mediated anxiolysis by CBD within the nucleus accumbens shell (NASh). Consequently, it appears that the inhibition of fear-related memory consolidation by Limonene-CBD is mediated by a unique mechanism.

One reason for these results could be due to the wide-spread effects of inhaled limonene affecting structures such as, the basolateral amygdala, which has been greatly implicated in the modulation of fear memory (Davis, 1992; Etkin et al., 2004; Reznikov et al., 2018). Thus, it is possible that the localized administration of NAD299 into the NASh is unable to effectively block the effects of limonene within the BLA. Conversely, given that 5-HT_{2A} signalling is associated with the formation of fear memory (Clinard et al., 2015; Jiang et al., 2020), it is probable that 5-HT_{2A} and not 5-HT_{1A} is modulating the anxiolytic effects in the CFC task.

4.3 5-HT_{1A}-Mediated EE-Potentiated Anxiolytic Mechanism

While the investigation of the combinatorial effects of CBD and limonene is unique to this thesis, previous literature has confirmed the 5-HT_{1A} agonistic properties of individual CBD and limonene administrations within these assays (Campos & Guimarães, 2008; Costa et al., 2013; Fogaça et al., 2014; Norris et al., 2016; Szkudlarek et al., 2019). Norris et al. (2016) demonstrated that microinfusions of CBD into the nucleus accumbens shell (NASh) led to increased GABAergic activity within the ventral tegmental area (VTA), which subsequently decreased VTA

dopaminergic activity, with these effects being mediated by 5-HT_{1A}R. Similarly, limonene has been shown to increase GABA neurotransmission, and consequently decrease dopamine levels within the nucleus accumbens (Yun, 2014; Zhou et al., 2009). Although the method of limonene administration in the study by Yun (2014) was intraperitoneal (i.p.), the transport of limonene to the brain is in fact, superior when inhaled rather than injected i.p. (Satou et al., 2017), suggesting that similar neurochemical effects are likely to be emulated within this study.

One possible mechanism of synergy is that sub-threshold doses of CBD and limonene act concurrently at 5-HT_{1A}R within the NASh either through allosteric modulation or direct substrate-receptor complex effects to increase GABAergic neurotransmission to the VTA, thereby decreasing DAergic activity to the NASh, PFC, and hippocampus (**Figure 3**). Given that all subtypes of anxiety are related to decreased GABA and 5-HT function, and subsequently enhanced DAergic activity within the mesocorticolimbic system (Nikolaus et al., 2010), this mechanism would explain the reduction in anxious phenotypes observed in the behavioural assays upon Limonene-CBD co-application.

4.4 Molecular Analysis: EE-Potentiated Anxiolysis is Mediated by MAPK and PI3K-Akt Signal Transduction Pathways

Table 4: Summary of the Ratio of Phosphorylated:Total Protein Expression from Western Blot Assays

	Brain Region		
Molecular Biomarker	**Prefrontal Cortex**	**Nucleus Accumbens Shell**	**Basolateral Amygdala**
p/T-ERK 1	↓ L2KCBD(5) compared to VV and VCBD(5) ↓ L2KCBD(5)NAD compared to VV	----	----
p/T-ERK 2	↓ L2KCBD(5) compared to VV and VCBD(5) ↓ L2KCBD(5)NAD compared to VV and VCBD(5)	↓ L2KCBD(5) compared to VV	----
p/T-Akt Ser 473	----	↓ L2KCBD(5) compared to VV	----

All CBD doses were 5 ng/0.5 µL, all volumes of limonene were 2000 µl, and the dose of NAD299 was 100 ng/0.5 µL. "----" denotes no significant differences in treatment groups "↓", denotes significantly lower protein expression. One-Way ANOVA; $p < 0.05$.

Based on the results from the behavioural assays demonstrating evidence of 5-HT_{1A} mediated synergistic anxiolysis between limonene and CBD, molecular analysis of the highest combinatorial formulation of L2KCBD(5) was conducted alongside VCBD(5) and L2KCBD(5)NAD. Expression levels of ERK1/2 of the MAPK pathway and Akt of the PI3K-Akt pathway within the prefrontal cortex, nucleus accumbens shell, and basolateral amygdala associated with the mesocorticolimbic system were analyzed.

