Evaluation of the use of cannabidiol in the treatment of anxiety related disorders by assessing changes in neurotransmitter levels and expression of CREB/BDNF in the rodent brain

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A thesis submitted to the School of Health Sciences, College of Health Sciences, University of KwaZulu-Natal, Westville, for the degree of Master of Medical Sciences.

This is the thesis in which the chapters are written as a set of discrete research publications with an overall introduction and final discussion. Typically, these chapters would have been published in internationally recognized, peer-reviewed journals.

This is to certify that the contents of this thesis are the original research work of Miss Advaitaa Meera Haripershad, carried out under my supervision at the Catalysis and Peptide Research Unit, University of KwaZulu-Natal, Westville campus, Durban, South Africa and Biomedical Resource Unit, University of KwaZulu-Natal, Westville campus, Durban, South Africa.

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Abstract

Anxiety and anxiety-related disorders are common psychiatric disorders that are responsible for high disease burden. The pathogenesis of anxiety involves dysfunction in the limbic brain regions including the amygdala, prefrontal cortex, and hippocampus. The current pharmacological treatments for anxiety target the modulation of the activity monoamine neurotransmitters such as dopamine, serotonin, gamma-aminobutyric acid, norepinephrine and glutamate. These neurotransmitters are key in the regulation of the maladaptive responses of anxiety. Primary pharmacotherapies demonstrate limitations in drug efficacy as well as adverse side effects, highlighting the need for novel therapeutics for anxiety and anxiety-related disorders. Cannabidiol (CBD), a non-psychoactive cannabinoid from the *Cannabis sativa* plant, has been considered a potential anxiolytic treatment as a result of its interaction in the endocannabinoid system, which regulates synaptic plasticity and neuronal activity implicated in the anxiety response. The therapeutic potential of CBD against neuropsychiatric disorders have been reported in preclinical and clinical studies. Since the global increase in cannabis legalization, there remains a need to supplement the available literature related to the neural effect of cannabis use on behavioural, neurochemical and biochemical changes. There are gaps in the knowledge of the pharmacokinetics and behavioural effects of CBD. This study will contribute to increasing the knowledge of the effect of cannabis on neurotransmitters and molecular changes in the brain.

In this thesis, chapter 1 is a literature review focusing on the neurobiology and pharmacological treatments of anxiety, cannabidiol as a treatment for anxiety, and the neurotransmitters and genes implicated in anxiety. In addition to this, chapter 1 also reviews the theory of the experimental processes performed in this study. Chapter 2 is the publication **"Evaluation of the use of cannabidiol in the treatment of anxiety related disorders by assessing changes in neurotransmitter levels and expression of CREB/BDNF in the rodent brain"** submitted to The Journal of Neuroscience Research. Chapter 3 is the summary and conclusion of the thesis.

Declaration 1 – Plagiarism

I, Advaitaa Meera Haripershad hereby declare that the research reported in this thesis, except where otherwise stated, is my original work and has not been submitted for any degree or examination at any other university.

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Declaration 2 – List of Publications

Advaitaa M. Haripershad, Sanelisiwe P. Xhakaza, Leon J. Khoza, Terisha Ghazi, Shanel Dhani, Cosmas Mutshima, Molopa J. Molopa, Krishnan Anand, Nithia P. Madurai, Lorna Madurai, Sanil D. Singh, Thavendran Govender, Hendrik G. Kruger, Anil Chuturgoon, Tricia Naicker, Sooraj Baijnath. Evaluation of the use of cannabidiol in the treatment of anxiety related disorders by assessing changes in neurotransmitter levels and expression of CREB/BDNF in the rodent brain. ***The Journal of Neuroscience Research.*** **Submitted 2020. ID: jnr-2020-Oct-9123.**

Contributions:

Advaitaa Meera Haripershad contributed to the study design, conducted the animal study, prepared samples for LC-MS analysis and prepared the manuscript.

Sanelisiwe P. Xhakaza and Leon. J. Khoza assisted with the animal study and sample preparation for LC-MS analysis and qPCR.

Terisha Ghazi and Shanel Dhani performed the molecular analysis.

Krishnan Anand extracted and prepared cannabidiol used in the study.

Nithia P. Madurai and Lorna Madurai assisted with access to LC-MS facilities. Cosmas Mutshima and Molopa J. Molopa conducted all LC-MS data analysis and interpretation.

Sanil D. Singh assisted with animal ethics and the animal study.

Sooraj Baijnath supervised the study, contributed to the study design and revised the manuscript.

The remaining authors are supervisors and revised the manuscript.

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2-AG: 2-arachidonoyl-glycerol

5-HT: 5-hydroxytryptamine/ serotonin

5-HT1A receptor: serotonin receptor subtype 1A receptor

5-HT2A receptor: 5-HT subtype 2A receptor

ACC: Anterior cingulate cortex

AEA: Anandamide

AUC: Area under curve

BDNF: Brain-derived neurotrophic factor

BLA: Basolateral amygdala complex

BNST: Bed nucleus of the stria terminalis

cAMP: Cyclic adenosine monophosphate

CB1: Cannabinoid-1-receptor

CB2: Cannabinoid-2-receptor

CBD: Cannabidiol

CeA: Central amygdala

C_{max}: Maximum concentration in a profile

CNS: Central nervous system

CREB: Cyclic AMP response element binding protein activation

DA: Dopamine

EP: Epinephrine

ESI: Electrospray ionization

FAAH: Fatty acid amide hydrolase

GABA: Gamma-aminobutyric acid

GAD: Generalized anxiety disorder

Glu: Glutamate

GPCR: G-protein-coupled receptors

HESI: Heated Electrospray ionization

i.p.: Intraperitoneal

K_{el}: Elimination rate constant

LC: Locus coeruleus

LC-MS/MS: Liquid chromatography – tandem mass spectrometry

mPFC: Medial prefrontal cortex

mRNA: Messenger RNA

NE: Norepinephrine

NT: Neurotransmitter

PCC: Posterior cingulate cortex

PCR: Polymerase chain reaction

PD: Panic disorder

PFC: Prefrontal cortex

PKA: Protein kinase

SAD: Social anxiety disorder

SD: Standard deviation

SNRI: Serotonin norepinephrine reuptake inhibitor

SSRI: Serotonin reuptake inhibitor

$T_{1/2}$: Terminal half-life

TCA: Tricyclic antidepressant

T_{max}: Time of maximum concentration

Δ-9-THC: Delta-9-tetrahydrocannabinol

CHAPTER 1

INTRODUCTION

1. Anxiety

Anxiety is characterized by an emotional response that occurs in anticipation of potential threats or dangers (Sartori & Singewald, 2019). Anxiety is an incessant feeling of dread, trepidation and imminent disaster (Kaur & Singh, 2017; Pilkington, 2010). Most people experience anxious feelings when faced with stress, however this anxiety is adaptive to inform and prepare the person for the latent threat (Sartori & Singewald, 2019). Anxiety is deemed pathological when it becomes uncontrollable, persistent and maladaptive (Sartori & Singewald, 2019). According to the World Health Organisation, anxiety disorders are estimated to have a global prevalence of 3.6 % and is more common in females (4.6 %) than in males (2.6 %) (WHO, 2017). In 2017, there were 1 768 851 reported cases of anxiety disorders in South Africa, which represents 3.4 % of the national population (WHO, 2017). The disease burden of anxiety disorders is reported as 2.8% of the total years living with disability in the South African population (WHO, 2017).

Anxiety disorders may arise from a multifaceted set of risk factors including genetics, brain chemistry, personality and life events (Kaur & Singh, 2017). Anxiety disorders are categorized by the International Statistical Classification of Diseases and Related Health Problems into generalized anxiety disorder (GAD), social anxiety disorder (SAD), and panic disorder (PD) (Kogan et al., 2016; Reed et al., 2019). Social Anxiety Disorder, also known as social phobia, is a common anxiety disorder characterized by an individual fearing and avoiding the scrutiny by others (Stein & Stein, 2008). Patients with SAD have a persistent fear in social situations with exposure to unknown people (Stein & Stein, 2008). The individuals experience intense emotional and physical anxiety symptoms which may lead to them avoiding social situations, which could interfere with their personal life (Stein & Stein, 2008). Panic disorder is diagnosed by repeated unpredicted panic attacks, anxiety about imminent panic attacks, or significant behavioural changes because of the panic attacks (Craske & Barlow, 2014). Generalized Anxiety Disorder is one of the most common and debilitating conditions. GAD is described by an uncontrollable, multifocal chronic worry that persistently occurs for periods of longer than 6 months (Stein & Sareen, 2015).

Patients with anxiety display a variety of cognitive, physiological and behavioural symptoms (Lang & Schlien, 1968). The cognitive factor of anxiety is associated with cognitive distortions in the components of attention, interpretation, and memory for information processing (Hollon & Beck, 2013; Kaur & Singh, 2017). The physiological symptoms of anxiety incorporate autonomic or somatic sensations, including sleep avoidance, insomnia, headaches, muscle

tension, gastrointestinal problems nightmares, heart palpitations, tachycardia and shortness of breath (Alfano, Ginsburg, & Kingery, 2007). The behavioural component of anxiety indicates the actions performed by an individual to prevent exposure to the feared stimuli. The behavioural symptoms involve avoidance of a particular stimuli or situation by the individual, thus impairing ones daily routines as well as family, academic and social functions (Hayes, Villatte, Levin, & Hildebrandt, 2011). Anxiety related disorders are accompanied by psychological symptoms including depression, alcohol and substance abuse (Sareen et al., 2006; Stein & Sareen, 2015). Anxiety-related disorders are associated with a decreased sense of well-being, elevated rates of unemployment and relationship breakdown, and increased suicide risk (Stein & Sareen, 2015)

1.1. Neuronal circuitry of anxiety

There are many brain regions implicated in the identification and modulation of adverse emotional stimuli and in the generation of cognitive, behavioural or somatic responses to the stimuli (Nuss, 2015). Human and rodent studies have contributed to identifying brain regions implicated in anxiety by developing worry, tension and apprehension due to an aversive stimulus (Duval, Javanbakht, & Liberzon, 2015). The amygdala nuclei situated within the temporal lobe and is considered as the central orchestra in the control of anxiety related responses (Linsambarth, Moraga-Amaro, Quintana-Donoso, Rojas, & Stehberg, 2017; Nuss, 2015). The basolateral amygdala complex (BLA) and the central amygdala (CeA), within the central nucleus, are two groups of nuclei that are critical in anxiety (Etkin, 2009). The BLA receives incoming information and determines the threat value of potentially negative emotional signals from the thalamus and the sensory association cortex (Davis & Whalen, 2001; Nuss, 2015). The CeA is vital in the species-specific defensive responses associated with fear (Davis & Whalen, 2001).

The CeA is directly activated by the BLA through an excitatory glutamatergic pathway (Pitkänen, Savander, & LeDoux, 1997). The BLA initiates a relay of inhibitory GABAergic interneurons situated between the BLA and the CeA, which employs an inhibitory effect on the CeA (Royer, Martina, & Pare, 1999). The somatic manifestation of anxiety occurs due to activation of the GABAergic neurons from the CeA to the hypothalamus and brainstem (Jongen-Rêlo & Amaral, 1998). Inhibitory GABAergic neuron projections from the CeA to the locus coeruleus (LC) and other forebrain nuclei may be involved in anxiety-related dysphoria (Forster, Novick, Scholl, & Watt, 2012). The bed nucleus of the stria terminalis (BNST), adjacent to the amygdala, is activated by the inhibitory neurons from the BLA which also may

have a role in dysphoria (Forster et al., 2012; Linsambarth et al., 2017). Studies suggest that the pharmacological activation of the BLA and CeA are anxiogenic, whereas its inhibition is anxiolytic (Flores-Gracia et al., 2010; Truitt, Johnson, Dietrich, Fitz, & Shekhar, 2009).

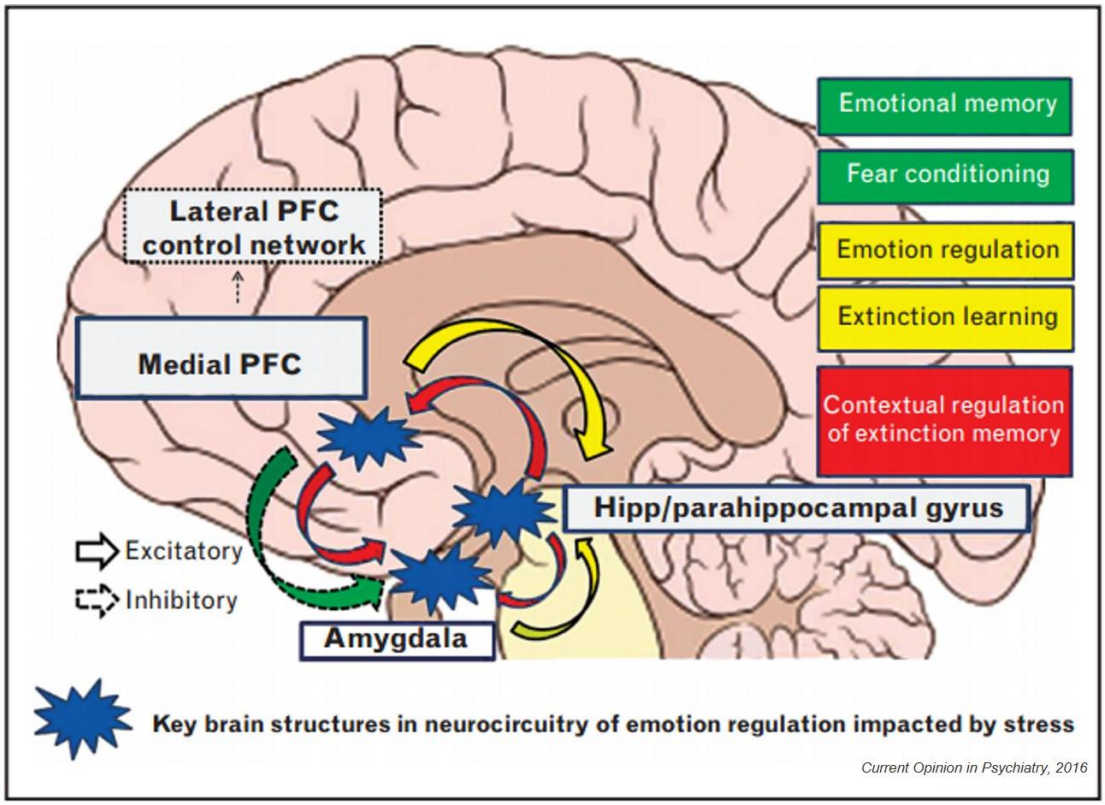

Figure 1: Structure of amygdala involved in the regulation of anxiety (sourced from (Mah, Szabuniewicz, & Fiocco, 2016).

Forebrain areas, including the medial prefrontal cortex (PFC) and the anterior cingulate cortex (ACC) are activated concurrently with the amygdala during the presence of an emotional stimuli (Figure 1) (Kober et al., 2008; Mah et al., 2016). These areas receive and convey excitatory glutamatergic projections to and from the BLA (Kober et al., 2008). The expression of anxiety is controlled by the PFC through the changes of neuronal activity in the BLA (Etkin, 2009; M. J. Kim et al., 2011). The neuronal circuitry of anxiety includes bottom-up activity from the amygdala, showing the presence of potentially threatening stimuli, and top-down control mechanisms originating in the PFC, signalling the emotional salience of stimuli as depicted in Figure 2 (Nuss, 2015). It is critical in understanding the factors that control these bottom-up/top-down mechanisms so that more effective anxiolytic interventions can be developed (Nuss, 2015).

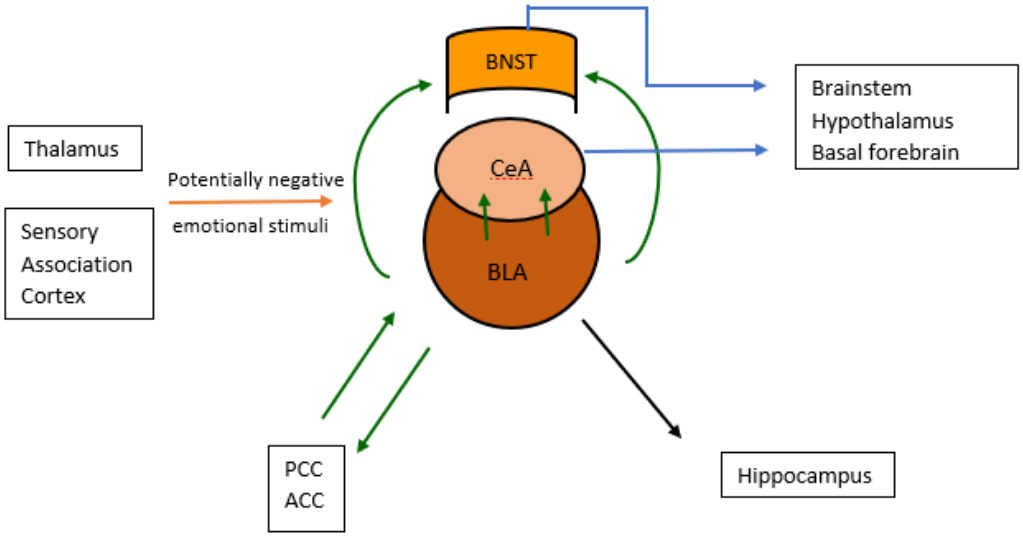

Figure 2: Neuronal circuitry of anxiety disorders highlighting the role of the amygdala *(adapted from (Nuss, 2015).*
Key: *orange arrow: negative emotional stimulus received from thalamus and sensory association cortex; green arrow: excitatory glutamatergic pathways; blue arrows: GABAergic inhibitory neurons are activated leading to the somatic manifestation of anxiety in the brainstem, hypothalamus and basal forebrain*

Abbreviations*: ACC, anterior cingulate cortex; PCC, posterior cingulate cortex; BLA, basolateral amygdala; BNST, bed nucleus of the stria terminalis; CeA, central nucleus of the amygdala; PFC, prefrontal cortex.*

2. Pharmacological treatment of anxiety

The treatment of anxiety disorders should include psychological therapy, pharmacotherapy, or a combination of both (Bandelow, Michaelis, & Wedekind, 2017). The current pharmacological treatments include serotonin reuptake inhibitors, benzodiazepines, monoamine oxidase inhibitors, tricyclic antidepressant (TCA) drugs, and partial 5-hydroxytryptamines (5-HT) 1A receptor agonists (Bandelow et al., 2017). The serotonin reuptake inhibitors (SSRIs) and the serotonin norepinephrine reuptake inhibitors (SNRIs) are the recommended first-drugs as a result of their positive benefit to risk balance ratio (Bandelow et al., 2017; Sartori & Singewald, 2019). SSRIs produce side effects such as jitteriness at the onset of therapy, gastrointestinal problems, insomnia, and sexual dysfunction which may or may not improve over time (Stübner et al., 2018). The TCAs are second-generation drugs which include imipramine and clomipramine (Thanacoody & Thomas, 2005). The TCAs have

a higher frequency of adverse effects and tolerability issues in comparison to the SSRIs and SNRIs (Bandelow et al., 2017). One of the shortcomings of the SSRIs and the TCAs is the delayed therapeutic action, rendering antidepressants ineffective as an acute anxiolytic treatment (Gomez et al., 2018). Benzodiazepines are efficacious in treating GAD, an acute anxiety disorder, but produces a range of side effects in long-term treatment, for example, dependence, withdrawal symptoms, impaired cognition and overdose deaths (Baandrup et al., 2018; Bachhuber, Hennessy, Cunningham, & Starrels, 2016). Therefore there is a need to develop or investigate novel pharmacological anxiolytic treatments due to the many shortcomings of the current treatments including increased cost of drug development, a diminished rate of successful drug discovery and development and long-term adverse side effects (Guina & Merrill, 2018; Nutt & Attridge, 2014; Sartori & Singewald, 2019).

3. *Cannabis sativa*

Cannabis sativa, also commonly known as marijuana, is a plant with a wide range of medicinal properties which has been extensively used throughout human history (Zou & Kumar, 2018). The *Cannabis* plant is classified into three species, *C. sativa*, *C. indica* and *C. ruderalis* (Akatan, 2012). The extracts of this ancient medicinal plant were reported to be first used as cramp and pain relief in China as early as 6000 BC (Mechoulam, 1986). Cannabis has a wide range of therapeutic uses including anti-inflammation, anti-nociception, anti-emetic and anticonvulsant activities (Iversen, 2003; Mechoulam, 1986; Wallace, Wiley, Martin, & DeLorenzo, 2001). However, the clinical application of the therapeutic action of *Cannabis* is limited due to the recreational use (Zou & Kumar, 2018). *Cannabis* contains in excess of 400 chemical constituents, including cannabinoids and other psychoactive phytochemicals, which are responsible for its biological effects (Atakan, 2012). Cannabinoids are found in the stalk, leaves, flowers and seeds of the cannabis plant (C. H. Ashton, 2001). Delta-9-tetrahydrocannabinol (Δ-9-THC) is one of the principle psychoactive component of approximately 70 phytocannabinoids identified in the plant (Atakan, 2012; Pacher, Bátkai, & Kunos, 2006). Some of the other identified phytocannabinoids also have individual biological effects which may mediate the psychoactive effects of THC (Mechoulam & Parker, 2013). It has been suggested in preclinical studies that the individual effects of phytocannabinoids are multiphasic and dose-dependent, which can be demonstrated by Δ-9-THC which has anxiolytic effects at low doses and anxiogenic effects at higher doses (Rey, Purrio, Viveros, & Lutz, 2012). There are minor cannabinoids which also display a range of biological activities such as cannabigerol which has antibacterial activity, cannabinol has sedative properties, and

tetrahydrocannabivarin has antiepileptic effects (Aizpurua-Olaizola et al., 2014), however the therapeutic roles of many of them remain unknown.

3.1. Cannabidiol (CBD)

Cannabidiol is a metabolic by-product and major cannabinoid of *Cannabis sativa* (Citti et al., 2018). Cannabidiol, shown in Figure 3, is the principle non-intoxicating component of Cannabis sativa, which is produced in high concentrations in the plant making up approximately 40% of the total cannabinoid content (Deiana et al., 2012). In contrast to THC, cannabidiol does not exhibit psychotropic effects (Alline Cristina Campos, Moreira, Gomes, Del Bel, & Guimaraes, 2012). Cannabidiol is a promising therapeutic agent which can be observed in its anxiolytic (Crippa et al., 2009), anti-psychotic (Moreira and Guimaraes, 2005), anti-inflammatory (Juknat, Rimmerman, Levy, Vogel, & Kozela, 2012), anti-convulsant (Wallace et al., 2001), and immunomodulatory (Costa, Trovato, Comelli, Giagnoni, & Colleoni, 2007) properties of CBD. The properties of cannabidiol allow it to be utilized in the treatment of a wide range of psychiatric and non-psychiatric disorders such as psychosis, anxiety and depression(Alline Cristina Campos et al., 2012). The anxiolytic and anti-psychotic effects of CBD can offset the anxiety and psychotomimetic effects induced by THC (Douglas Lee Boggs, Peckham, Boggs, & Ranganathan, 2016).

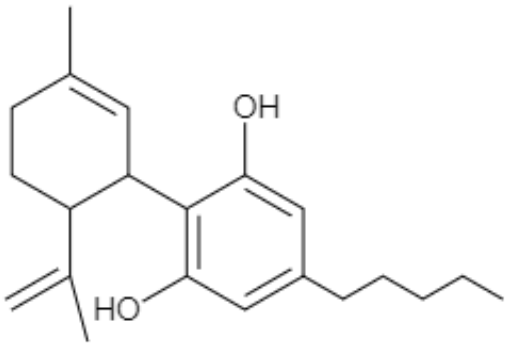

Figure 3: Structure of cannabidiol (CBD)

Previous literature has suggested that this non-psychotomimetic compound has a relatively low affinity for cannabinoid (CB) receptors (Pertwee, 1997; Pertwee et al., 2010). However, studies by (Thomas et al., 2007) reported that CBD has the ability to act as a partial antagonist at cannabinoid-1 (CB1) receptors and as an inverse agonist at cannabinoid-2 (CB2) receptors. Cannabidiol has a complex pharmacological profile as more than 20 different mechanisms of action has been described (Douglas L Boggs, Nguyen, Morgenson, Taffe, & Ranganathan,

2018; Deiana et al., 2012). There are two well-researched mechanisms of action by which CBD exerts its antipsychotic effects: the facilitation of endocannabinoid signalling, and the administration of an exogenous CB receptor agonist. There is an increase in research depicting the beneficial effects of cannabidiol in the brain, concluding CBD as a promising novel therapy for various neurological disorders.

3.2 Delta-9-tetrahydrocannabinol

The discovery of THC resulted in the generation of a range of synthetic cannabinoids that are similar in structure to phytocannabinoids, which finally led to the identification and successful cloning of the CB1 receptor, and the CB2 receptor (Pertwee et al., 2010). THC, depicted in Figure 4, is a partial agonist of the CB1 and CB2 receptors, that are G protein coupled receptors which are part of the endocannabinoid system (Atakan, 2012). THC produces hypothermia, hypoactivity, spatial and verbal short-term memory impairment, in a dose-dependent manner (Hayakawa et al., 2008). It is suggested that the pharmacological effects of Δ -9-THC can be potentiated by CBD via a CB1R-dependent mechanism (Hayakawa et al., 2008). Delta-9-THC has the ability to activate the CB1 and CB2 receptors, similar to the endocannabinoids (Pertwee, 2008). THC produces *in vivo* effects in healthy rodents via the activation of the CB1 receptors which inhibit the ongoing neurotransmitter release (Howlett et al., 2002; Pertwee, 2008). In mice, Δ-9-THC generates a 'tetrad' of effects including the suppression of locomotor activity, hypothermia, immobility during the ring test and antinociception in the tail-flick test (Pertwee, 2008). In a study performed by (Atakan, 2012), it was reported that Δ-9-THc caused transient psychotic symptoms, augmented the levels of anxiety, intoxication and sedation in healthy volunteers whilst CBD had no significant effect on these parameters. During brain imaging, THC reduced the action of brain regions that typically mediate response inhibition, whilst CBD controlled activity in regions not implicated during verbal learning tasks (Borgwardt et al., 2008).

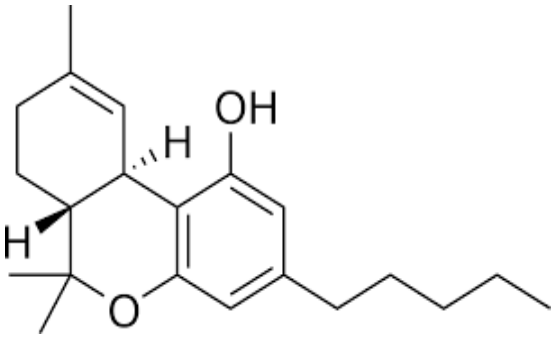

Figure 4: Structure of delta-9-tetrahydrocannabinol

4. The Endocannabinoid System

The endocannabinoid system is responsible for mediating the physiological changes, such as motor function, pain perception, cognition, appetite, and sleep cycles, that occur after cannabis administration (Mechoulam & Parker, 2013). This system is comprised of endogenous cannabinoids (endocannabinoids), anandamide (AEA) and 2-arachidonoyl-glycerol (2-AG), cannabinoid-1 receptors and cannabinoid-2 receptors, and enzymes which synthesize and degrade endocannabinoids (Dhopeshwarkar & Mackie, 2014). Anandamide is synthesized by the enzyme, N-acylphosphatidylethanolamine-selective phospholipase D, and degraded by fatty acid amide hydrolase (FAAH) (De Aquino et al., 2018). The other endocannabinoid, 2-arachidonoylglycerol, is synthesized by diacylglycerol lipase and monoacylglycerol, whereas 2-arachidonoylglycerol hydrolase degrades 2-AG (De Aquino et al., 2018). These endocannabinoids differ from other neurotransmitters as they are synthesized on demand which is initiated by the activation of G-protein- coupled receptors or depolarization (Mechoulam & Parker, 2013). The precursors of endocannabinoid are found in the lipid membranes of post synaptic neurons (De Aquino et al., 2018; Mechoulam & Parker, 2013). Cannabinoids interact with cannabinoid receptors, as well as, other G protein-coupled receptors, nuclear receptors and ion channels (Howlett et al., 2002). The endocannabinoids act as endogenous agonists of the cannabinoid receptors and have varying intrinsic efficacies to the cannabinoid receptors (Lu & Mackie, 2016). Anandamide is a low efficacy agonist at CB1 receptors and an extremely low efficacy agonist at CB2 receptors (Lu & Mackie, 2016; Luk et al., 2004). 2-Arachidonoyl glycerol is a high efficacy agonist at both CB1 and CB2 receptors (Gonsiorek et al., 2000; Luk et al., 2004).

The cannabinoid receptors are members of the superfamily of G-protein-coupled receptors (GPCR's) which mainly couple to Go and Gi protein classes(Howlett et al., 2002). Hence, the activation of these receptors exerts various effects on cellular physiology such as the inhibition of adenylyl cyclases and voltage dependent calcium channels, as well as the activation of many MAP kinases (Howlett et al., 2002). The regulation of cannabinoids is primarily based on retrograde signalling where the endocannabinoids are synthesized and released on demand, which differs from other neurotransmitters that are produced in advanced and stored in vesicles, from post synaptic sites to active the CB receptors on the presynaptic membranes in neurons (Figure 5) (Alger, 2002; Navarro et al., 2016). The synthesis of endocannabinoids is promoted by depolarization or the activation of the G-protein-coupled receptors (De Aquino et al., 2018).

Exogenous cannabinoids have a distinction from endocannabinoids as they are metabolized over several hours before being excreted (De Aquino et al., 2018).

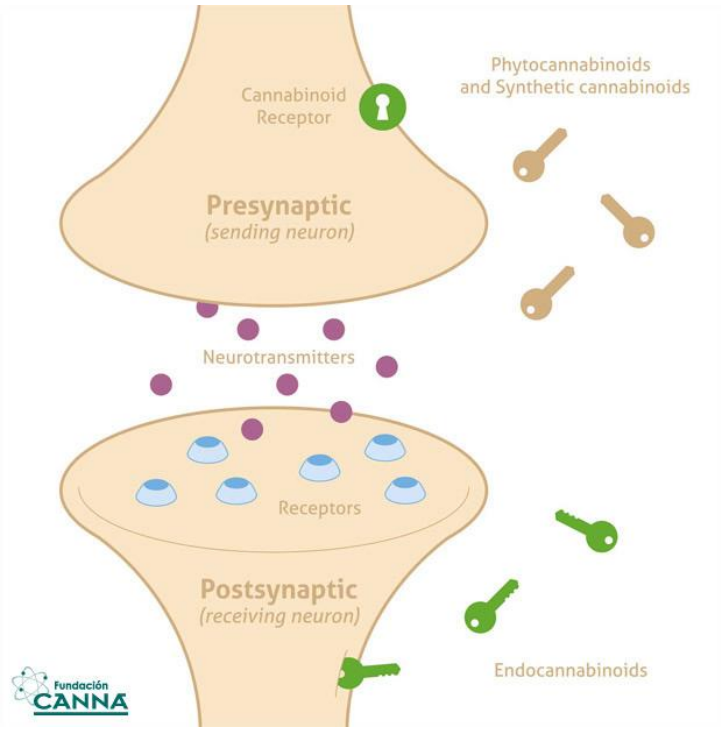

Figure 5: The endocannabinoid system (Fundacion, CANNA (https://www.fundacion-canna.es/en/endocannabinoid-system)

Endocannabinoids act as neuromodulators that inhibit the release of other neurotransmitter systems such as GABA and glutamate. The endocannabinoids are retrogrades which are released on demand from the postsynaptic sites and exert their effects by binding to CB1Rs on the presynaptic membrane. The CB receptors are activated and inhibit the release of neurotransmitters through the various ion channels. The interaction between endocannabinoid system with multiple neurotransmitters, such as acetylcholine, dopamine, GABA, serotonin, glutamate, and norepinephrine, mediates many pharmacological effects of cannabinoids.

4.1 Cannabinoid-1-receptor

The CB1 receptors are the most abundant cannabinoid receptors and are primarily expressed in the central and peripheral nervous system. These receptors have a fundamental role in the mammalian central nervous system (CNS) in regulating neuronal activity as endocannabinoids rely on the receptors for higher brain function activity (Marcu & Schechter, 2016; Navarro et al., 2012). In rodent brain models, CB1 receptors are found in higher densities in the basal ganglia, substantia nigra, globus pallidus, cerebellum and hippocampus (Mechoulam & Parker, 2013). CB1 receptors are also expressed in the heart, bones, lung, thyroid liver, uterus,

testicular tissue and the vascular endothelium (Pertwee, 2006; Russo & Guy, 2006). CB1 receptors are located at both the GABAergic and glutamatergic terminals of the central and peripheral neurons, suggesting their involvement on GABA and glutamate neurotransmission and both inhibitory and excitatory activity (Atakan, 2012; Howlett et al., 2002; Mechoulam & Parker, 2013). They are also found in the central nervous system of the brain areas that are related to the body's stress response (Atakan, 2012; Kaur & Singh, 2017). These brain areas include the central amygdala, basal ganglia, limbic system, hippocampus, frontal cortex, substantia nigra and the cerebellum (Pertwee, 2006). In the brain, CB_1 receptors mediate inhibitory actions by the ongoing release of excitatory and inhibitory dopaminergic, gamma-aminobutyric acid (GABA), glutamatergic, serotoninergic, noradrenalin and acetylcholine neurotransmitter systems (Atakan, 2012). The involvement of the neurotransmitter systems and the high density of CB1 receptors in the sensory and motor brain regions contribute to the motor movement, pain perception and cognition functions (Howlett et al., 2002). The activation of CB1 receptors results in a decline in the accumulation of cyclic adenosine monophosphate (cAMP), subsequently the inhibition of cAMP-dependent protein kinase (PKA) (Mechoulam & Parker, 2013). The potential of CB1 receptors as targets for CNS diseases are limited by the psychotropic side effects of its natural agonists, such as delta-9-tetrahydrocannabinol, on the animal models (Navarro et al., 2012). Therefore, there is an increasing research performed on CB2 receptors as targets for diseases of the CNS (Navarro et al., 2012).

4.2 Cannabinoid-2 receptors

The CB2 receptors are abundantly expressed on the surface of cells involved in the peripheral immune system (Atkinson & Abbott, 2018). In the brain, the CB2 receptors are localized to some extent in the neurons of the brainstem, the cerebellum, internal and external segments of the *globus pallidus,* substantia nigra, and in the microglial cells (Atkinson & Abbott, 2018; Lanciego et al., 2011; Stella, 2004). However, the CB2 receptors are expressed at a lower level compared to CB1 receptors in the central nervous system (J. C. Ashton, Friberg, Darlington, & Smith, 2006; Mechoulam & Parker, 2013). Consequently, there are fewer side effects that are expected when drugs are targeting CB2 receptors, which has limited expression in the CNS, compared to drugs targeting the abundantly expressed CB1 receptors in the CNS (Navarro et al., 2016). CB2 receptors are upregulated in the activated microglial cells in a variety of CNS diseases, making it a promising candidate in diseases with neuroinflammatory components (Navarro et al., 2016). There is some controversy pertaining to the degree of CB2 receptor expression in resting verses activated microglial cells as there is a difference in the phenotypes

of microglial that are filtered from blood into the CNS, and in resident microglial which becomes activated by accumulation of proteins (Navarro et al., 2016). An improved understanding of the expression and the role of CB2 receptors in the various microglial phenotypes will assist in designing CB2 receptor ligands with the potential to induce the anti-inflammatory-skewed phenotypes (Franco & Fernandez-Suarez, 2015). A review by (Mechoulam & Parker, 2013) discussed the possibility of CB2 receptors as a component of a general protective system of the mammalian body, particularly the immune system. The CB2 receptors are also involved in CNS inflammation which is an increasingly researched aspect of the pathophysiology of schizophrenia (Müller, Weidinger, Leitner, & Schwarz, 2015).

Cannabinoids, such as cannabidiol, that target CB2 receptors have potential therapeutic properties such as the preservation of neuronal integrity and survival (Atwood, Straiker, & Mackie, 2012). Accordingly, cannabidiol has a promising potential in pain, and acute and chronic neuroinflammatory conditions (Micale, Mazzola, & Drago, 2007). The neuroprotective potential of the CB2 receptor-targeting cannabinoids is mediated by their various locations in the CNS (Chung et al., 2016; Navarro et al., 2016). This enables the cannabinoids to selectively activate the CB2 receptors to apply a selective control over the particular functions performed by these cells in protection, degeneration and repair (Fernández-Ruiz et al., 2014). The pharmacological action of anxiolytic treatments, including cannabidiol and standard drugs such as benzodiazepines, implicate the neurotransmitters within the central nervous system (Bandelow et al., 2017; Sartori & Singewald, 2019).

5. Neurotransmitters and anxiety

Neurotransmitters (NT's) are signalling molecules, which have vital roles in neuronal communications within the central nervous system (CNS) (Liu, Zhao, & Guo, 2018). Previous literature has reported that modifications in the quantification of NT's in many brain regions involve the development of several neurodegenerative and psychiatric diseases such as Parkinson's disease, Huntington's disease, multiple sclerosis (Bandelow & Michaelis, 2015; Hussain, Zubair, Pursell, & Shahab, 2018). Neurotransmitters are categorized based on their chemical structures: (i) small molecules and (ii) neuropeptide (Liu et al., 2018). The small molecules consist of dopamine (DA), norepinephrine (NE), glutamate (Glu), serotonin (5-HT), epinephrine (EP), gamma-aminobutyric acid (GABA) and endocannabinoids (Kaur & Singh, 2017). The neuropeptides include enkephalin, endorphin and substance P (Kaur & Singh, 2017). The irregular functioning of neurotransmitters such as dopamine, GABA,

norepinephrine, serotonin, acetylcholine and, chemoreceptor activity results in anxiety (Kaur & Singh, 2017).

5.1 The function of dopamine in anxiety

Dopamine (Figure 6), is the principle catecholamine in the mammalian brain and is vital in various cerebral functions including reward, motor control, learning, cognition, and emotion (Ko & Strafella, 2012; Zarrindast & Khakpai, 2015). The dopaminergic system is also involved in the pathogenesis of many psychiatric and neurological disorders such as Parkinson disease (PD), depression, anxiety, schizophrenia, Huntington disease and behavioural/chemical addiction (Ko & Strafella, 2012). The dopaminergic system plays a vital role in anxiety-like behaviour via the transmission in the mesolimbic, mesocortical and nigrostriatal pathways (Melis & Pistis, 2012). Dopamine is produced in the substantia nigra and is important in the reward system for anxiety (Nasehi, Mafi, Oryan, Nasri, & Zarrindast, 2011). This neurotransmitter is released from the substantia nigra and the VTA, via the dopaminergic pathways, to all brain regions involved in anxiety such as the amygdala, hippocampus, septum, prefrontal cortex and nucleus accumbens (Melis & Pistis, 2012; Zarrindast & Khakpai, 2015).