4.4.1 ERK1/2 Signalling Modulates Limonene-CBD Anxiolysis

Within the prefrontal cortex, the VCBD(5) group did not display significantly lower levels of p/T-ERK1/2 expression (**Figure 19C**) relative to the VV group. This outcome is consistent with data from Hudson et al. (2019) exhibiting no differences in p/T-ERK1/2 levels between intra-hippocampus CBD (100 ng) and untreated subjects and correlates with the lack of behavioural differences (relative to VV) observed in this thesis in the assays from the Baseline Cohort. It should be noted, however, that chronic administration of CBD (i.p. injection; 10.0 mg/kg) has been shown in previous literature to decrease p-ERK1/2 (without affecting T-ERK1/2) within the PFC and these effects have been linked to anxiogenic symptoms, as opposed to the anxiolytic effects typically observed with acute administrations of CBD at the same dose (ElBatsh et al., 2012). Notably, this discrepancy highlights the reported biphasic therapeutic efficacy of orally and intraperitoneally delivered cannabidiol (Campos & Guimarães, 2008, 2009; Guimarães et al., 1990).

For instance, Guimarães et al. (1990) observed that i.p. injections of 2.5, 5.0, and 10.0 mg/kg significantly increased the open arm entry ratio in the elevated plus maze, while 20.0 mg/kg was ineffective. Similar findings in the EPM task were documented by Campos and Guimarães (2008) upon delivery of CBD into the

dorsolateral periaqueductal gray midbrain structure. This bimodal activity has been proposed to be due to the differential activation of receptors by cannabidiol, with anxiolytic effects induced by 5-HT_{1A}R activation at low doses (Campos & Guimarães, 2008) and anxiogenic effects resulting from $TRPV_1$ activation at high doses and subsequent increase in glutamate transmission (Campos & Guimarães, 2009). Interestingly, this bell-shaped response curve appears to be overcome by CBD extracts enriched with other components, with increasing doses corresponding to increasingly more effective anti-inflammatory and anti-nociceptive effects (Gallily et al., 2015). Consequently, Gallily and colleagues (2015) have proposed that potential synergistic effects between various phytocannabinoids may make combinatorial extracts more clinically effective than isolated CBD, thereby, providing additional support for the proposition that combining CBD and limonene may result in greater anxiolytic efficacy than increasing doses of CBD alone.

p/T-ERK1/2 levels were significantly reduced in the PFC in the L2KCBD(5) group relative to the VV and VCBD(5) groups (**Table 4; Figure 19C**) suggesting that limonene is likely reinforcing the effects of sub-threshold CBD in decreasing phosphorylated ERK1/2 levels. Given that phosphorylated ERK within the PFC is linked to high levels of anxiety (Ailing et al., 2008), these results correlate with outcomes from our behavioural assays (namely, the elevated plus maze and light dark box) in the Baseline Cohort in which the L2KV group displayed greater anxiolysis relative to the VV group, and wherein Limonene-CBD groups exhibited the greatest anxiolytic efficacy.

Potential mechanisms by which limonene may synergize the anxiolytic effects of CBD within the MAPK pathway include i) interactions at 5-HT_{1A}R via allosteric or direct receptor complexes, ii) alteration of molecular targets upstream of ERK1/2

(such as, Ras, Raf, and MEK1-2), iii) alteration of downstream nuclear localization effectors such as, cAMP-response-element-binding protein (CREB), iv) modulation of proteins and other associated pathways outside the scope of this thesis, and iv) a combination of these effects (**Figure 21**).

While there are currently no studies on the influence of d-limonene on MAPK proteins as it relates to anxiety, the assertion that limonene affects the MAPK pathway is supported by *in-vivo* and *in-vitro* data examining other disease conditions. Essential oils from the citrus plant (composed of 52.44% limonene) have been found to decrease the levels of phosphorylated MAPKs (JNK and ERK) *in-vitro* resulting in anti-inflammatory effects (Kim et al., 2013), while Younis (2020) revealed that limonene pre-treatment reduced the expression of MAPK proteins and p/T-ERK levels in rats experiencing myocardial injury. Moreover, Chaudhary et al. (2012) demonstrated that limonene decreased the expression of Ras, Raf, and phosphorylated ERK1/2 levels in a mouse model of skin tumorigenesis supporting the proposition that the reduction in p-ERK1/2 levels observed in this thesis may be a result of Ras or Raf inactivation upstream of ERK1/2.