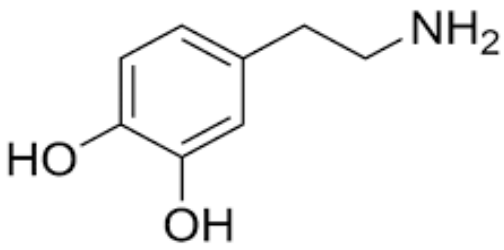

Figure 6: Structure of Dopamine

5.2 The function of serotonin in anxiety

Serotonin (5-hydroxytryptamine; 5-HT) (Figure 7) is involved in the regulation of emotion (Kaur & Singh, 2017). This neurotransmitter system has complex and multifaceted functions in cognition, learning, memory and reward (Young, 2007). Serotonin has an essential role in the development of anxiety disorders which is modulated by its effect on the locus coerulus and amygdala in the brain (Graeff, 2002; Jia & Pittman, 2014). The serotonergic pathways are activated by components of anxiety including fear and stress (Akimova, Lanzenberger, & Kasper, 2009). Previous literature demonstrates an increase in 5-HT levels in the brain corresponds to an increase in anxiety, whereas an attenuation in the levels of 5-HT reduces anxiety (Murphy et al., 2013).The extensive range of actions of 5-HT neurons are regulated at

a series of 5-HT receptors (5-HTR) (Staes et al., 2019). In humans, genetic polymorphisms in the serotonin receptor subtype 1A receptor (5-HT1A receptor), 5-HT subtype 2A receptor (5-HT2A receptor) and the 5-HT transporter (SLC6A4) are associated with anxiety disorders, impulsivity and neurotic personalities (Golimbet, Alfimova, & Mityushina, 2004; Gordon & Hen, 2004; Lesch & Gutknecht, 2005; Staes et al., 2019).

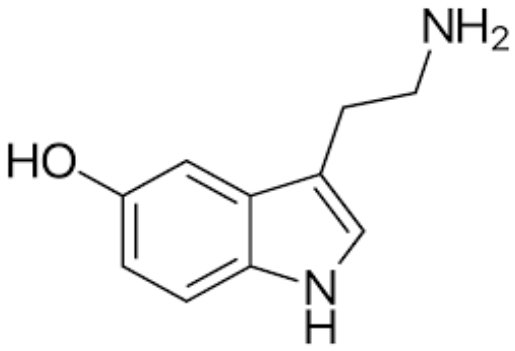

Figure 7: Structure of Serotonin

5.3 The function of glutamate in anxiety

Glutamate (Figure 8), is the principle excitatory neurotransmitter ubiquitous in the mammalian central nervous system, specifically in the cortex and subcortical brain regions (Lujan, Shigemoto, & Lopez-Bendito, 2005). The glutamatergic system has pivotal roles in various brain functions such as, neurodevelopment, learning, information transfer, acute and chronic neurodegeneration, response to stress and anxiety disorders (Kew & Kemp, 2005). The actions of glutamate are regulated via four glutamate receptors: N-methyl-d-aspartate (NMDA), kainite, G protein-coupled metabotropic receptors (mGluR1-8) and a-amino-3-hydroxy-5-methyl-4-isoxazole (AMPA) (Kew & Kemp, 2005; Zhou & Danbolt, 2014). Recent studies have demonstrated the important function of the glutamatergic transmission in the pathogenesis of anxiety disorders (Zhou & Danbolt, 2014). Glutamate excitotoxicity is the excessive activation of glutamate receptors that can excite nerve cells to their death, and this occurs during extreme stress and anxiety exposure (Zhou & Danbolt, 2014). An increase in glutamate results in excitotoxicity, which subsequently results in anxiety due to cell death. Anxiolysis can be stimulated by a reduction in endogenously released glutamate (Kaur & Singh, 2017; Zhou & Danbolt, 2014).

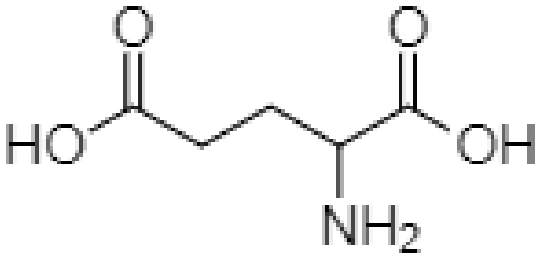

Figure 8: Structure of Glutamate

5.4 The function of Gamma-Aminobutyric acid (GABA) in anxiety

GABA (Figure 9), is a principle inhibitory neurotransmitter which is abundantly distributed throughout the mammalian brain (Watanabe, Maemura, Kanbara, Tamayama, & Hayasaki, 2002). Neurons containing GABA establish interneuronal populations in the cortex regions (Rudy, Fishell, Lee, & Hjerling-Leffler, 2011). The neurotransmission of GABA is believed to strongly modulate excitatory neurotransmission, regulate the processing of information and neuroplastic events through polysynaptic communication with glutamatergic neurons (Kelsom & Lu, 2013) This neurotransmitter system is considered to be the core of the regulation of anxiety and is the primary target of anxiolytic drugs and benzodiazepines (Lydiard, 2003). The regulation of neuronal activity is influenced by GABA controls the excitability states in brain areas (Lydiard, 2003). Anxiety disorders are linked to the dysfunction of the GABA system (Lydiard, 2003). The pathogenesis of anxiety has been associated with the GABAergic brain regions such as the amygdala, hippocampus and hypothalamus(Kalueff & Nutt, 2007). The onset of anxiety in animal models were correlated with a decrease in the expression of GABA receptors and the synthesis of GABA (Mei et al., 2005). Clinical and pre-clinical studies have shown that anxiolytic effects are produced from positive modulators of GABA receptors, whereas the negative modulators cause anxiogenic activity (Nutt, 2001). An early study by (Study & Barker, 1981) proved that GABA receptors are a target for benzodiazepines, which augment the inhibitory action of GABA.

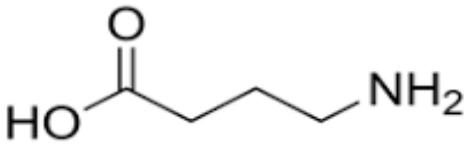

Figure 9: Structure of GABA

5.5 The function of norepinephrine in anxiety

Norepinephrine (NE) (Figure 10) is an essential monoamine neurotransmitter that has broad effects across brain areas to regulate arousal and environmental and internal stress responses (Goddard et al., 2010). The central norepinephrine system is labelled as a modulator with anxiogenic or anxiolytic effects which changes according to acute of chronic conditions of stress (Goddard et al., 2010). Norepinephrine neurons are distributed in small clustered groups in the brainstem, including the locus coeruleus (LC) (Pacak & Palkovits, 2001). The LC-NE system provides an optimal and adaptable mechanism for offsetting vigilance with focused attention based on the novelty of environmental stimuli (Goddard et al., 2010). The response of norepinephrine to acute and chronic stress involve neuroendocrine and autonomic adaptations (Goddard et al., 2010). According to early literature, the activity of LC-NE neuronal firing transpires in phasic bursts whilst responding to an acute threat signal (Abercrombie & Jacobs, 1987). An unexpected surge in the autonomic and neuroendocrine response can elicit acute panic attacks in humans (Koob, 1999). Prolonged and repeated stress may result in the development of anxiety. The amygdala and the prefrontal connections are vital as neural substrates of fear and anxiety states in animals and humans (Bishop, 2007). Under chronic stress, the NE system activity dysregulation of various brain regions may change a homeostatic stress response into a pathological stress response (Goddard et al., 2010). The hyperactivity of norepinephrine in the CNS can cause symptoms of anxiety by the activation of the corticotropin-releasing factor (Martin, Ressler, Binder, & Nemeroff, 2009). Pharmacological treatments for anxiety disorder patients that affect the NE system result in anxiolytic outcomes (Goddard et al., 2010).

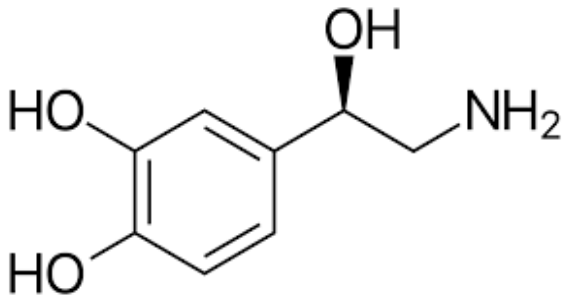

Figure 10: Structure of Norepinephrine

5.6 The function of acetylcholine in anxiety

Acetylcholine is pivotal to the learning and memory processes in the brain. Acetylcholine (Figure 11) levels are regulated by stress in many brain regions. Cognitive impairment is

induced by an increase in cholinergic transmission as a result of the activation of presynaptic (nicotine) and post-synaptic (muscarinic) cholinergic receptors (Dall'Acqua, 2013). The hyperactivity of the brain cholinergic systems can contribute to the pathophysiology of depression and anxiety (Mineur et al., 2013). Clinical and pre-clinical studies have demonstrated that cholinergic receptor blockers can induce anti-depressant-like and anti-anxiety-like responses (Furey & Drevets, 2006). The presynaptic nicotinic receptor facilitates GABAergic neurons which induced anxiety (Anderson & Brunzell, 2012).

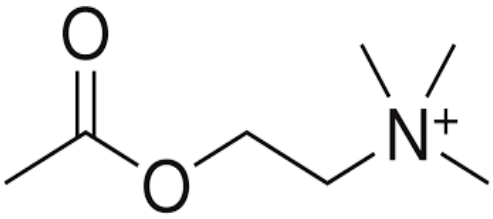

Figure 11: Structure of Acetylcholine

5.7 The implication of neurotransmitters in anxiolytic treatments

GABA agonists, or benzodiazepines, are one of the most recognized and commonly prescribed and anxiolytic treatments (Linsambarth et al., 2017). In the amygdala, the anxiety neuronal circuitry is considered to be comprised of GABAergic interneurons (Marowsky, Yanagawa, Obata, & Vogt, 2005; Palomares-Castillo et al., 2012). Infusions of GABA agonists into the amygdala reduces fear and anxiety in many animal species, whereas GABA antagonist infusions upsurge fear and anxiety (Barbalho, Nunes-de-Souza, & Canto-de-Souza, 2009). This is supported by studies by (Moghaddam, Roohbakhsh, Rostami, Heidary-Davishani, & Zarrindast, 2008) and (Jiménez-Velázquez, López-Muñoz, & Fernández-Guasti, 2010), which demonstrated the anxiolytic effects of microinjections of GABA agonists into the basolateral amygdala (BLA), whilst microinjections of GABA antagonists elicited anxiogenic effects. Anxiolytic drugs classes, such as benzodiazepines, bind to the allosteric sites on the $GABA_A$ receptors which permits the precise regulation of the neuron inhibition (Mody & Pearce, 2004; Nuss, 2015). The inhibition of GABAergic neurons is important for the maintaining the equilibrium between neuronal excitation and inhibition (Mody & Pearce, 2004). During anxiety states, the GABAergic neuronal inhibition is downregulated (Nuss, 2015). The mechanism of this downregulated may be explained by fluctuations in the levels of the endogenous modulators of the allosteric sites and alterations in the subunit structure of the $GABA_A$ receptors (Nuss, 2015).

Mononamine neurotransmitters mutually interact with each other in the central nervous system (Quesseveur, M Gardier, & P Guiard, 2013). NE is essential in the regulation of the release of 5-HT (Liu et al., 2018). Studies suggest that the 5-HT_{2A} receptors can enhance the release of NE under the anxiolytic SSRIs treatment (Sullivan et al., 2005).The serotonin 5-HT_{1A} receptor (5-HT_{1a}R) is a metabotropic G protein-coupled receptor which is involved in the pathogenesis of anxiety and depression (Rupprecht et al., 2009). The anxiolytic effect of SSRI's, a first-line clinical treatment for anxiety, is reliant on the activation of 5-HT_{1a}R (Rupprecht et al., 2009; Santarelli et al., 2003). The acute anxiolytic actions of acute CBD administration are proposed to be mediated by 5-HT_{1A} receptors (Alline C Campos et al., 2017). Acute and chronic CBD administration into brain regions such as the basal ganglia, the BNST, prelimbic PFC and the dorsal raphe nucleus appear to mediate CBD effects via the 5-HT_{1A} receptors (Espejo-Porras, Fernández-Ruiz, Pertwee, Mechoulam, & García, 2013; Fogaça, Reis, Campos, & Guimaraes, 2014; Gomes, Resstel, & Guimarães, 2011; Katsidoni, Anagnostou, & Panagis, 2013). This was supported by the 5-HT_{1A} receptor antagonist, WAY-100635, preventing the anxiolytic effects of CBD administration (Alline C Campos et al., 2013; Zanelati, Biojone, Moreira, Guimaraes, & Joca, 2010). The mechanism of activation of 5-HT_{1A} receptor by CBD is unclear. Literature suggests that the mechanism may involve a rise in 5-HT release and/or reuptake inhibition, or an indirect formation of heterodimers consisting of 5-HT_{1A} and other receptors, such as CB1 (Linge et al., 2016; Mato et al., 2010).

6. Gene expression and anxiety

Brain-derived neurotrophic factor (BDNF) is a neurotrophin that is involved in many affective disorders including depression and anxiety (Bergami, Berninger, & Canossa, 2009; Martinowich, Manji, & Lu, 2007). BDNF has a neuroprotective effect, supports the proliferation, differentiation, maturation and survival of neurons in the nervous system, as well as stimulates and regulates neurogenesis (Binder & Scharfman, 2004; Huang & Reichardt, 2001). BDNF levels are reduced in neurodegenerative diseases including multiple sclerosis, Parkinson's diseases, depression and anxiety (Bathina & Das, 2015; Scalzo, Kümmer, Bretas, Cardoso, & Teixeira, 2010). BDNF enhances neurogenesis via the tyrosine kinase B (TrkB) receptor, which activates a variety of downstream signalling pathways (Palomares-Castillo et al., 2012). The serum and plasma BDNF levels are reduced in patients with an anxiety disorder compared to patients without an anxiety disorder (Dell'Osso, Buoli, Baldwin, & Altamura, 2010; dos Santos et al., 2011; Maina et al., 2010; Ströhle et al., 2010; Wang, Mathews, Li, Lin, & Xiao, 2011). The expression of BDNF messenger RNA (mRNA), in the hippocampus and

prefrontal cortex, can be considerably increased by multiple classes of anti-depressant and anxiolytic treatments (Duman & Monteggia, 2006). CBD induces anti-depressant-like effects associated with elevated BDNF levels in the medial prefrontal cortex (mPFC) and hippocampus (Sales et al., 2019). The effects may be related to rapid changes in synaptic plasticity in the mPFC through activation of the BDNF-TrKB signalling pathway (Sales et al., 2019).

Cyclic AMP response element binding protein activation (CREB) is one of the long-term transcriptional factors that is considered to mediate the effects of anti-depressants on BDNF expression (Malberg & Blendy, 2005). CREB is well-known for its implication in memory and learning (Carlezon Jr, Duman, & Nestler, 2005; Herdegen et al., 1997). Hippocampal CREB activity is enhanced due to a variety of anti-depressant treatments (Carlezon Jr et al., 2005; Gourley et al., 2008; Thome et al., 2000). Anxiety-like behaviours can be modulated by changing the function or expression of CREB as studies demonstrated that CREB knock-out mice displayed an increase in anxiety-like behaviours (Valverde et al., 2004; Vogt et al., 2014). The mechanism of the modulation of anxiety-like behaviours are poorly understood. A report by demonstrated that anxiety-like behaviours can be modulated by the 5-HT_{1A} activation via CREB in the hippocampus which promotes neurogenesis and synaptogenesis (Zhang et al., 2016). A disruption in CREB activity stopped the anxiolytic effect of the 5-HT_{1a}R agonist, whereas an increase in CREB activity reversed the anxiogenic effect of the 5-HT_{1a}R antagonist (Zhang et al., 2016). There is inadequate understanding of the effects of CBD administration on the expression of BDNF and CREB.

7. Liquid Chromatography- Mass Spectrometry

Liquid chromatography – tandem mass spectrometry (LC-MS/MS) is a specific and sensitive technique used to detect and identify organic and inorganic compounds (Bianchi et al., 2018). It is widely used in analytical laboratories as it can be used to provide molecular weight and structural information of targeted analytes, and simultaneously determine endogenous compounds (Kind & Fiehn, 2010). Mass spectrometers work by converting the analyte molecules into an ionized state (Pitt, 2009). Electrospray ionization (ESI) is a soft ionization technique that produces ions suitable for mass analysis of biological molecules (Bianchi et al., 2018). Heated electrospray ionization (H-ESI) is a modification of ESI whereby the ionization efficiency of analytes is augmented by the increasing the heating vapourizer temperature to quickly evaporate droplets (Kourtchev et al., 2020). The liquid samples are pumped through a metal capillary maintained at 3 to 5 kV and nebulized at the tip of the capillary to form a fine

spray of charged droplets (Pitt, 2009). The triple quadrapole mass spectrometer is comprised of a collision cell between two quadrapole mass analysers (Figure 12) (Pitt, 2009). Each mass quadrapole analyser contains a set of four parallel metal rods (Pitt, 2009). The transmission of a narrow band of m/z along the axis of the rods is a result of constant and varying voltages (Pitt, 2009). Collision induced dissociation (CID) is a process where ions can be induced to undergo fragmentation by collision with an inert gas (Pitt, 2009). There are various operational modes of the triple quadrapole mass spectrometers such as product scan, precursor scan and multiple reaction monitoring (MRM) (Pitt, 2009). The triple quadrapole analysers are widely used in LC-MS applications due to the ease of scanning and the good quality quantitative data obtained (Bianchi et al., 2018).

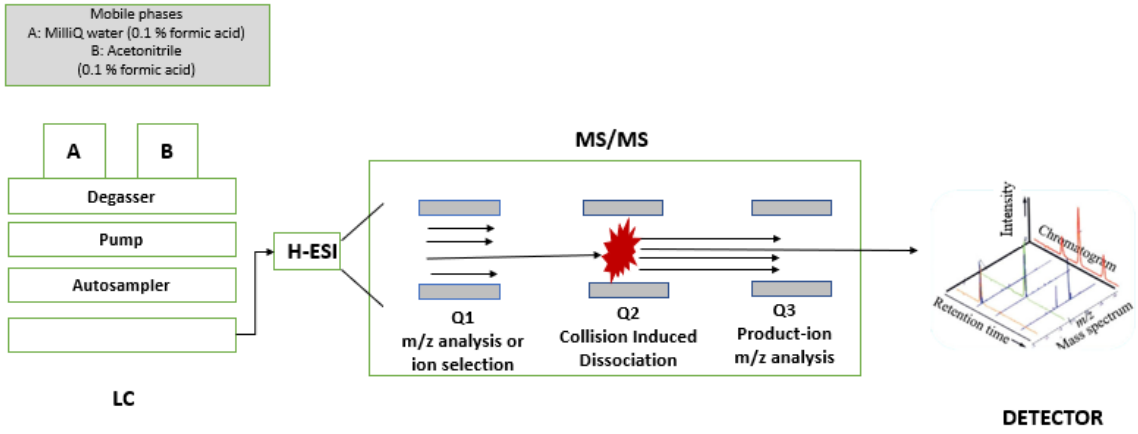

Figure 12: Schematic representation of the LC-MS/MS process (adapted from (Mertens, 2016)

Key: LC: Liquid Chromatography; MS/MS: Mass Spectrometer/Mass Spectrometer; Q1: Quadrapole 1; Q2: Collision Cell; Q3: Quadrapole 3.

8. Polymerase Chain Reaction (PCR)

Polymerase chain reaction is a technique used to amplify specific DNA fragments a billionfold (Hindson et al., 2013; Pierce, 2012). PCR is based on replication catalysed DNA polymerase to synthesis a new complementary strand of DNA to the original template (Y. Kim, Flynn, Donoff, Wong, & Todd, 2002). PCR uses a temperature modulation process consisting of heating and cooling cycles, called thermal cycling. PCR usually consists of three steps: denaturation, annealing and elongation, which is performed in repeated cycles as shown in Figure 13 (Chen et al., 2019). During denaturation, the DNA solution is heated at 94°C - 96°C for 1 – 2 minutes to separate double-stranded DNA complexes and produce single strands (Chen et al., 2019). The strands are then rapidly cooled to between 30°C - 65°C to allow single-

stranded primers to anneal to their complementary sequences (Chen et al., 2019). In the final step of elongation, the solution is heated to 72°C and DNA polymerase synthesizes new DNA strands by allowing the primers to extend into the new complementary strands (Chen et al., 2019). After one cycle of PCR, there are two double-stranded DNA molecules produced for each original molecule of target DNA, doubling the amount of target DNA in each cycle (Pierce, 2012). As PCR progresses, the cycles are repeated, and the amount of target DNA are exponentially increased (Pierce, 2012).

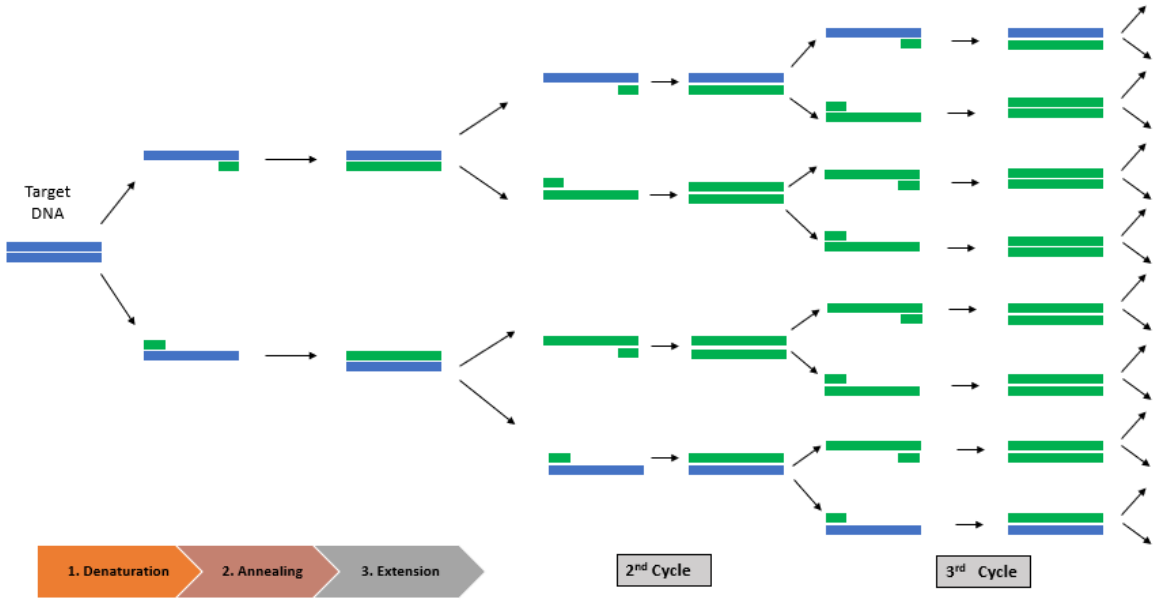

Figure 13: The three steps of PCR and exponential amplification of the target DNA (*adapted from (Pierce, 2012)*)
Key: Blue bars: target DNA; Green bars: New DNA; Green blocks: Primers

9. Aims and objectives:

Aim: to investigate the potential use of CBD in the treatment of anxiety by assessing NT levels and molecular changes in BDNF and CREB signalling in a healthy rodent model.

Objectives:

1. To develop an LC-MS/MS method for the simultaneous detection of dopamine, GABA, serotonin, norepinephrine and glutamate.
2. To determine the pharmacokinetic parameters (pK) of CBD in a healthy rodent brain.
3. To investigate the neurotransmitter changes in response to CBD administration.

4. To determine the expression levels of BDNF and CREB in response to CBD administration.

10. Thesis outline

Chapter 2 presents the results of the research study.

Chapter 3 presents the general discussion and conclusion.

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CHAPTER 2

Evaluation of the use of cannabidiol in the treatment of anxiety related disorders by assessing changes in neurotransmitter levels and expression of CREB/BDNF in the rodent brain

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Evaluation of the use of cannabidiol in the treatment of anxiety related disorders by assessing changes in neurotransmitter levels and expression of CREB/BDNF in the rodent brain

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Abstract

Cannabidiol (CBD), a non-psychoactive component of the *Cannabis sativa* plant, has gained pharmacological interest due to its therapeutic potential in the treatment of neurological disorders. Anxiety-related disorders are some of the most prevalent mental health disorders. However, the current pharmacological anxiety treatments display shortcomings in drug efficacy, side effects and therapeutic action. Studies have reported the use of CBD in the management of anxiety-related disorders however its exact neurological action remains unknown, making it crucial to understand the effect of CBD on neurotransmitter levels and the expression of CREB/BDNF expression in the brain. Twenty-seven male Sprague-Dawley rats received an acute treatment of 10 mg/kg body weight of CBD via intraperitoneal injection. The pharmacokinetics of CBD, neurotransmitter concentrations, and CREB/BDNF expression in the brain were measured in the brain over 24 hours via LC-MS and qPCR respectively. CBD reached a C_{max} of 152.801 ± 1.541 ng/ml at 1-hour post drug administration. The results showed that GABA and glutamate were significantly decreased between 4 hours and 24 hours in response to CBD administration with norepinephrine diminishing over the 24 hours period. The concentration of serotonin peaked at 1-hour post drug administration, whereas dopamine levels increased at 30 minutes and 2 hours. CBD significantly increased BDNF at 2 hours following administration, and CREB expression was upregulated when compared to the control over the 24 hours. This study contributes to the understanding of the pharmacodynamic effects of CBD in the brain by demonstrating its effects on target neurotransmitters and important expression factors.

Significance statement

The legalization of cannabis products in many regions of the world has seen it receive interest in the treatment of mental health disease, neurodegenerative disorders and inflammatory diseases. Despite showing clinical efficacy in the management of these disorders, the exact mechanisms remain to be fully elucidated. Therefore assessing the pharmacodynamic effects of CBD in the brain, by evaluating neurotransmitter changes, and its effect on the expression of CREB/BDNF will contribute to increasing the knowledge of the neurological action of CBD and can assist clinicians in better understanding its effects allowing them to make more informed choices in the management of patients suffering from neurological disorders.

1. Introduction

Anxiety disorders are the most prevailing neuropsychiatric disorders and are concomitant with a high burden of disease and substantial health care expenses (Bandelow & Michaelis, 2015). Patients with anxiety experience an immense burden on societal resources which contribute to the pathogenesis of depression and the development of physical illnesses (Kariuki-Nyuthe & Stein, 2015). Anxiety-related disorders are associated with a decreased sense of well-being, elevated rates of unemployment and relationship breakdown, and increased suicide risk (Stein & Sareen, 2015). Anxiety can be conceptualized by the inappropriate emotional responses to potential threats or danger and sub-divided based on the type of anxious stimuli (A Rabinak & Phan, 2014; Sartori & Singewald, 2019). Anxiety disorders include Generalized Anxiety Disorder (GAD), Panic Disorder (PD), Social Anxiety Disorder (SAD), and specific phobias (Kogan et al., 2016; Reed et al., 2019). Social anxiety disorder is the most prevalent anxiety disorders where people display fear and avoidance of the scrutiny of others (Stein & Stein, 2008). The onset of social anxiety disorder by age 11 is 50 % prevalence and reaches up to 80 % prevalence in 20-year olds (Stein & Stein, 2008). Generalized Anxiety Disorder is one of the most common and most impairing anxiety conditions, it is defined by an insistent, multi-focal chronic worry (Stein & Sareen, 2015). Neurological disorders, such as depression and anxiety, are a result of impairments in the modulation of neural circuits in many limbic brain regions including the amygdala, bed nucleus of the stria terminalis, hippocampus and thalamus (Nuss, 2015).

Dysfunction in various brain areas viz. the amygdala, hippocampus and the prefrontal cortex are responsible for anxiety (Bremner, 2002). The etiology of anxiety disorders also involve dysfunction of the mono-aminergic neurotransmitter systems such as the norepinephrine (NE), dopamine (DA) and serotonin (5-HT) systems and their receptors (Liu, Zhao, & Guo, 2018). These neurotransmitter systems have a mutual interaction in the central nervous system, modulating human emotion, anxiety and depression (Quesseveur, M Gardier, & P Guiard, 2013). The serotoninergic system consists of differentially expressed multiple 5-HT receptors which produce both anxiolytic and anxiogenic effects via differential neurotransmission (Albert, Vahid-Ansari, & Luckhart, 2014). Anxiety may be caused by the suppression of the postsynaptic 5-HT receptors, upregulation of 5-HT1A receptors or a decreased 5-HT neurotransmission (Albert et al., 2014). The 5-HT1A receptor plays an important role in mediating the effects of 5-HT in the etiology and treatment of anxiety (Akimova, Lanzenberger, & Kasper, 2009). The hyperactivity of norepinephrine in the central nervous

system is proposed to cause anxiety symptoms (Liu et al., 2018). During stress conditions, the activation of the NE energy pathway by corticotrophin-releasing factors, releases NE, inducing anxiety symptoms (Liu et al., 2018). Studies have also demonstrated DAergic neurotransmission in the processing of anxiety (Vicario, Rafal, Martino, & Avenanti, 2017). The emergence of fear and anxiety occurs due to a deficiency in DA receptor function (Liu et al., 2018). The DA receptors are inhibited in the prefrontal cortex and the amygdala which induces the hyperexcitability of the amygdala (Liu et al., 2018). Patients with anxiety disorders are reported to have decreased DA transporter density and D2 receptor binding in the striatum (Shin & Liberzon, 2010).

A combination of pharmacotherapy and psychotherapy is used to treat anxiety disorders (Bandelow, 2017). Traditional pharmacotherapeutic approaches target specific neurochemical imbalances (Sartori & Singewald, 2019). Monoaminergic neurotransmitter systems such as the endocannabinoid, GABA-ergic and glutamatergic systems, as well as their receptors are responsible for the regulation of anxiety and fear ((Bandelow, Michaelis, & Wedekind, 2017); Maron et al., 2018). Recent pharmacological anxiolytic treatments include selective serotonin (5-HT) reuptake inhibitors (SSRIs), benzodiazepines, selective noradrenalin reuptake inhibitors (SNRIs) such monoamine oxidase inhibitors, tricyclic antidepressant drugs, and partial 5-hydroxytryptamines (5-HT) 1A receptor agonists (Bandelow et al., 2017). SSRIs are reported to be the first-line pharmacotherapeutics due to their favourable benefit/risk ratio (Bandelow et al., 2017). Acute administration of SSRIs stimulates the presynaptic 5-HT receptors, which subsequently yield anxiogenic-like effects (Liu et al., 2018) The current pharmacological anxiolytic treatments are suboptimal with regards to efficiency and tolerability of the drug, abiding adverse side effects, expensive drug development and a reduced rate of successful drug discovery (Guina & Merrill, 2018; Sartori & Singewald, 2019). These shortcomings highlight the need for improved and novel pharmacological treatments for anxiety disorders (Sartori & Singewald, 2019).

Cannabidiol (CBD), a non-psychoactive phytocannabinoid, is a constituent of the *Cannabis sativa* plant, which has become increasingly popular as a proposed treatment for a spectrum of neuropsychiatric disorders (Blessing, Steenkamp, Manzanares, & Marmar, 2015). Existing preclinical evidence displays the extensive range of action of CBD: anxiolytic (Campos, Moreira, Gomes, Del Bel, & Guimaraes, 2012; Izzo, Borrelli, Capasso, Di Marzo, & Mechoulam, 2009) reduction of conditioned fear expression (Resstel, Joca, Moreira, Corrêa, & Guimarães, 2006), augmentation of fear extinction (Bitencourt, Pamplona, & Takahashi,

2008), the prevention of the abiding anxiogenic effects from stress (Campos, Ferreira, & Guimarães, 2012) and the reconsolidation blockade of adverse memories (Stern, Gazarini, Takahashi, Guimarães, & Bertoglio, 2012). The anxiolytic action of cannabidiol is mediated by the Cannabinoid-1-receptors (CB_1R), 5-HT1A receptors and the TRPV1 receptors (Blessing et al., 2015). The complex activation of CB_1R produces anxiolytic effects relevant to multiple anxiety disorder symptoms (McLaughlin, Hill, & Gorzalka, 2014; Ruehle, Rey, Remmers, & Lutz, 2012). CB_1R activation in different brain loci may augment or reduce fear expression, enhance fear extinction and prevent the reconsolidation of fear (Llorente-Berzal et al., 2015). CBD functions as an indirect agonist of CB_1R by either decreasing its constitutional activity, or increasing the endocannabinoid, anandamide, through the inhibition of fatty acid amide hydrolase (FAAH) (McPartland, Duncan, Di Marzo, & Pertwee, 2015). The 5-HT1A receptor is an anxiolytic target which prevents the negative effects of stress and increases fear extinction (Saito et al., 2013; Zhou et al., 2014). The anxiolytic effects of 5-HT1A have complex mechanisms that vary between brain region and pre-versus postsynaptic locus (Celada, Bortolozzi, & Artigas, 2013). In vitro studies suggest that CBD acts as a direct 5-HT1AR agonist, whilst in vivo studies are more consistent with CBD acting as an allosteric modulator, or facilitator of 5-HT1A signalling (Rock et al., 2012; Russo, Burnett, Hall, & Parker, 2005). Brain derived neurotrophic factor (BDNF) is a neurotrophin implicated in anxiety and depression. BDNF exhibits a neuroprotective effect and has decreased levels in patients with anxiety. Studies have shown that anti-depressant effects induced by CBD are associated with an upregulation in BDNF levels. The nuclear transcription factor, cyclic AMP response element binding protein (CREB) is crucial in memory and learning (Bourtchuladze et al., 1994; Josselyn et al., 2001), drug addiction (Pliakas et al., 2001), and anti-depressant effects implicated in mood disorders (Gourley et al., 2008; Nestler et al., 2002) The expression and function of CREB in the hippocampus, amygdala, hypothalamus and thalamus is increased as a result of anti-depressant and anti-anxiety therapeutics such as SSRIs (Carlezon Jr, Duman, & Nestler, 2005; Pandey, Zhang, Roy, & Xu, 2005; T. L. Wallace, Stellitano, Neve, & Duman, 2004). SSRIs induces anxiolytic effects via the 5-HT1A receptors, which elevates CREB expression in the hippocampus (Zhang et al., 2010) Despite the demonstrated clinical efficacy and increasing knowledge surrounding the use of CBD as an anxiolytic treatment, the effects of CBD on neurotransmitter systems contributing to the treatment of anxiety, BDNF and CREB expression remain unknown.

Therefore, this study aimed to evaluate the pharmacodynamic effects of acutely administered cannabidiol (10 mg/kg body weight) by assessing brain neurotransmitter levels and the expression of BDNF and CREB in the healthy rodent brain in order to better understand its role in the treatment and management of anxiety-related disorders.

2. Materials and Methods

2.1 Experimental subjects

Twenty-seven male Sprague-Dawley rats (110 – 120 g) were sourced from the University of KwaZulu-Natal Biomedical Resource Unit (Westville campus). The animals were collected two weeks prior to the experimental procedures to allow for acclimatization. The animals were housed in well-ventilated cages, located in temperature and humidity-controlled housing units, with a 12-hour/ 12-hour light-dark cycle. Environmental enrichment, in the form of shredded paper and plastic tunnels, were added to the cage. The animals were provided with commercially pelleted feed and clean water *ad libtium.*

2.2 Ethical approval

The experimental protocols used in this study were approved by the University of KwaZulu-Natal institutional Animal Research Ethics Committee (approval reference: AREC/010/019M). This was in accordance with the regulations of the South African National Legislations for animal husbandry, welfare and experimentation using laboratory animals.

2.3 Experimental design and procedure

2.3.1. Drug treatment and animal experiments

The animals were treated with 10 mg/kg body weight of pure cannabidiol (CBD) was extracted from the *Cannabis sativa* plant and its identity confirmed via LC-MS and NMR. The CBD was dissolved in a 1:9 mixture of hexane: ultrapure water (Milli-Q water). A volume of 1 ml/ 250g body weight was administered via intraperitoneal (i.p.) injection to the animals (Deiana et al., 2012; Long et al., 2010; Taffe, Creehan, & Vandewater, 2015; Wiley, O'Connell, Tokarz, & Wright, 2007). The animals were sacrificed at different time points (Table 1) to determine the pharmacokinetics of CBD in the brain, as well as associated neurotransmitter changes at different time points. These time intervals were determined based on previous pharmacokinetic studies involving CBD following various lengths of exposure (Deiana et al., 2012).

Table 1: Acute exposure group and the number of animals to be terminated at the time points of euthanasia

	Time post CBD administration (hours)									Total number of animals
	0	0.25	0.5	1	2	4	6	8	24	
N	3	3	3	3	3	3	3	3	3	27

Experimental animals were euthanized by decapitation, following which the brain tissues were surgically removed, cooled on ice for 15 minutes, and subsequently frozen using liquid nitrogen vapour. Blood was collected into heparinized tubes and centrifuged at 10 000 rpm for 7 minutes to obtain the blood plasma. The brain tissues and plasma samples were stored at -80 °C until analysis.

2.3.2. Liquid chromatography – Mass Spectrometry/ Mass Spectrometry (LC-MS/MS) analysis

2.3.2.1 Neurotransmitter analysis

LC-MS/MS analysis was conducted using a Thermo Scientific Quantis Triple Quadrupole Mass Spectrometer (Thermo Scientific, Massachusetts, USA) coupled to a Thermo Scientific Vanquish Ultra High-Performance Liquid Chromatography (UHPLC) System (Thermo Scientific, Massachusetts, USA). The system was controlled using the Thermo Scientific SII Xcalibur 1.3 (version: 3.0.20389) and Thermo Trace finder General (version: 4.1 SP5) software packages. Liquid chromatographic separation was achieved using the Poroshell 120 EC-C18 (50 mm x 4.6 mm, 2.7 µm) column (Agilent technologies) to assay serotonin (5-HT), GABA, norepinephrine (NE), glutamate (GLU), and dopamine (DA). The column compartment temperature was maintained at 25 °C. Mobile phase A was ultrapure water (0.1 % Formic Acid) and mobile phase B was methanol (0.1 % Formic acid). A flow rate of 0.800 ml/min was utilized, with a sample injection volume of 20 µl. The Heated Electrospray Ionizer (H-ESI) mass spectrometer was operated in the positive mode. The source parameter MS settings included: Spray voltage, 4809 V; Sheath gas, 50 Arbitrary units; Auxiliary gas, 15 Arbitrary units; Sweep gas, 1.2 Arbitrary units; vapourizer temperature, 400 °C; and ion transfer tube temperature, 325 °C. The MS scan parameters were as following: cycle time, 0.6 seconds; Q1 and Q3 resolution, 0.7; collision induced dissociation (CID) gas pressure, 1.5 mTorr; and chromatographic peak width, 12 seconds. The mass transitions monitored via selected reaction

monitoring (SRM) and ion optic parameters are defined in Table 2. The gradient profile used for the elution of neurotransmitters is -1.00 min(pre-injection equilibration): 15 % B; 0.00 mins: 15 % B; 1.00 min: 70 % B; 1.10 mins: 95 % B; 5.50 mins: 95 % B; 5.60 mins:15% B; 5.60 – 8 mins: 15 % B; with a total run time of 8 mins.