In contrast, the anxiolytic behavioural effects of Limonene-CBD could be a result of direct inactivation of ERK1/2 affecting downstream target effectors such as CREB, and subsequent changes in mRNA production (**Figure 4**). Within the hippocampus, the 5-HT_{1A}-mediated induction of CREB by activated ERK1/2 is associated with the modulation of anxiety behaviours (Zhang et al., 2016), while drug or stress-induced activation of CREB within the NAc or amygdala results in depressive and anxiety-like behaviors (Carlezonjr et al., 2005; Pandey et al., 2003). Therefore, a long-term consequence of Limonene-CBD administration may be a decrease in the phosphorylation and consequently, the inactivation of PFC-ERK1/2,

thereby, leading to reduced CREB activity and anxiety. That said, within the context of this thesis, the assertion that these formulations are inducing long-term changes through nuclear localization of downstream effectors should be made with caution, as drug administrations in this study were acute, or at most sub-chronic, in nature. Thus, anxiolysis via rapid-acting, short-term changes are important to consider.

One possible immediate alteration is the formation of stress-induced free radicals and reactive oxygen species (ROS) that are implicated in the prognosis and maintenance of neurogenerative disease. In rats, conditions of acute (1 hour) (Nadeem et al., 2006) and chronic stress (21 days) (Zafir & Banu, 2009) result in the formation of reactive oxygen species, with increased ROS resulting in the phosphorylation and activation of ERK (Cao & Kaufman, 2014). Intracellular accumulation of ROS within the cerebral cortex, cerebellum, and hippocampus has been linked to anxious phenotypes in mice (Rammal et al., 2008) with increased intracellular ROS positively correlating with measures of anxiety in the light-dark box (Bouayed et al., 2007). Given the acute/sub-chronic quality of the stress assumed by the subjects in this thesis, the notion that high ROS levels are mediating anxious behaviour is not unlikely. Accordingly, the decreased p/T-ERK levels observed in the Limonene-CBD group could arguably be a consequence of the 5-HT_{1A}-mediated reduction in ROS activity, given the *in-vitro* evidence demonstrating the antioxidant properties (via the inhibition of ROS) of both cannabidiol and limonene (Campos et al., 2016; Mechoulam et al., 2007; Shah & Mehta, 2018)

In addition to revealing changes in downstream effectors, a principal objective of this thesis was to elucidate the chief receptor targeted by the Limonene-CBD formulations. As expected from the NAD299 Challenge behavioural assays (namely, EPM and LDB) illustrating reversal of anxiety-related behaviour upon administration

of NAD299, the L2KCBD(5)NAD group displayed higher levels of p/T-ERK1/2 than the L2KCBD(5) group (**Figure 19C**), suggesting that the Limonene-CBD mediation of ERK is occurring at the 5-HT_{1A} receptor. That said, the possibility that limonene is modulating these effects by interactions with other receptor targets should not be excluded.

For instance, the antioxidant properties of limonene have been linked to its modulation of 5-HT_{2A} signalling (Yun, 2014) and 5-HT_{2A}R activation is associated with the potent inhibition of the tumor necrosis factor-alpha (TNF-α) (Nau et al., 2013). Moreover, citrus essential oils containing a high concentration of limonene have been found to dose-dependently suppress TNF-α (Kim et al., 2013), while administration of cannabidiol reduces serum TNF-α expression in a biphasic manner (Gallily et al., 2015). These findings are noteworthy as blocking TNF-α reduces anxiety and depressive-like behaviors (Alshammari et al., 2020). Given that TNF-α phosphorylates and activates ERK proteins (Sabio & Davis, 2014), the reduction in p-ERK1/2 expression observed in the Limonene-CBD group could allude to potential inhibition of TNF-α activity. Collectively then, these findings suggest that limonene and/or CBD inhibits TNF-α signalling via 5-HT_{2A}R, leading to decreased p/T-ERK1/2 expression and a reduction in anxious phenotypes. Conversely, an alternative mechanism pertains to the downregulation of p-ERK1/2 (by limonene) resulting in decreased 5-HT_{2A}R expression as expected from data by Xiang et al. (2017) reporting these effects. Given that 5-HT_{2A}R expression competitively inhibits 5-HT_{1A}R in the hippocampi of PTSD mice (Xiang et al., 2017), this reduction in 5-HT_{2A}R could facilitate the therapeutic effects of 5-HT_{1A}-mediated anxiolysis by CBD. Regardless of which mechanism of action underlies the observed 5-HT_{1A}R associated anxiolysis,

the prospect of additional targets aiding in this process should be examined by the use of respective receptor antagonists.

According to findings from this thesis, ERK levels appear to be modulated specifically within the PFC as indicated by the lack of significant differences in p/T-ERK levels observed within the BLA and NASh (aside from the significant reduction in NASh p/T-ERK2). Given that drug delivery did not include intra-PFC administration, these results highlight the functional connections between the PFC and NASh, specifically NASh-mediated inhibition of PFC-MAPK/ERK signalling and the PFC-mediated GABAergic and glutamatergic input to the NASh; thus, some contributory effects via GABA or glutamate neurotransmission are likely modulating the observed EE-potentiated anxiolysis. Specifically, synaptic GABA release in neurons has been found to lower levels of p-ERK, while the inhibition of MEK1/2 (an upstream activator of ERK) reduces p-ERK and increases $GABA_AR$ peak current amplitudes (Brady et al., 2018). Therefore, at the systems level, intra-NASh drug delivery may modulate PFC neurotransmission indirectly (via associations with the VTA), and the consequent increase in PFC GABAergic neurotransmission to the NASh decreases PFC-p-ERK1/2 and anxious behaviour.

In summary, the reduction in p/T-ERK1/2 expression observed in the Limonene-CBD group offer a few 5-HT_{1A}-mediated mechanisms that may underlie the EE-potentiated anxiolysis established in the behavioural assays including, joint reduction in ROS activity, modulation of GABAergic transmission, long-term alterations in CREB activity, or a combination of these effects. Moreover, interactions with other receptors such as the 5-HT_{2A}R-mediated reduction in TNF-α or competitive inhibition of 5-HT_{1A}R, are necessary to consider given the reported diversity in the properties associated with CBD and limonene. The lack of anxiolytic

behavioural efficacy detected by administrations of isolated CBD and often, in isolated limonene doses, together with the fact that CBD alone did not significantly alter ERK1/2 expression, underscore a unique mechanism of action that likely modulates the synergistic efficacy of combinatorial limonene and CBD via the MAPK pathway.

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4.4.2 Akt Signalling Modulates Limonene-CBD Anxiolysis

Data by Renard et al. (2016) utilizing a higher dose of CBD (100 ng) and the same intra-NASh delivery method employed in this thesis found significantly decreased levels of p-Akt Ser473 and p/T-Akt Ser 473 in CBD-treated rats. It should be noted that while these findings by Renard et al. (2016) were reported in AMPH-sensitized rats and thus, may not represent an ideal model of comparison, similar trends have been documented in CBD-treated glioblastoma cells by Ivanov et al. (2017). Conversely, the VCBD(5) group of this study did not display a significant reduction in this biomarker in any of the brain regions analyzed (**Figure 20**) correlating with the absence of anxiolysis observed in the behavioural assays. Together, these findings support the assertion that 5 ng of CBD is a sub-threshold dose and that the observed L2KCBD(5) reduction of p/T-Akt Ser 473 in the NASh and the associated reduction in anxiety-related behaviour is a consequence of limonene modulation.

Although yet to be examined in mood and anxiety-related states, limonene inhibition of p-Akt Ser 473 has been well-established in human cancer cells (Chidambara Murthy et al., 2012; Jia et al., 2013; Reddy et al., 2017). Moreover, the contention that limonene may synergize the effects of CBD is corroborated by data from Saunders et al. (2014) revealing that KO mice with ablated Akt phosphorylation at Ser473 display increased 5-HT_{1A}R binding and wherein, *in-vitro* pharmacological inhibition of Akt subsequently enhances 5-HT_{1A}R cortical expression. Collectively, these findings offer a mechanism of interaction whereby, limonene reduction in p-Akt could potentially increase the available 5-HT_{1A} receptor pool for CBD to exert its anxiolytic effects. Moreover, the observed trend in increased NASh-p/T-Akt Ser 473 in the L2KCBD(5)NAD group (**Figure 20F**) correlating with the reversal to anxious

behaviour observed in the NAD299 Challenge Cohort support this proposition of 5-HT_{1A}R mediated synergy.