Table 2:Selected Reaction Monitoring (SRM) and Ion optic parameters of neurotransmitters and CBD

Compound	Precursor (m/z) $[M+H]^+$	Product (m/z)	Quantifier/ Qualifier Ions (m/z)	Collison Energy (V)	RF Lens Voltage (V)	Dwell Time (msec)
Dopamine	154.08	137	**Quantifier**	14.55	78	9.473
		90.946	Qualifier	27.51	78	9.473
		118.929	Qualifier	15	78	9.473
GABA	104.05	87	**Quantifier**	13.41	30	9.473
		46	Qualifier	55	30	9.473
		85.875	Qualifier	15	30	9.473
Glutamate	148.05	84.071	**Quantifier**	18.98	30	9.473
		131	Qualifier	23.72	30	9.473
Norepinephrine	170.088	152.054	**Quantifier**	10.23	79	9.473
		107.018	Qualifier	23.99	79	9.473
		135.018	Qualifier	14.63	79	9.473
Serotonin	177.000	159.982	**Quantifier**	10.23	30	9.473
		114.929	Qualifier	46.7	30	9.473
		132.929	Qualifier	55	30	9.473
D4-Serotonin	181.175	164.02	**Quantifier**	10.23	192	9.473
		78.557	Qualifier	34.57	192	9.473
		118.042	Qualifier	26.49	192	9.473
Cannabidiol (CBD)	315.125	193.083	**Quantifier**	32.56	95	13.115
		259.137	Qualifier	27.17	95	13.115
		135.173	Qualifier	29.64	95	13.115
		235.167	Qualifier	28.16	95	13.115
		123.071	Qualifier	48.37	95	13.115

2.3.2.2. Cannabidiol analysis

The liquid chromatographic separation of CBD was achieved using the Biphenyl Pinnacle DB column (50 mm x 2.1 mm, 5 µm) (Restek, USA). The column compartment temperature, mobile phases and injection volume, and MS source and scan parameters are according to section 2.3.2.1. The gradient profile for the elution of CBD was -1.00 min: 50 % B; 0.00 mins: 50 % B; 1.50-5.00 mins: 98 % B, 5.10 mins; 50 % B, 5.10-8 mins: 50 % B; with a flow rate of 0.300 ml/min and a total run time of 8 mins.

2.3.3. Sample preparation of brain tissue

Frozen brain samples were swiftly dissected on an ice bath, into hemispheres, using surgical blades. One hemisphere of the brain was subsequently homogenized using the OMNI tissue homogenizer (OMNI international, Kennesaw Georgia, USA). The brain tissue homogenates were diluted with a 1:1 v/v of MilliQ water. 100 µl of brain tissue homogenate was added to 850 µl methanol spiked with 50 µl of internal standard (10 µg/ml). The solutions were centrifuged at 10 000 rpm for 10 minutes at 4°C. The supernatants were carefully transferred to the conditioned Solid Phase Extraction (SPE) Discovery® DSC18-100mg cartridges (Merck, Gauteng, South Africa) for extraction.

2.3.4. Solid Phase Extraction

A SPE diaphragm vacuum pump manifold with Discovery® DSC18-100mg 1 ml cartridge tubes (Merck, Gauteng, South Africa) were utilized. Prior to the elution of samples, the C18 cartridges were conditioned with 1000 µl of 100% methanol under vacuum pressure. The supernatants were eluted at a rate of 1 ml/minute under vacuum pressure and the flow through collected for LC-MS/MS analysis. 400 µl of the collected eluent samples were dried in a nitrogen evaporator, ZipVap (Glass-Col, Indiana, USA) at 55°C for 15 – 17 minutes at a constant flow of nitrogen gas at 2 bars. The samples were then reconstituted in 400 µl of ultrapure water and vortexed for 30 seconds.

2.3.5. Preparation of neurotransmitter and internal standards stock solutions

A 1 mg/ml multi-mix stock solution of neurotransmitters (DA, GABA, GLU, NE and 5-HT) and their respective internal standards was prepared by accurately weighing 1 mg of each compound and dissolving it in 100 µl water: 900 µl methanol. The multi mix solution of neurotransmitters and internal standards was then diluted with methanol to 10 µg/ml and 1 µg/ml working solutions

2.3.6. Preparation of calibration standards for the calibration curve

The samples for the calibration curve were prepared by spiking the blank solution (methanol) with the appropriate volume of the multi-mix neurotransmitter stock solution. Yielding final concentrations of 50, 150, 250, 500, 750, 1000 ng/ml which were used to construct a calibration curve. The neurotransmitter multi-mix was spiked with internal standard at a concentration of 500 ng/ml in each calibration sample. Cannabidiol (CBD) calibration standards were prepared in solution at final yielding concentrations of 0.98, 1.95, 3.9, 7.8, 15.625, 31.25, 62.5, 125, 250

and 500 ng/ml. The CBD samples in solution were prepared by spiking methanol with the appropriate volume of CBD.

2.4. Gene expression analysis

2.4.1. RNA extraction

Brain tissue homogenates were centrifuged at 10 000 x *g* at 4°C for 10 minutes. The supernatants were transferred into autoclaved micro-centrifuge tubes containing 500 µl of Qiazol reagent (Qiagen, Hilden, Germany) and stored at -80°C until extraction. RNA extractions were performed by adding 100 µl of chloroform to the tissues stored in Qiazol. The solutions were then vortexed for 15 seconds and incubated at room temperature for 2 – 3 minutes. The tubes containing the organic phase in Qiazol and chloroform were centrifuged at 12 000 x *g* for 12 minutes at 4 °C, and 250 µl of the aqueous phase, containing crude RNA, was transferred to a micro-centrifuge tube. 250 µl of isopropanol was added to each aqueous sample and mixed by flicking. The samples were incubated overnight at -80 °C. The samples with isopropanol were centrifuged at 12 000 x *g* for 20 minutes at 4 °C. The supernatant was discarded and 500 µl of 75% cold ethanol was added to release the pellet. The pellet in ethanol was centrifuged (7 400 x*g*, 15 minutes, 4°C). The ethanol was discarded, and the pellet allowed to air dry. The pellets were resuspended in 15 µl of nuclease-free water and incubated at room temperature for 2-3 minutes. The samples were stored at -80 °C and the Nanodrop 2000 spectrophotometer (Thermo-Fisher Scientific, Massachusetts, USA) was used to quantify RNA, which was standardized to 1000 ng/µl and the A260/ A280 absorbance ratio was used to assess RNA purity.

2.4.2. cDNA synthesis

The RNA templates were reverse transcribed into cDNA using Maxima H Minus First Strand cDNA Synthesis Kit (Thermo-Fisher Scientific, California, USA). The master mix (excluding template RNA) consisted of 0,25 µl Oligo $(dT)_{18}$ primer (25 p mol), 1 µl 10 mM dNTP mix and 12,75 µl nuclease-free water. 1000 ng/µl template RNA was added and gently mixed and incubated for 5 minutes at 65 °C. The samples were stored on ice. The second master mix consisted of 4 µl of 5x RT Buffer and 1 µl Maxima H Minus enzyme mix. This master mix was added to all samples and incubated in the Applied Biosystems Viia real time PCR system thermo-cycler (Thermo-Fisher Scientific, USA) (10 minutes at 25°C, then 15 minutes at 50 °C). The reaction was terminated by incubating at 85 °C for 5 minutes. 60 µl of nuclease-free water was added to the samples and stored (-80 °C) until qPCR processing.

2.4.3. qPCR analysis

The primers used for qPCR analysis were BDNF: Sense: 5'-GAATTCATGACCATCCTTTTCCTTACTATG-3', Anti-sense: 5'-AAGCTTTCTTCCCCTTTTAATGGTCAG-3'; and CREB Sense: 5'-CCAAACTAGCAGTGGGCAGTATATT-3', Anti-sense: 5'-GGTACCATTGTTAGCCAGCTGTATT-3' were used at a final concentration of 25 µM. The PowerUp™ SYBR™ Green Master Mix (Thermo-Fisher Scientific, California, USA) contained 5 µl SYBR Green, 2 µl nuclease-free water, 1 µl sense primer (25 µM), and 1 µl anti-sense primer (25 µM). The master mix was added to the well, along with 1 µl of 1000 ng cDNA. The plate was centrifuged at 1000 *xg* at 24 °C for 1 minute. The mRNA amplification of BDNF and CREB were performed on the Applied Biosystems Viia 7 Real-Time PCR system (Thermo-Fisher Scientific, California, USA). The thermal cycling profile for PCR for BDNF and CREB was as follows: initial denaturation (1 cycle) at 95 °C for 8 minutes, PCR (40 cycles) consisting of denaturation at 95 °C for 15 seconds, annealing at 60 °C for 40 seconds, and extension at 72 °C for 30 seconds . The samples were run as triplicates. The housekeeping gene GAPDH (Sense: 5'-GGCACAGTCAAGGCTGAGAATG-3',Anti-Sense: 5'–ATGGTGGTGAAGACGCCAGTA-3') was used to normalized the variances in gene expressions and the data was presented as fold change (2^CCT) relative to the control (Livak & Schmittgen, 2001).

2.5. Materials

The neurotransmitter standards were purchased from Sigma-Aldrich (St. Louis, USA): (-)-Norepinephrine, Serotonin, L-Glutamic acid monosodium salt monohydrate, γ-Aminobutyric acid (GABA), and Dopamine hydrochloride (Steinheim, Germany). The internal neurotransmitter standard Serotonin D_4 hydrochloride was obtained from ClearSynth® Research Chemicals Inc (Ontario, Canada). Hexane was obtained from Merck (Darmstadt, Germany) and formic acid (Merck, Gauteng, South Africa). LC-MS grade Methanol was purchased from Honeywell (Steinheim, Germany). Milli-Q water was purified using the Milli-Q® water purifying system (Merck Millipore, Burlington, MA).

2.6 Statistical Analysis

Statistical analysis was performed using GraphPad Prism v8.4.3 (GraphPad Software, San Diego, California USA, www.graphpad.com). GraphPad Prism Software was used to analyse data using the unpaired multiple t-test with statistical significance determined using the Holm-

Sidak t-test method (alpha = 0.05). Results were expressed as mean ± standard deviation (SD). Level of significance (p) was established at a $p < 0.05$ The pharmacokinetic (pK) parameters were calculated using Stata/IC 15.0 (StataCorp LLC, Texas, USA, www.stata.com).

3. Results

Following i.p. administration of 10 mg/kg b.w. CBD to Sprague-Dawley rats, the concentration of CBD, and neurotransmitters (DA, 5-HT, NE, GLU and GABA) were measured using LC-MS/MS analysis. The results of the drug concentration, neurotransmitter levels and molecular changes in the brain were analysed using unpaired multiple t-tests to determine the statistical significance between the time intervals post administration compared to the control.

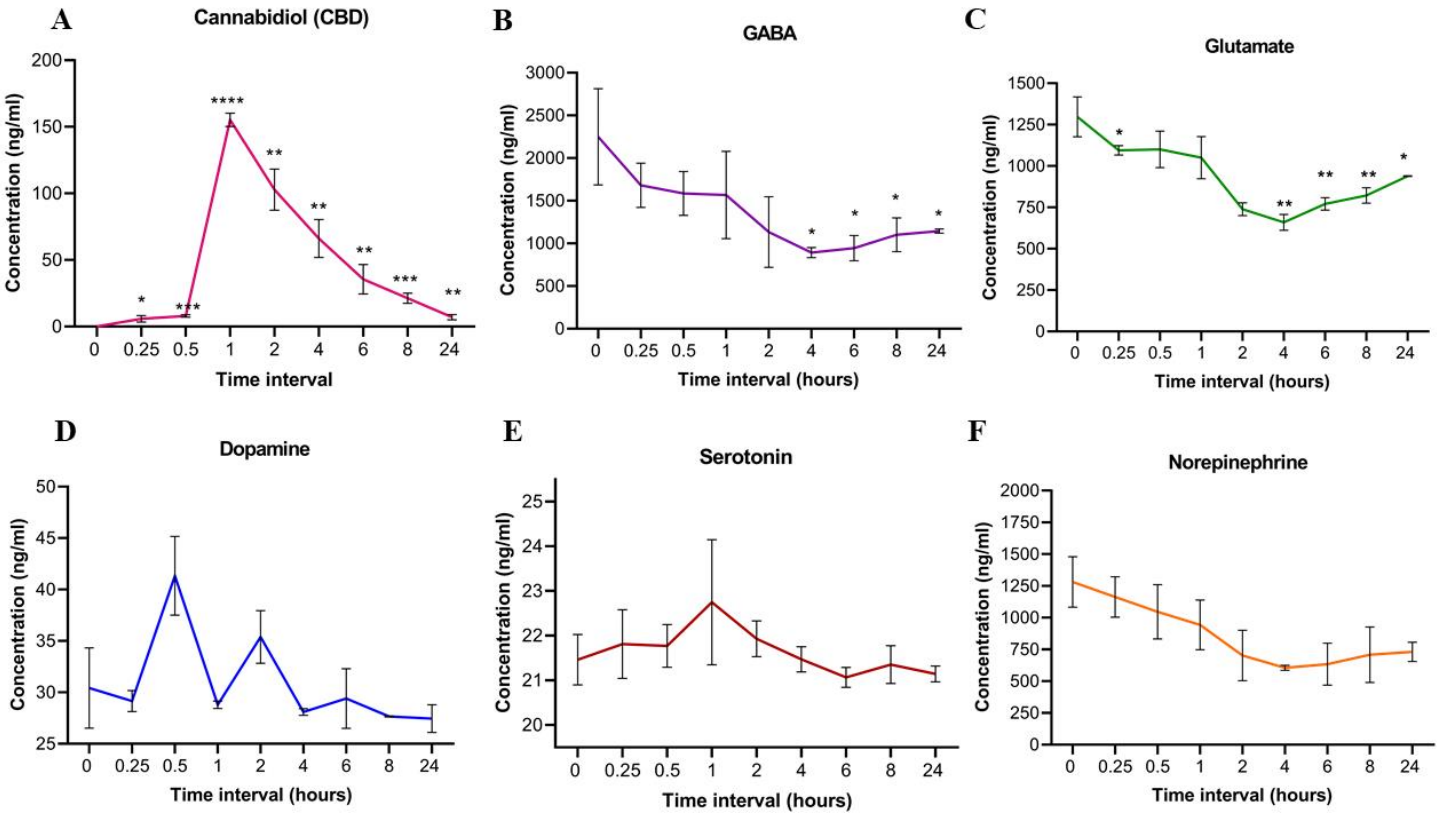

Figure 14: Mean brain concentration-time profile of cannabidiol and neurotransmitters following i.p. administration of CBD

*(A) the concentration-time curve of CBD over time following i.p. administration (B) GABA brain concentrations following acute i.p. CBD administration. (C) Glutamate brain concentrations over 24 hours post i.p. CBD administration (D) DA brain concentrations following i.p. CBD administration (E) the effect of CBD on brain Serotonin concentration over 24 hours (F) Brain NE concentration against time post CBD administration. Unpaired multiple t-tests were conducted using the Holm-Sidak t-test method. * P < 0.05, ** P < 0.01, *** P <*

*0.001, **** P < 0.0001 compared to the control, where T = 0 hours represented the control in all graphs.* ***(data presented as mean ± SD, n = 3).***

In Figure **14A**, CBD was first detected in the brain at (0.25 hours) post-administration (5.87 ± 2.441 ng/ml, $P < 0.05$). The T_{max} of CBD (the time at which the maximum concentration was reached) was at 1-hour post administration with a C_{max} of 152.801 ± 1.541 ng/ml ($P < 0.0001$) was reached (**Table 3)**. After 1 hour, the concentration of CBD in the brain steadily decreased until it reached a concentration of 6.904 ± 2.174 ng/ml ($P < 0.01$) at 24 hours post-administration. The CBD concentration in brain is statistically different among the groups where $P < 0.05$ for all time points against the control.

The effect of cannabidiol on the level of neurotransmitters in the brain is depicted in Figure 14. GABA **(14B)** is downregulated, with the lowest concentration of 891.98 ± 58.71 ng/ml at 4 hours ($P < 0.05$). The levels of GABA in the brain were then elevated from 6 hours (944.35 ± 146.65 ng/ml, $P < 0.05$) to 24 hours (1144.49 ± 24.99 ng/ml, $P < 0.05$). Glutamate **(14C)** levels were significantly decreased from the control (1297.25 ± 120.04 ng/ml, $P > 0.05$) to 4 hours (659.96 ± 47.91 ng/ml, $P < 0.001$), 6 hours (771.10 ± 37.80 ng/ml, $P < 0.01$) and 8 hours (822.72 ± 47.54 ng/ml). Glutamate experienced a decline from 1 hour to 4 hours post CBD administration, and thereafter displayed a rise in levels from 6 hours onwards. The C_{max} of dopamine **(14D)** in the brain was 41.34 ± 3.82 ng/ml at 0.5 hours, followed by a decrease that occurred at 1 hour (T_{max} of CBD) with a concentration of 28.797 ± 0.36 ng/ml. The levels of dopamine spiked at 2 hours (35.39 ± 2.57 ng/ml), and thereafter diminished at 4 hours (28.10 ± 2.90 ng/ml) and remained at similar levels until 24 hours (27.45 ± 1.34 ng/ml) post dosing. The C_{max} of serotonin **(14E)** (22.746 ± 1.40 ng/ml) was achieved at 1 hour, which corresponds to the T_{max} of CBD. Serotonin brain concentration over time did not have a significant difference against the control. Norepinephrine **(14F)** was decreased over time from the control (1281 ± 199.19 ng/ml) to 24 hours (775.82 ± 76.43 ng/ml) post administration, without any significant difference.

Table 3: A summary of the pharmacokinetic parameters of CBD following acute i.p. administration of 10 mg/kg b.w. CBD

C_{max} (ng/ml)	**152.8**
T_{max} (hour)	1
$T_{1/2}$ (hour)	8.35
K_{el}	0.0830
$AUC_{0\text{-}inf}$ (ng min/ml or ng min/g)	605.015

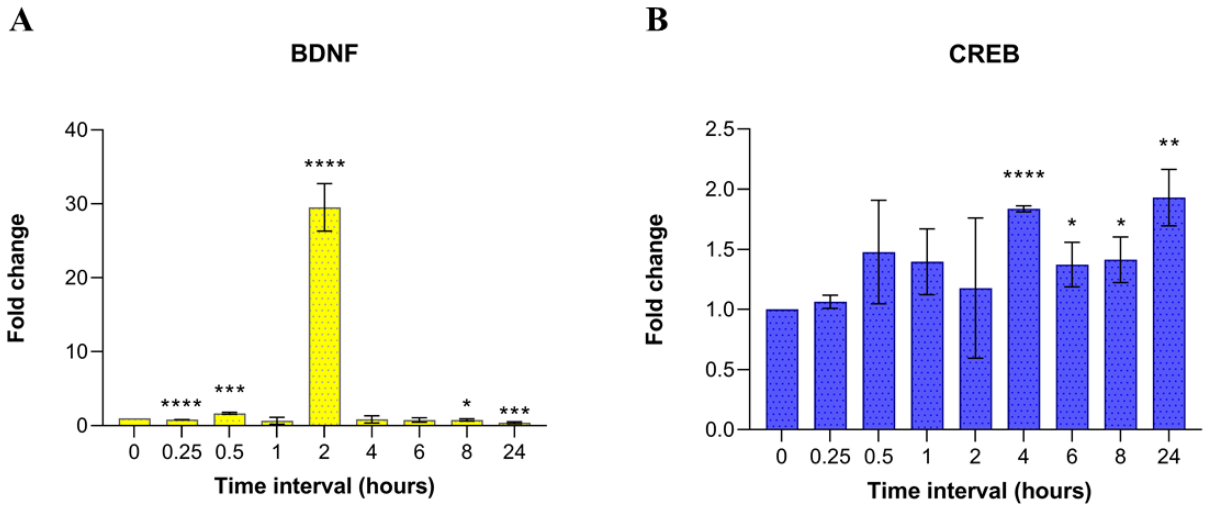

Figure 15: The effect of CBD administration on the expression of CREB and BDNF at various time intervals.