Although not analyzed in this thesis, the study by Renard and colleagues (2016) mentioned previously revealed that in addition to p-Akt downregulation, CBD concurrently inhibited the expression of GSK3β (a downstream target of Akt), which is in contrast to the reported effects of traditional antipsychotics that exhibit a reciprocal increase in GSK3β upon Akt inactivation (Beaulieu et al., 2009). Accordingly, Renard et al. (2016) have proposed that a novel molecular pathway likely underlies the therapeutic effects of CBD within the mesolimbic system which bypasses the conventional PI3K-Akt-GSK3 pathway of conventional antipsychotics.

Given the assertion that limonene is a key player modulating the anxiolytic properties of these formulations, it is worthwhile understanding how olfactory nervous system activation by limonene can lead to alterations at the molecular level. The presence of limonene in the brain after 30-minute inhalation has been documented by Satou et al. (2017), and when combined with the fact that chemical compounds activate the olfactory bulb neurons which transmit signals to the olfactory cortex, as well as the amygdala, hypothalamus, and hippocampus involved in emotion, hormone secretion, and memory, respectively (Koyama & Heinbockel, 2020), alterations in behaviour are a plausible consequence of odourous limonene. That said, electrophysiological recordings analyzing changes in activity within specific brain regions following limonene inhalation would aid in extending the work of this thesis beyond the speculated correlations made between biomarker expression, neurotransmission, and behavioural phenomena.

Taken together, the MAPK and PI3/Akt molecular changes observed within the PFC and NASh, respectively, offer two different 5-HT_{1A}-associated mechanistic

pathways that are altered by these Limonene-CBD formulations. Importantly, although this data highlights the circuit-level connections between the brain regions of the MCL system (namely, the NASh and PFC), the exact manner in which these regions communicate with each other to evoke these changes remains unclear. Moreover, given the reciprocal interactions between the NASh and VTA and the indirect modulation of the PFC via this connection, further investigations involving functional disconnection between these brain regions (alongside the application of specific inhibitors) would aid in clarifying the observed EE-potentiated anxiolytic mechanism of Limonene-CBD. Finally, the examination of supplementary pathways such as the ADCY/cAMP/PKA cascade, and additional biomarkers such as JNK and GSK3, would better inform the conclusion that Limonene and CBD regulate anxiety via the 5-HT$_{1A}$ receptor.

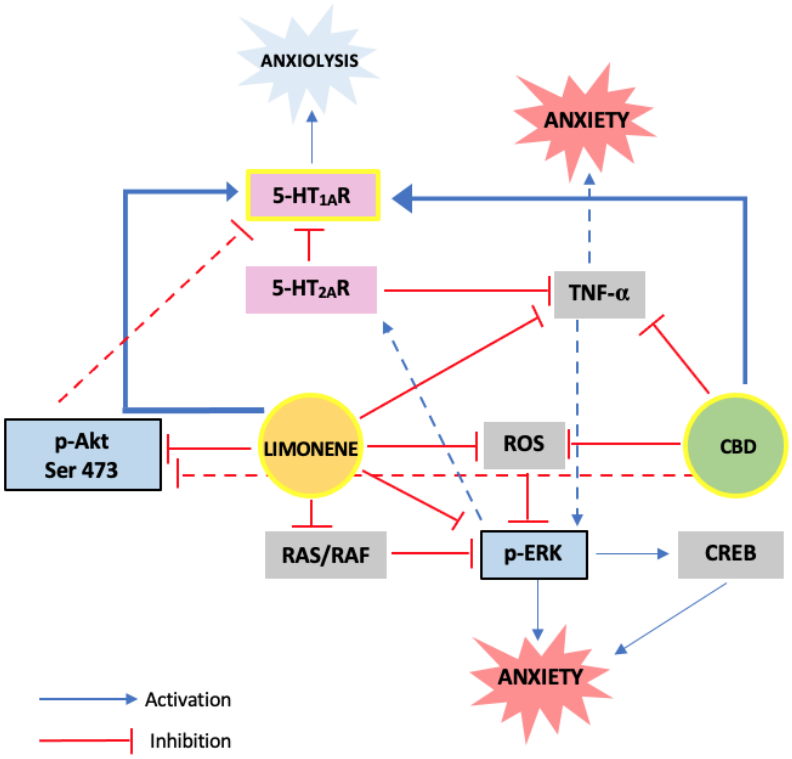

Figure 21: Schematic of Potential Proteins Targeted by Limonene and CBD in Modulating Anxiety (as presented in *Discussion*).