*Figure 15A displays the fold changes in BDNF expression in the brain over 24 hours post i.p. CBD administration. Figure 15B shows the CREB expression levels in the brain over 24 hours post i.p. CBD administration. T = 0 hours is the control. Unpaired multiple t-tests were performed to detect differences in BDNF expression (15A) and CREB expression (15B) respectively, between the control and time points post CBD administration. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.* ***Data are expressed as mean ± SD (n = 3).***

In **figure 15A**, CBD (10 mg/kg body weight significantly augmented BDNF at 30 minutes (1.637 ± 0.13, $P < 0.001$) and at 2 hours (29.517 ± 3.21, $P < 0.0001$). The drastic increase of BDNF at 2 hours can be correlated with a high CBD brain concentration (102.82 ng/ml ± 15.44) at 2 hours (Figure 14A). BDNF levels were significantly decreased at 15 minutes (0.810 ± 0.020, $P < 0.0001$), 8 hours (0.770 ± 0.142, $P < 0.05$) and 24 hours post administration (0.387

± 0.126, $P < 0.001$). CREB levels **(15B)** in the brain were significantly increased at 4 hours (1.837 ± 0.025, $P < 0.0001$), 6 hours (1.373 ± 1.37, $P < 0.05$), 8 hours (1.413 ± 0.189, $P < 0.05$) and 24 hours (1.930 ± 0.235, $P < 0.01$) post administration.

4. Discussion

The therapeutic use of CBD in the treatment of anxiety has been increasingly studied in both preclinical and clinical studies (Bandelow et al., 2017). The primary objective of this study was to investigate the neurochemical effects induced by acute exposure of CBD. The mechanism of action of CBD in anxiety related disorders was evaluated by assessing the changes in brain neurotransmitter concentration, BDNF and CREB expression in male Sprague Dawley rats. The C_{max} of CBD at 1 hour (Figure 14A) is supported by previous studies (Deiana et al., 2012) and (Hložek et al., 2017), who showed that varying routes of CBD administration have a half-life of 60 – 120 minutes, thereafter experiences a continuous decline towards 24 hours. Based on these findings CBD has been shown to attenuate anxiety levels in healthy individuals 2 hours post-administration (Martin-Santos et al., 2012).

Cannabidiol acts as an indirect agonist at the CB_1R and may inhibit the release of neuromodulatory systems such as, 5-HT, NE and DA, thus reducing its concentration in the brain (Rubino et al., 2008). A deficiency of serotonin (5-HT) in the brain is strongly associated with negative emotions involved in anxiety disorders and major depressive disorders (Liu et al., 2018). The overlap of pharmacological treatment of anxiety and depression, such as SSRIs which are 5-HT partial agonists, exert their therapeutic potential by facilitating 5-HT neurotransmission and upregulating extracellular 5-HT concentrations (Artigas, 2013; Gartside, Umbers, Hajos, & Sharp, 1995). The agonistic action of CBD at the 5-HT1ARs causes a suppression in glutamatergic and GABAergic transmission (Russo et al., 2005). A study by (De Gregorio et al., 2019) concluded that acute dosing of CBD (0.1 – 1.0 mg/kg) reduced the firing rate of 5-HT neurons. However, repeated administration of CBD resulted in an increase in 5-HT activity and reduced anxiety-like behaviour through the agonism of 5-HT1A receptors (De Gregorio et al., 2019; Russo et al., 2005). The reduced concentration of serotonin in this study can be attributed to the acute administration of CBD, thus only increasing when CBD was at its maximum concentration (Figure 14E). The anxiolytic effects of the 5-HT1A receptors are activated during chronic treatment of SSRIs and CBD, whereas anxiogenic effects may be produced in response to acute SSRIs and CBD administration (De Gregorio et al., 2019; Liu et al., 2018). In a previous study, an acute, single dose of CBD produced an increase in 5-HT levels through a 5-HT1A receptor-dependent mechanism in the

OBX murine model of depression (Linge et al., 2016). Behavioural and neurochemical studies by (Sales, Crestani, Guimarães, & Joca, 2018) suggest that the anti-depressant and anti-anxiety like effects of CBD are dependent on the concentration of serotonin.

CBD acts on molecular targets that correspond to targets of neurological disorder treatments, such as benzodiazepines (Bih et al., 2015). The inhibition and activation of the main inhibitory neurotransmitter system, GABA, can be facilitated by CBD agonism at the transient receptor potential vanilloid type 1 (TRPV1) receptor (Ho, Ward, & Calkins, 2012; Musella et al., 2009). Our findings reported a gradual decrease in the GABA levels in healthy rats as the concentration of CBD decreased over time (Figure 14B). A study by (Kaplan, Stella, Catterall, & Westenbroek, 2017) demonstrated an anti-seizure effect of CBD in mice with Dravet syndrome which was associated with an upregulation of GABA neurotransmission. It is suggested that CBD may upregulate the neurotransmission of GABA through the antagonist action in the basal ganglia, at the G protein-coupled receptor 55 (GPR55) (Kaplan et al., 2017). The exact role of GABA remains unclear as an *in vivo* study reported an increase in GABA levels in healthy control patients, and a decrease of GABA in patients with autism spectrum disorder 5 hours post CBD administration (Pretzsch et al., 2019).

A study by (Pretzsch et al., 2019) also presented a decrease in of glutamate in the dorsomedial prefrontal cortex in both healthy and autism spectrum disorder individuals, following the administration of 600 mg of CBD p.o. The decline of glutamate levels may have been a result of inhibition of the activity of the prefrontal glutamatergic neurons by CBD via the 5-HT1A receptors (Russo et al., 2005; Santana, Bortolozzi, Serrats, Mengod, & Artigas, 2004). The levels of glutamate also decreased as the concentration of CBD in the brain decreased 2 hours post administration (Figure 14C). However, a preclinical study by (Linge et al., 2016) showed an augmentation of glutamate in OBX mice. The increase of glutamate release was mediated by the action of CBD on 5-HT1A receptors (Linge et al., 2016). The anti-seizure properties of CBD were investigated by co-administration of CBD with cocaine (Gobira et al., 2015). The results of the study by (Gobira et al., 2015) indicated that CBD attenuates glutamate levels in the hippocampus via 5-HT1A receptors..

During stressed conditions, the hyperactivity of norepinephrine (NE), by corticotrophin releasing factors, in the CNS can result in the manifestation of anxiety symptoms (Liu et al., 2018). The NE levels showed no significant changes after CBD administration (Figure 14F), thus postulating that CBD has no significant effect on NE in a healthy rodent model. This is

supported by a study by (Sales et al., 2018) which showed that NE levels were reduced by DSP-4, a noradrenalin neurotoxin. The effect of CBD on anxiety-related behaviour during the forced-swimming test was dependent of the facilitation of 5-HT levels rather than NE itself (Sales et al., 2018). These results were corroborated as a DSP-4 did not inhibit the anti-depressive effects of CBD (Ross & Stenfors, 2015). There is a mutual interaction between NE and 5-HT neurotransmission as the NE mediated activation of the α2- adrenergic receptors can prevent the release of 5-HT (Liu et al., 2018; Quesseveur et al., 2013). 5-HT systems can also negatively influence NE neurotransmission through the action of 5-HT2A and 5-HT2C systems (Hamon & Blier, 2013). The mutual interaction between NE and 5-HT is a possible explanation for the results obtained in this study, as there is a high concentration of NE, whilst the concentration of 5-HT remains fairly low (Figure 14E and 14F).

The dopaminergic system, consisting of different dopamine receptors, is crucial in the modulation of anxiety in the amygdala (de la Mora, Gallegos-Cari, Arizmendi-García, Marcellino, & Fuxe, 2010) . Previous literature mostly reports the effect of CBD on the dopamine receptors, D1 and D2, rather than the concentration of dopamine in the brain (Seeman, 2016; Shin & Liberzon, 2010). Studies have provided evidence that the activation of the dopamine D1 receptor elicits anxiogenic effects, whereas antagonists induce anxiolytic effects in models of anxiety (de la Mora et al., 2010; Kupferschmidt, Newman, Boonstra, & Erb, 2012; Zarrindast, Sroushi, Bananej, Vousooghi, & Hamidkhaniha, 2011). The dopamine D2 receptors are involved in anxiety-like behaviour in the ventral tegmental area (de la Mora et al., 2010; de Oliveira et al., 2011). A decrease in dopamine levels can induce anxiety and depression-like behaviours (Jaunarajs, George, & Bishop, 2012). An insufficient function of DA receptors may result in failure in inhibition from the PFC to the amygdala, promoting hyperexcitability of the amygdala which results in the pathogenesis of anxiety (Liu et al., 2018). However, elevated levels of DA are related to an increase in anxiety and depression in patients with paraganglinomas (Bonomaully, Khong, Fotriadou, & Tully, 2014). A study by (Seeman, 2016) reported the partial agonist activity of CBD on dopamine D2 receptors as CBD prevented the binding radio-domperidone, contributing to the anti-psychotic effect of CBD. A study by (Murillo-Rodríguez, Palomero-Rivero, Millán-Aldaco, Mechoulam, & Drucker-Colín, 2011) demonstrated that perfusion of CBD in a sleep deprived rats increased extracellular DA concentrations during and after sleep deprivation, as sleep deprivation is associated with diminished DA levels. It can be speculated from our results that the anti-anxiety action of CBD as a dopamine agonist occurs at 0.5 and 2 hours post CBD administration as the DA levels are

increased (Figure 14D). At the 1-hour following drug dosing, there was a sudden decrease of DA (Figure 14D) with a concentration of 28.797 ± 0.356 ng/ml, whilst the level of 5-HT (Figure 14F) reached its C_{max} of 22.746 ± 1.404 ng/ml. This could be explained by the action of 5-HT system via 5-HT2A and 5-HT2C receptors which can inhibit the DAergic system and induce short-term motor changes (Clausius, Born, & Grunze, 2009; Liu et al., 2018).

Diminished BDNF levels are associated with stress, anxiety and depression (Castrén & Rantamäki, 2010). CBD, as an anti-depressant therapeutic, has been reported to increase *BDNF* levels in the brain (Giacoppo, Pollastro, Grassi, Bramanti, & Mazzon, 2017). Our results show a significant 25-fold increase of BDNF level at 2 hours, which could be a result of the high CBD concentration between 1 and 2 hours (Figure 15A). This rapid increase in BDNF is supported by (Sales et al., 2019) as BDNF levels in the prefrontal cortex and hippocampus, were increased 30 minutes following acute CBD administration (10 mg/kg). The augmentation of BDNF in neurodegenerative rodent models is also presented by (Mori et al., 2017) as acute CBD treatment increased BDNF levels in the hippocampus. The mechanism of action of CBD causing the upregulation in BDNF mRNA levels is unknown, however it is suggested to be a result of CBD demonstrating agonism at 5-HT1A receptors (Jiang et al., 2016). The activity of CREB in the hippocampus is upregulated in response to anti-anxiety and anti-depressant therapies (Carlezon Jr et al., 2005). The mRNA levels of CREB are upregulated post CBD administration when compared to the control (Figure 15B). At 1 hour following the administration of CBD, 5-HT reached the highest concentration and the expression of CREB increased from the control. This is supported by an increase in CREB expression in response to anti-depressants, such as SSRIs. The modulation of CREB expression can affect anxiety-like behaviour as the anxiolytic effect of the 5-HT1A receptor agonist, such as CBD, is inhibited by a decrease in CREB activity (Zhang et al., 2016). The agonistic action of SSRIs and CBD at 5-HT1A receptors upregulates the phosphorylation of CREB in the hippocampus, which results in anti-anxiety behaviour by promoting neurogenesis and synaptogenesis (D. L. Wallace et al., 2009; Zhang et al., 2016; Zhang et al., 2010).

5. Conclusion

This study demonstrates the pharmacokinetic properties and pharmacodynamic effects of CBD in a healthy rodent brain. The findings show that CBD significantly alters the glutamatergic and GABAergic neurotransmitter systems in the brain while also significantly increasing BDNF expression. The findings of this study are important in not only improving the

understanding of the anxiolytic mechanisms of action of CBD but also by contributing to our currently limited knowledge on the neurological effects of CBD.

Conflict of interest

The authors declare that they have no known conflict of interest.

Author contributions

A.M. Haripershad, L.J. Khoza, S. Xhazaka, S. Baijnath, assisted with the treatment and sacrifice of rats. L. Khoza and S. Xhakaza assisted with sample preparation for LC-MS and genetic analysis. T. Ghazi and S. Dhani performed qPCR analysis. C. Mutsimhu and Molopa J. Molopa performed LC-MS analysis and interpretation. A. Krishnan provided extracted and purified CBD. N.P. Madurai and L. Madurai provided access to LC-MS instrumentation. S. D. Singh assisted with animal ethics application and the animal study. H.G. Kruger, T. Govender and T. Naicker were supervisors. A.A. Chuturgoon provided access to molecular biology facilities. A. Haripershad composed the manuscript and conducted data analysis. All authors reviewed the manuscript.

CRediT authorship contribution statement

A.M.H.: Conceptualization, Methodology, Investigation, Formal analysis, Writing – original draft, Visualization. S.X and L.J.K.: Investigation. T.G.[b] and S.D.: Investigation, Formal Analysis. C.M. and M.J.M.: Methodology, Investigation, Formal Analysis. A.K.: Resources. N.P.M.: Resources. L.M.: Resources. S.D.S.: Resources. H.G.K.: Supervision. T.G.[e]: Supervision. A.A.C: Resources, Supervision. T.N.: Supervision. SB.: Supervision, Conceptualization, Writing- review & editing.

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CHAPTER 3

GENERAL DISCUSSION AND CONCLUSION

Chapter 3 – General discussion and Conclusion

The anti-anxiety properties of CBD have been reported in many clinical and preclinical studies (Bandelow, Michaelis, & Wedekind, 2017). Since the legalization of *Cannabis* in many countries, CBD is receiving more attention for its medical properties and as an alternative treatment for many ailments, including neurological disorders. The pharmacological profile of the psychoactive cannabinoid, THC has been extensively studied, whilst the mechanism of CBD is not fully understood (Morales, Hurst, & Reggio, 2017). This study examined the pharmacodynamic properties of CBD following acute i.p. administration by evaluating the brain concentration of neurotransmitters and BDNF/CREB mRNA expression in a healthy rodent model. Our findings show that CBD reached its C_{max} at 1-hour post drug administration which is consistent with literature as (Deiana et al., 2012) and (Hložek et al., 2017) reported the T_{max} to be between 1 – 2 hours. A low dose, such as 10 mg/kg used in this study, CBD has been reported to induce anxiolytic effects in neurobehavioural studies. This mechanism of action can be explained by the agonism of CBD on serotonin 5-HT1A receptors (Campos, Ferreira, & Guimarães, 2012; Lee, Bertoglio, Guimarães, & Stevenson, 2017; Russo, Burnett, Hall, & Parker, 2005). The anti-anxiety and anti-depressant-like effects of CBD are associated with increased BDNF levels, which are usually reduced in anxiety and depression (Castrén & Rantamäki, 2010; Sales et al., 2019). The significant 25-fold increase of BDNF found in this study, 2 hours post CBD administration is indicative of the rapid effect of CBD on BDNF signalling. This increase in BDNF enhances neurogenesis, neuroplasticity and synaptic transmission through the activation of the TrKB-mTOR signalling pathway, contributing to the sustained anxiolytic effects of CBD (Autry & Monteggia, 2012; Palomares-Castillo et al., 2012; Sales et al., 2019). The results of this study found an increase in CREB expression following acute CBD exposure (Figure 15B). The elevated levels of CREB are known to promote synaptogenesis and neurogenesis in response to CBD agonism at 5-HT1A receptors (Zhang et al., 2016), while the upregulation of hippocampal CREB expression results in anti-anxiety effects (Zhang et al., 2016).

This study demonstrated an increase in 5-HT levels 1-hour post CBD, thereafter, declining over time. This could be explained by the anxiolytic effects of CBD being mediated by 5-HT during chronic drug exposure (De Gregorio et al., 2019). Acute treatment of CBD reacts similarly to SSRIs, by attenuating 5-HT neuron firing which decreases 5-HT levels as reported in our study (Figure 14E), whereas chronic treatment increases 5-HT transmission via desensitization of 5-

HT1A receptors (De Gregorio et al., 2019; Russo et al., 2005). Norepinephrine is not significantly implicated during acute CBD administration, which is also seen in our study (Figure 14F) (Sales, Crestani, Guimarães, & Joca, 2018). 5-HT has been reported to adversely affect NE transmission via the 5-HT receptors (Hamon & Blier, 2013). Modifications in dopaminergic systems that are responsible for anti-anxiety effects may be mediated by increases or decreases in 5-HT and NE signalling (Liu, Zhao, & Guo, 2018). Since diminished DA levels are related to anxiety-like behaviour, the increase in DA concentrations at 0.5 hours and 2 hours following drug exposure (Figure 14D) suggests that CBD exhibits anxiolytic effects by partial agonist action at dopamine D2 receptors and could be extremely beneficial in the treatment of anxiety (Seeman, 2016). The primary inhibitory, GABA, and excitatory, glutamate, neurotransmitters were significantly reduced from 4 hour following CBD exposure (Figure 14B and 14C). The decline in GABA is supported by a decrease in GABA seen in autism spectrum disorder (ASD) patients (Pretzsch et al., 2019), whereas an increase in GABA promotes anti-seizure affects observed in a Dravet syndrome murine model (Kaplan, Stella, Catterall, & Westenbroek, 2017). There are opposing theories regarding the mechanisms of action of CBD on GABA sand need to be supplemented with further research. Reports show that CBD has contradicting effects on glutamate as reduced levels are reported in ASD patients (Pretzsch et al., 2019) and elevated levels in a murine model after CBD p.o. (Linge et al., 2016). Glutamate attenuations and elevations are mediated via the 5-HT1A receptors as CBD either inhibits or activates the glutamatergic neurons (Gobira et al., 2015; Linge et al., 2016; Russo et al., 2005).

The pharmacological action of CBD is dependent on many mechanisms of action, such as the mediation of the 5-HT1A receptors. However, from this study, the implications of other neurotransmitter systems have been highlighted. Further pre-clinical studies are required to investigate the pathways and receptors involved in the anxiolytic effects of CBD on neurotransmission and molecular expression. The acute administration of CBD, without the addition of anxiety behavioural models remains a limitation of this study. Future studies into the mechanism of action of CBD in the brain should consider the: (1) acute and chronic dosing of CBD at varying low-dose concentrations; (2) behavioural tests in anxiety models, such as elevated-T-maze, open field test, elevated plus maze, and forced swimming test; (3) administration of CBD with neurotransmitter receptor antagonists to confirm the action of CBD, such as WAY100635 antagonist of 5-HT1A receptors; and (4) investigations of other genes associated with anxiety, such as 5-HTT, 5-HT1A, and MAOA, to improve the

understanding of CBD as an anxiolytic therapeutic. Ultimately, the findings of this study contribute to understanding the pharmacological and neurological effects of CBD on the brain and its mechanism of action in the management of neurological disorders.

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APPENDIX

Evaluation of the use of cannabidiol in the treatment of anxiety related disorders by assessing changes in neurotransmitter levels and expression of CREB/BDNF in the rodent brain

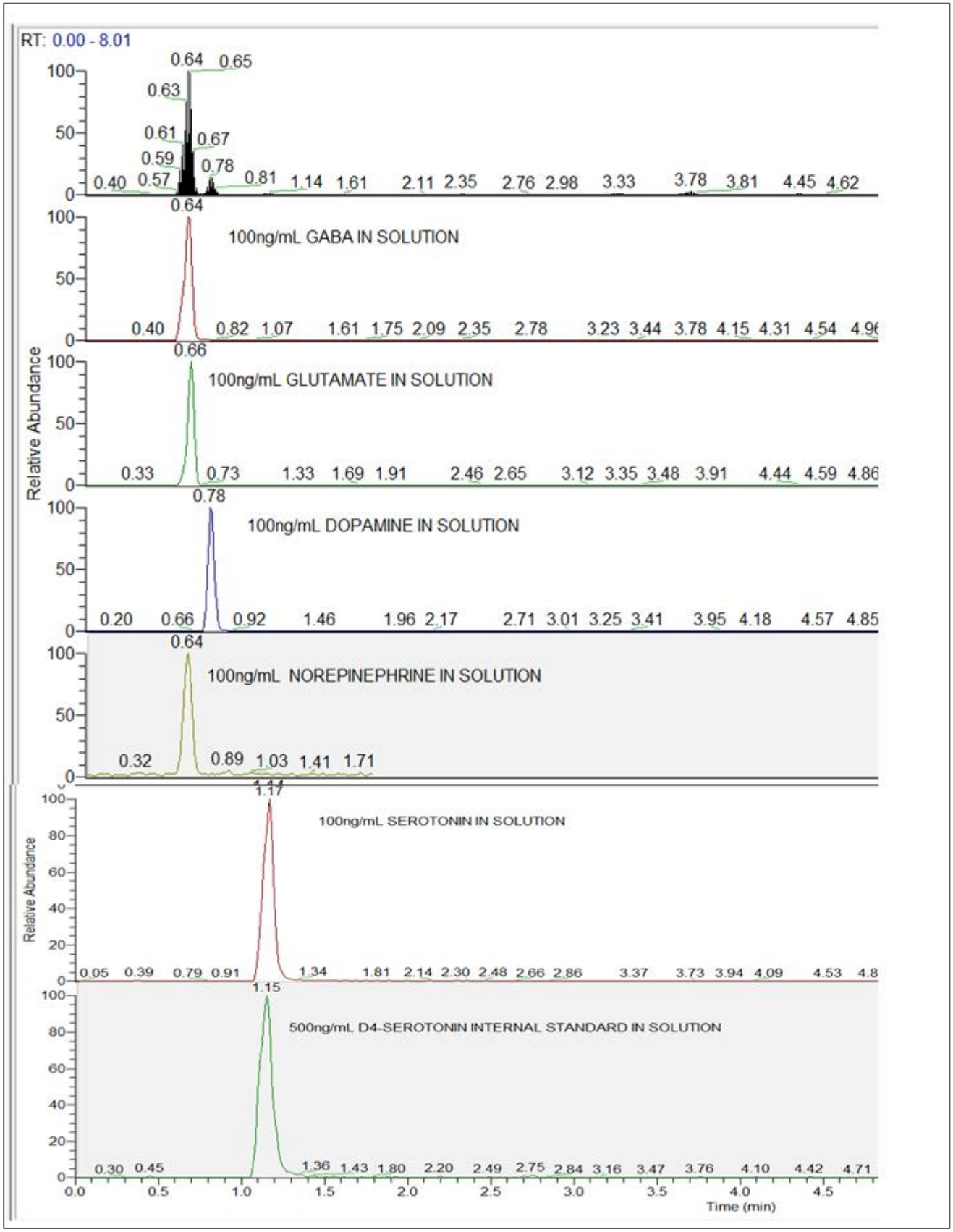


Figure 16: Chromatograms of neurotransmitters and internal standard in solution.

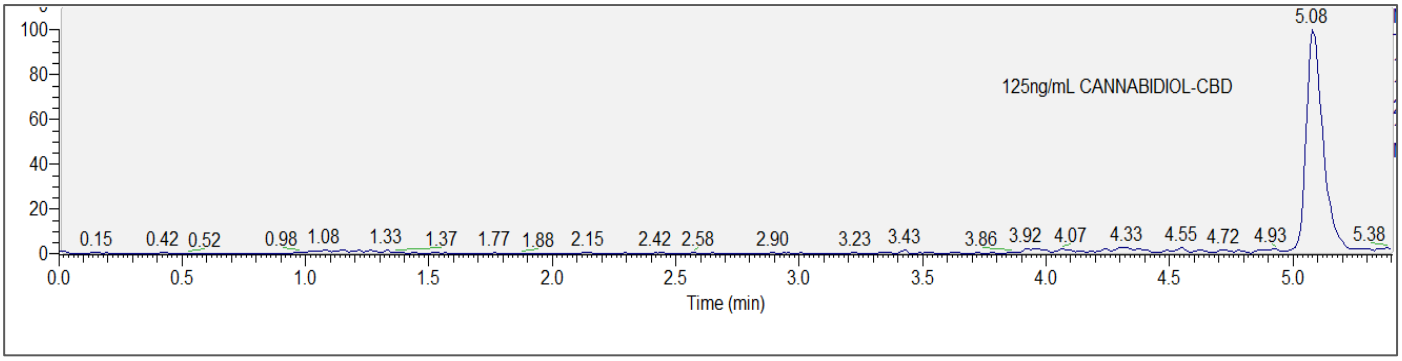


Figure 17: Chromatogram of cannabidiol in solution.

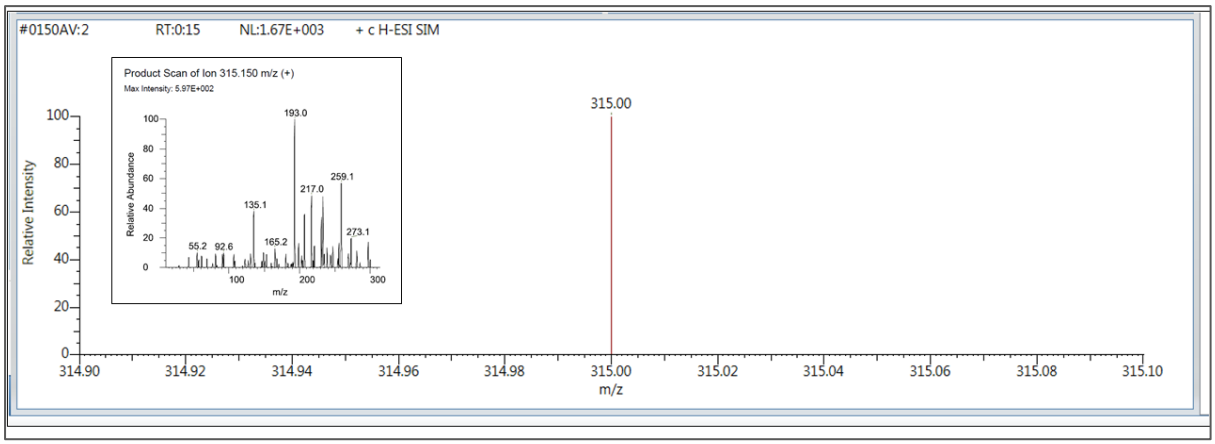


Figure 18: Precursor and product ion $[M+H]^+$ mass spectra of cannabidiol.

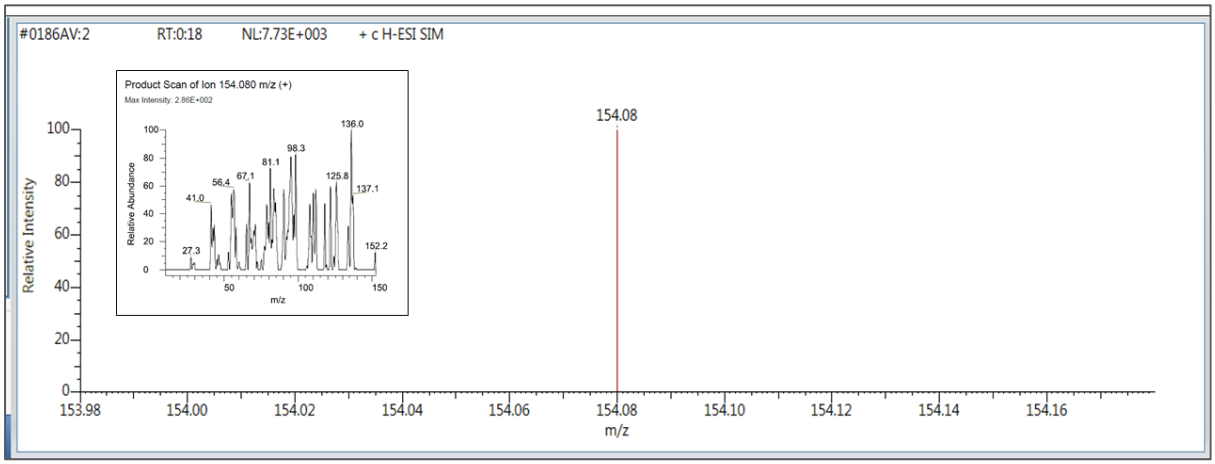


Figure 19: Precursor and product ion $[M+H]^+$ mass spectra of dopamine.

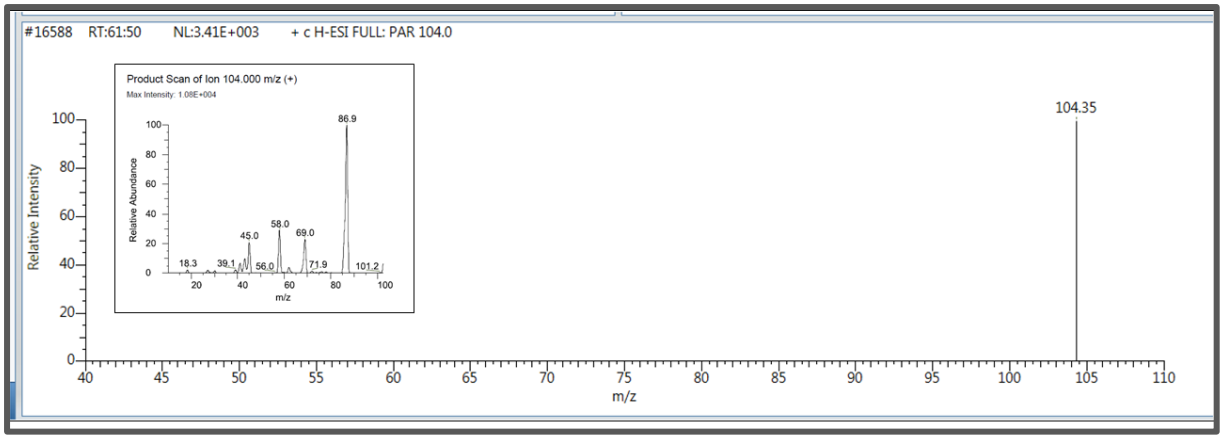


Figure 20: Precursor and product ion[M+H]$^+$ mass spectra of GABA

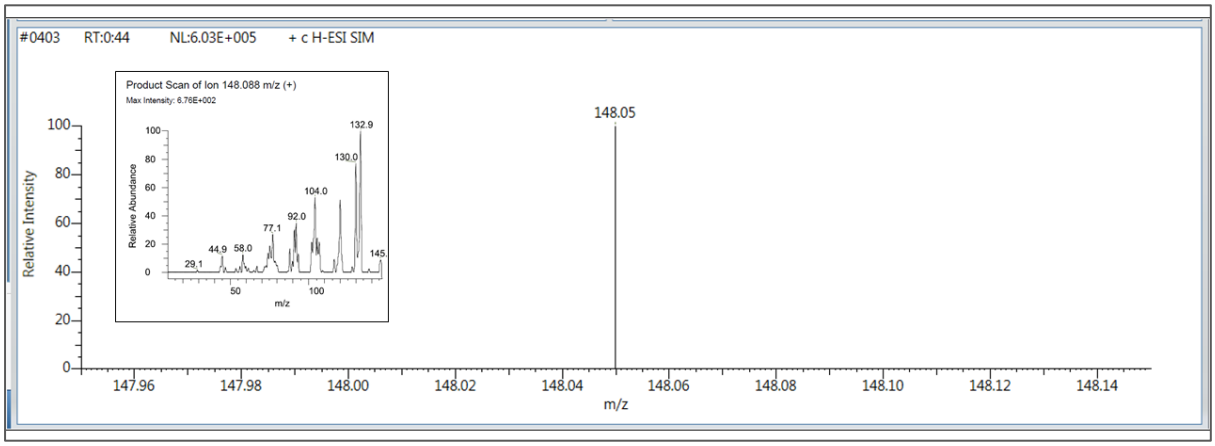


Figure 21: Precursor and product ion [M+H]$^+$ mass spectra of glutamate.

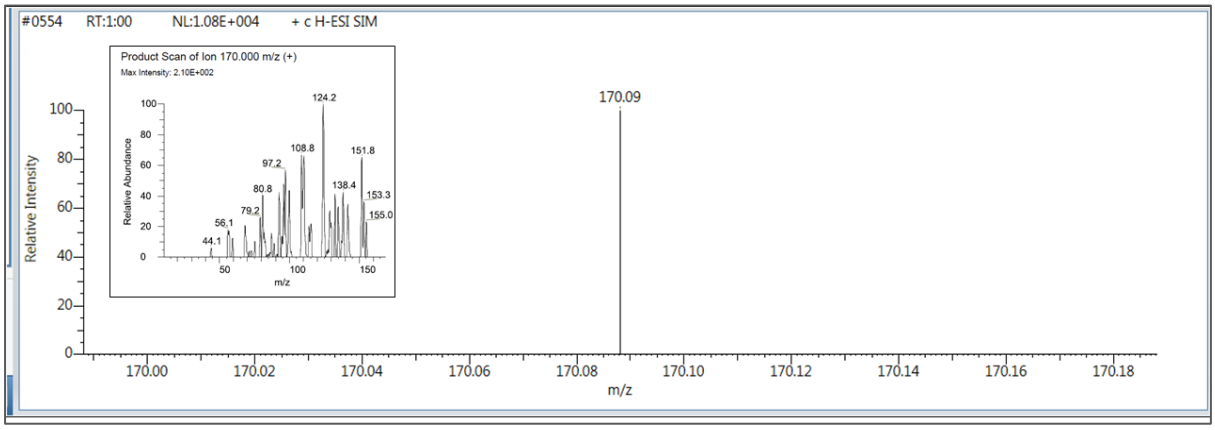


Figure 22: Precursor and product ion [M+H]$^+$ mass spectra of norepinephrine.

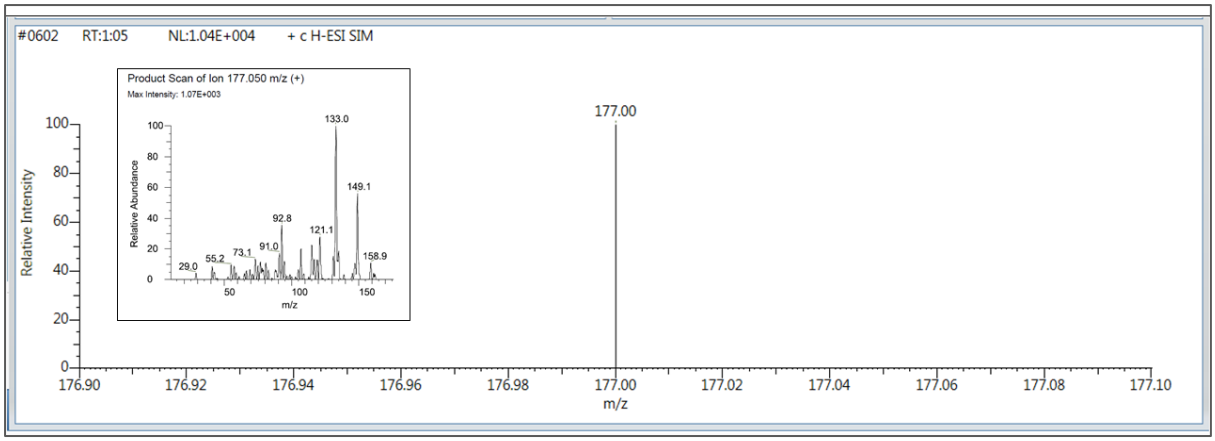


Figure 23: Precursor and product ion $[M+H]^+$ mass spectra of serotonin.

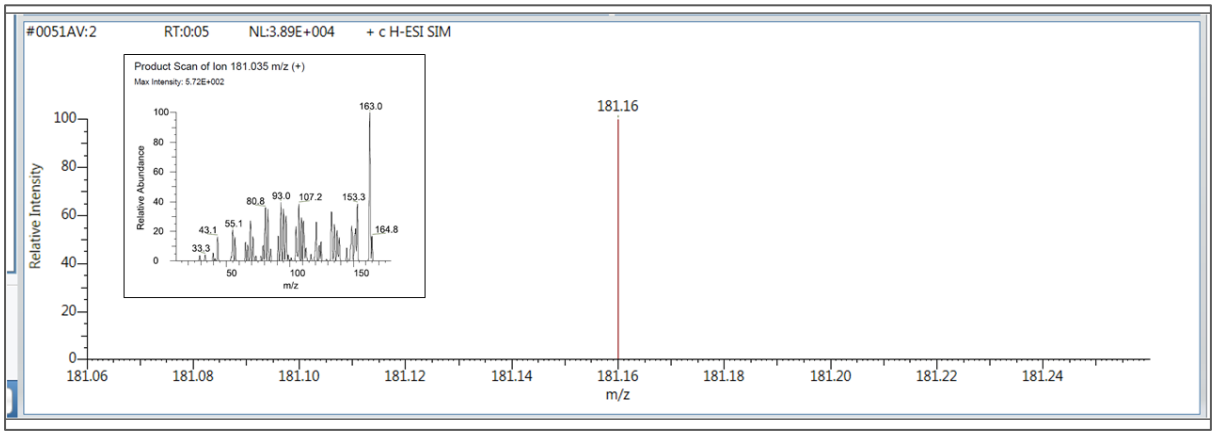
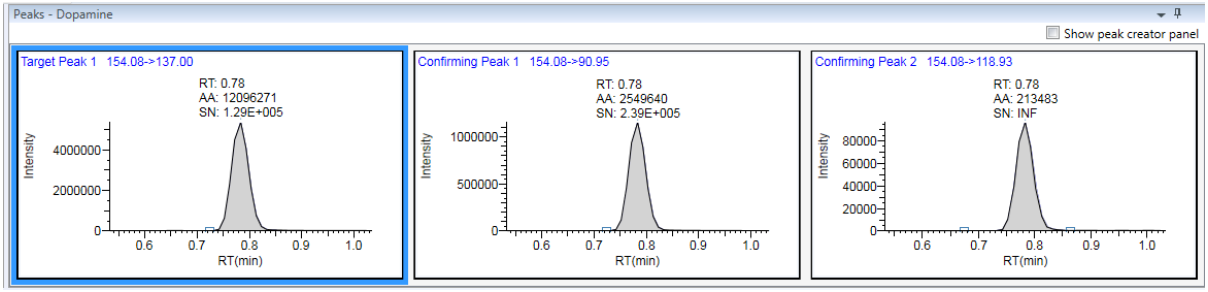
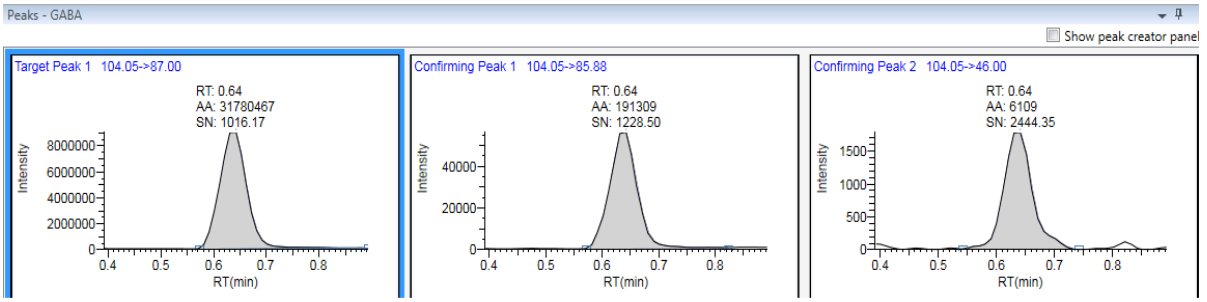


Figure 24: Precursor and product ion mass $[M+H]^+$ spectra of D4-serotonin internal standard.