5. Conclusions and Future Directions

Changes in the legal landscape surrounding recreational and non-prescriptive cannabis practices have prompted scientific investigation to better inform safe and responsible usage. Moreover, advances in pre-clinical and clinical research elucidating the divergent effects of different phytocannabinoids (i.e., CBD and THC) and the remedial properties of terpenes, have highlighted the therapeutic potential of cannabis-derived components. In particular, the discovery that combinatorial formulations of different phytocannabinoids are more effective than isolated administrations, termed the Entourage Effect, have provoked scientific inquiry on whether specific phytocannabinoid-terpene combinations could elicit greater therapeutic efficacy. Given the documented anxiolytic properties of individual administrations of the monoterpene d-limonene and the phytocannabinoid cannabidiol, this thesis explored the presence of potential Entourage Effect-potentiated anxiolysis in combinatorial doses of Limonene-CBD.

Utilizing a rodent model of open-space and bright-space anxiety, and fear-related memory formation, this study demonstrated for the first time the synergistic and consequently, enhanced anxiolytic efficacy of concurrently inhaled limonene (200 μL or 2000 μL) and intra-nucleus accumbens shell cannabidiol (1 ng/0.5 μL or 5 ng/0.5 μL) in conditions of acute stress, as well as the absence of anxiolysis in a model of social anxiety; dose-dependent effects could not be conclusively determined, however, given that combinations of the lowest dose of each component (200 μL of limonene + 1 ng/0.5 μL or 5 ng/0.5 μL of CBD) evoked similar anxiolysis compared to the highest dose combination treatment (2000 μL of limonene + 5 ng/0.5μL of CBD).

Moreover, correlations between behavioural outcomes and the detected changes in the L2KCBD(5) group in ERK1/2 expression within the PFC and Akt Ser473 within the NASh (relative to CBD alone), not only substantiated the facilitative effects of limonene in synergizing the anxiolytic effects of CBD but also highlighted the functional connections between the associated brain regions of the mesocorticolimbic system in this regulation. Although questions remain on precisely how intra-NASh delivery is communicating with the PFC and VTA and whether various receptor targets may be aiding in the synergistic anxiolysis of Limonene-CBD dosages, outcomes from this thesis support the well-established role of the NASh, and specifically CBD within the NASh, in regulating affective processing, thereby, emphasizing this brain region as a useful target in treating mood and anxiety disorders. Furthermore, delivery of these formulations with co-applied NAD299 hydrochloride and the consequent reversal of behavioural effects and related biomarker activity validated the role of 5-HT_{1A}R in mediating Limonene-CBD synergistic anxiolysis.

While further work involving functional disconnection and electrophysiological analysis would aid in clarifying the mechanisms presented in this thesis, data obtained from this investigation adds to the growing body of knowledge on the Entourage Effect observed amongst cannabis-derived phytochemicals. Moreover, given the enhanced pharmacological safety and relative lack of adverse side-effects associated with limonene and CBD when compared to conventional anti-anxiety medications, this study opens up a potential avenue to consider in terms of naturally-derived anxiolytics that may aid or substitute current pharmacotherapies.

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Curriculum Vitae

Name: Nathashi Jayawardena

Post-secondary Education and Degrees:

Western University
London, Ontario, Canada
2019-2021 MSc.

University of Toronto
Mississauga, Ontario, Canada
2012-2016 H.BSc.

Honours and Awards:

MITACS Accelerate Internship
01/2019-12/2020

Western Graduate Research Scholarship
01/2019-12/2020

Natural Sciences and Engineering Research Council of Canada (NSERC) – Undergraduate Student Research Award
2016

Dean's Honour List
2014, 2015, 2016

Related Work Experience:

Graduate Teaching Assistant
Western University
01/2019-12/2020

Undergraduate Teaching Assistant
University of Toronto
2016

Research Assistant (USRA)
University of Toronto Mississauga (Dr. Bryan Stewart)
2016

Research Assistant
University of Toronto Mississauga (Dr. Tina Malti)
2014-2016