A



B



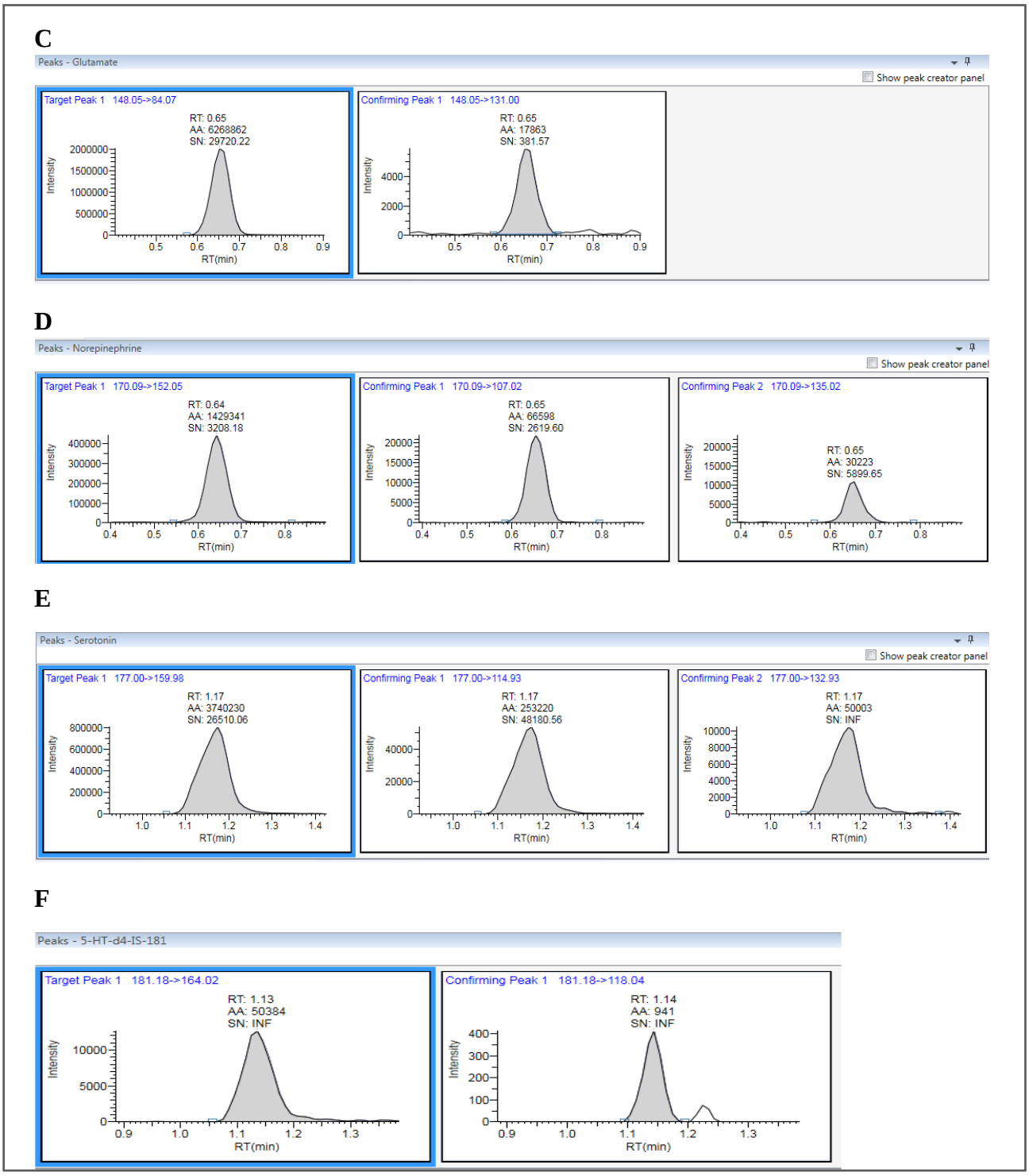


Figure 25: : Quantifier and confirming ion chromatograms of (A) dopamine, (B) GABA, (C) glutamate, (D) serotonin, (E) norepinephrine and (F) serotonin internal standard

UNIVERSITY OF KWAZULU-NATAL ETHICS COMMITTEE

ANIMAL ETHICS SUB-COMMITTEE

APPLICATION FOR APPROVAL OF RESEARCH PROTOCOLS USING ANIMALS

Please note that approval must be obtained for ALL work involving animals irrespective of the source of funding. DO NOT CHANGE THE FORMAT OF THIS FORM.

This form is to be completed in typescript and one signed, hard copy to be submitted to Animal Ethics, Research Office, Govan Mbeki Bldg, Westville Campus AND an electronic copy submitted to animalethics@ukzn.ac.za. Please enter your surname between the marks at the top of each page.

1. TITLE OF PROJECT

The in vivo effects of cannabinoids on brain neurotransmitter levels and tissue epigenetic changes

2. DETAILS OF APPLICANT

2.1 Title (e.g. Dr): Miss

2.2 Surname: Haripershad

2.3 Full name: Advaitaa Meera

2.4 Student / Staff Nr: 216001483

2.5 Applicant's Race & Gender: Indian Female

2.6 Existing Qualifications: BSc Biological Sciences, BSc (Hons)

2.7 Proposed Qualifications / Position: MSc Medical Sciences

2.8 School: Health Sciences

2.9 Campus: Westville

2.10 Internal mailing address for sending hard copy of approval letter:

Catalysis and Peptide Research Unit, Department of Pharmacy, Rm 016-060, E-Block 6th – Floor, University of KwaZulu-Natal, Westville Campus

2.11 Cell No: 0745291707

2.12 Email: 216001483@stu.ukzn.ac.za

2.13 **Supervisor's Details (if applicable)**

Full Name: Dr. Sooraj Baijnath

Telephone Number:031 260 1799/ 084 562 1530 Email: Baijnaths@ukzn.ac.za

3. STAFF, RESEARCH ASSOCIATES, STUDENTS AND TECHNICIANS AUTHORISED TO CARRY OUT THE PROPOSED HANDS-ON ANIMAL STUDIES.		
Full name of team members (Exclude animal facility staff and technicians)	Academic qualification	Animal training (If Yes, PROOF* to be attached)
Advaitaa Haripershad	BSc, BSc (Hons)	Yes
Dr S. Baijnath	B Med Sci, B Med Sci Hons Med Sci, PhD	Yes

*Proof can be a certificate of animal training, a first authored publication using animals, reference letter from relevant authority etc.

4. EXPERIENCE IN WORKING WITH ANIMALS RELEVANT TO THE APPLICATION

The Catalysis and Peptide Research Unit (CPRU) has performed numerous experiments utilizing the same experimental design. Dr, S. Baijnath is registered with the SAVC (registration number AR18/17074) for para-veterinary procedures and worked under the supervision of Dr. S.D. Singh and Dr. L. Bester.

Publications by team member:

Naidoo, V., Mdanda, S., Ntshangase, S., Naicker, T., Kruger, H.G., Govender, T., Naidoo, P., Baijnath, S. 2019. Brain penetration of ketamine: Intranasal delivery VS parentetal routes of administration. *Journal of Psychiatric Research,* 112: 7 -11.

I have experience working with Sprague-Dawley rats during my BSc (Hons) in School of Life Science in 2018 under the approved ethical clearance number (AREC 040 018 H). I have attended the Animal Ethics Training course presented by University of Kwa-Zulu Natal's Biomedical Resource Unit (2018) which entailed: Introduction to laboratory animal sciences. Bioethics and Animal experimentation. Animal Research Methodology. Experimental design, environmental enrichment and occupational safety. I have successfully completed the Animal Handling course presented by the BRU.

5. ANIMAL HOUSING FACILITIES WHERE WORK WILL BE CARRIED OUT

5.1. University of KwaZulu-Natal Centres

Biomedical Resource Unit (Westville) ☑

School of Life Sciences (SLS) (PMB) Animal House ☐

Ukulinga Research Farm (PMB) ☐

Other (specify below under 5.2) ☐

5.2 Non-University of KwaZulu-Natal Centres*

PLEASE SPECIFY in FULL_____

***N.B. If ALL of your work involving animals is performed at a Non-University of KwaZulu-Natal Centre, you need not complete the rest of this form, but you HAVE TO attach a letter of ethical approval obtained from the relevant authority at the Non-University of KwaZulu-Natal Centre.**

6. BACKGROUND, OBJECTIVES AND POTENTIAL BENEFITS OF THE PROJECT

(Please give a clear and succinct statement of the background, objectives and potential benefits of the project under three separate headings - 3 pages allocated for this section including references)

Background

The plant, *Cannabis sativa,* comprises of more than 400 compounds, of that at least 60 are cannabinoids which mediate its wide range of effects (Atakan, 2012). Delta- 9 – tetrahydrocannabinol (THC) and cannabidiol (CBD) are the main phytocannabinoids which are the most commonly researched (Huestis, 2007). Cannabidiol (CBD) is the main non-psychoactive constituent and is reported to have both therapeutic and adverse effects (Alexander, 2016). The pharmacological action of CBD is elicited through the binding of these compounds to the cannabinoid-1 receptor (CB_1) and cannabinoid-2 receptors (CB_2), which are located in the cannabinoid system (Boggs et al., 2016). The CB_1 receptors are primarily expressed in the central and peripheral nervous system, as well as in the heart, bones, lung, thyroid liver, uterus, testicular tissue and the vascular endothelium (Pertwee, 2006; Russo and Guy, 2006). CB_1 receptors are located at the GABAergic terminals and on glutamatergic terminals of the central and peripheral neurons and, in the central nervous system of the brain areas that are related to the body's' stress response (Atakan, 2012; Kaur and Singh, 2016). These brain areas include the central amygdala, basal ganglia, limbic system, hippocampus, frontal cortex, substantia nigra and the cerebellum (Pertwee, 2006). Cannabinoid-2 receptors are mainly expressed in the gastrointestinal system, immune cells and spleen, and are also expressed at low levels in the central and peripheral nervous system (Pertwee, 2006).

In the brain, CB_1 receptors mediate inhibitory actions by the ongoing release of excitatory and inhibitory dopaminergic, gamma-aminobutyric acid (GABA), glutamatergic, serotoninergic, noradrenalin and acetylcholine neurotransmitter systems (Atakan, 2012). The involvement of the neurotransmitter systems subsequently affects functions such as pain perception, memory, cognition and motor movements (Howlett et al., 2002). Cannabis influences the activities of neurotransmitters in the brain, as GABA, glutamate and dopamine are generally higher in rats treated with cannabis (Owolabi et al., 2017). Cannabidiol (CBD) has complex pharmacological mechanisms of action involving neurotransmitter systems and receptors. Most of the evidence suggests that CBD reduces many of the psychoactive effects of THC (Morgan et al., 2012; Boggs et al., 2016). Recent literature has described the anxiolytic and antipsychotic properties of CBD (Atakan, 2012; Deepak et al., 2012). A study performed by Owolabi et al. (2017) investigated the relationship

in the modifications in neurotransmitter levels and enzymes caused by cannabis exposure in rats. The findings of this study reported that higher doses of CBD can potentiate the lower doses of d-9-THC by increasing the level of expression of CB_1 receptor in the hippocampus and the hypothalamus (Owolabi et al., 2017).

In addition to cannabis-induced neurotransmitter modifications, the global increase in cannabis legalization has resulted in evolving scientific interest on the impact of cannabis on epigenome modification (Dobs and Ali, 2019). Epigenetics can be defined as the process that alters gene regulation, without changes in the DNA sequence, that may result in a specific phenotype (Berger et al., 2009). Molecules, that influence gene constitution, can express or supress genes if frequently exposed to a stimulus such as cannabis (Dobs and Ali, 2019).

There remains a need to supplement the available literature related to the neural effect of *Cannabis* use on behavioural changes, with the accompanying in situ neurochemical and biochemical changes (Owolabi et al., 2017). There are gaps in the knowledge of the pharmacokinetics and behavioural effects of THC and CBD, as well as its combination, across the various administration forms (Hlozek, et al., 2017). The dose effects of cannabinoids *in vivo* against *in vitro* have not been fully described (Hlozek et al., 2017). There are many unanswered questions relating to the use of epigenetics in cannabis use disorders and the extent of epigenetic modifications (Dobs and Ali, 2019). The outcome of cannabis exposure to adolescents and adults is unknown due to the limited knowledge pertaining to cannabinoid-mediated epigenome modulations (Dobs and Ali, 2019).

This study aims to investigate the effect of cannabinoids on the neurotransmitters in the brain.

AIM:

1. To determine the concentration of cannabidiol in the brain
2. To evaluate the effect of cannabinoids on neurotransmitters in the brain
3. To investigate the impact of cannabinoid exposure on epigenome modulation

OBJECTIVES:

1. To utilize LC-MS to quantify cannabidiol concentration in the brain
2. To identify the epigenetic biomarkers associated with cannabis exposure using Western Blots and PCR

POTENTIAL BENEFITS:

Recently, cannabidiol has been punted as a solution to many neuropsychiatric disorders including anxiety, depression, epilepsy and panic disorders. Neurotransmitters are implicated in the pathogenesis of these disorders, but this has not been substantiated with scientific evidence. This study will understand the role of CBD and its effects on neurotransmitters in the brain. This can be potentially used in studies to develop novel therapeutic strategies for mental health disease as well as leading to the identification of possible genetic targets for the treatment of substance abuse.

REFERENCES

Atakan, Z. 2012. Cannabis, a complex plant: different compounds and different effects on individuals. *Therapeutic Advances in Psychopharmacy,* 2(6): 241 – 254.

Alexander, S.P. 2016.Therapeutic potential of cannabis-related drugs. *Prog. Neuropsychopharmacol. Biol. Psychiatry*, 64: 157–166.

Berger, S.L., Kouzarides, T., Shiekhattar, R., Shilatifard, A. 2009. An operational definition of epigenetics. *Genes Dev.* 23, 781–783.

Boggs, DL, Peckham A, Boggs AA, Ranganathan M. 2016. Delta-9-tetrahydrocannabinol and cannabidiol: Separating the chemicals from the ‘‘weed,’’ a pharmacodynamic discussion*. Ment Health Clin.* 6(6):277-284.

Dobs, Y.E., Ali, M.M. 2019. The epigenetic modulation of alcohol/ethanol and cannabis exposure/co-exposure during different stages. *Open Biol.* 9: 180115.

Hložek, T., Uttl, L., Kadeřábek, L., Balíková, M., Lhotková, E., Horsley, R.R., Nováková, P., Šíchová, K., Štefková, K., Tylš, F., Kuchař, M., Páleníček, T. 2017. Pharmacokinetic and behavioural profile of THC, CBD, and THC+CBD combination after pulmonary, oral, and subcutaneous administration in rats and confirmation of conversion in vivo of CBD to THC. European Neuropsychopharmacology, 27: 1223 – 1237.

Huestis, M., 2007. Human cannabinoid pharmacokinetics. *Chemical Biodiversity*, 4: 1770 - 1804.

Kaur, S., Singh, R. 2017. Role of different neurotransmitters in anxiety: a systemic review. *International journal of Pharmaceutical Sciences and Research,* 8(2): 411 – 421.

Mechoulam, R., Hanus, L.O., Pertwee, R., Howlett, A.C. 2014. Early phytocannabinoid chemistry to endocannabinoids and beyond. *Nat.Rev.Neurosci.,* 15: 757–764.

Morgan C, Gardener C, Schafer G, Swan S, Demarchi C, Freeman TP, et al. 2012. Sub-chronic impact of cannabinoids in street cannabis on cognition, psychotic-like symptoms and psychological well-being. *Psychol Med.* 42(2):391-400.

Pertwee, R. 2006. The pharmacology of cannabinoid receptors and their ligands: an overview. *Int J Obes (Lond)* 30: S13–S18.

Owolabi, J.O., Olatunji, S.Y., Olanrewaju, A.J. 2017. Caffeine and Cannabis effects on vital neurotransmitters and enzymes in the brain tissue of juvenile experimental rats. Annals of Neurosciences, 73: 24 – 65.

7. DESCRIPTION OF YOUR PLAN OF WORK

(**Read the notes on this section** – Apart from detail description, a mandatory flow diagram of experimental design to be attached in separate sheets, 4 pages allocated for this section including references)

7.1. Source of Animals

The Sprague-Dawley rats are sourced from the University of KwaZulu-Natal (Westville campus). The animal requisition process has been established within the BRU and there will be random selection of the animals.

7.2. Animal care:

The animals will be provided with commercially pelleted food and clean water *ad libtium.* The well-being of the animals, as well as the food and water supplies, will be overseen by the facility veterinarian and laboratory supervisor including weekends and vacation periods. The animal holding area is visited daily by a registered veterinarian.

7.3. Housing and enrichment

The animals will be collected two weeks prior to the procedure to allow for acclimatization. The rats will be weighed to confirm that they weigh 110 – 120g. The

animals will be housed in well-ventilated cages, located in temperature and humidity-controlled housing units. Environmental enrichment, in the form of shredding paper and plastic tunnels, will be added to the cage.

7.4. Reagents

Cannabidiol will be administered via intraperitoneal injection at a dose of 10mg/kg body weight, which is reported to have no toxic effect on the animal according to literature. A chronic cannabinoid exposure study by Long et al. (2010) effectively administered cannabidiol in a saline solution vehicle, via intraperitoneal injection, at doses of 1, 5, 10 and 50 mg/kg body weight. Dieana et al. (2012) successfully intraperitoneally injected cannabidiol at a dose of 120 mg/kg body weight. The dose was based on the maximal concentration of cannabidiol that can be diluted in solvent (Dieana et al., 2012). There was no reported drug toxicity in both studies. Studies have shown that CBD administration causes slight sedation and slowed motor movements. CBD administration has not reported adverse psychological or cardiovascular effects.

7.5. Drug treatment

The animals will be treated with 10mg/kg body weight of pure cannabidiol which will be suspended in a 1:1:18 mixture of ethanol: Tween 80: saline (Long et al., 2010). A volume of 1ml/ 250g body weight will be administered via intraperitoneal injection to the animals in both the acute and chronic group. There are number of preclinical studies which have administered cannabinoids (d-9-tetrahydrocannabinol and cannabidiol) via intraperitoneal injection (Wiley et al., 2007; Long et al., 2010; Dieana et al., 2012; Taffe et al., 2015).

Number of animals	Cannabinoid exposure	Route of administration
27	Acute	Intraperitoneal injection
18	Chronic	Intraperitoneal injection

7.6. Humane endpoints

A humane endpoint sheet (attached) will be used to record the well-being of the animals for each experiment and kept on file. If any animal appears to be in severe discomfort prior to the experimental time point, that animal must be euthanized. Animals will be monitored twice daily. Should an animal display a sign of discomfort or abnormal behaviour, the supervising vet (Dr S. Singh. SAVC registration number DA

93/3378) will be consulted and deem if necessary, to euthanize the animal. This will then be formally reported to AREC via the Adverse Events Form.

7.7. Study design

Forty-five male Sprague-Dawley rats (110-120g) will receive 10mg/kg body weight via intraperitoneal injection. The animals will be randomly selected and assigned to the specified groups. The rats will be housed in polycarbonate cages, with a cage density of 646cm^2. There will be 15 cages with 3 rats per cage: 9 cages will be used for the acute treatment and 6 cages will be used for the chronic treatment.

The quantification of cannabidiol in the brain will be determined after acute and chronic dosing plans. This will allow the quantification of cannabidiol as well as its effect on the neurotransmitters at different time points following various lengths of exposure. The time intervals were determined based on previous pharmacokinetic studies involving cannabidiol (Deiana et al., 2011).

Acute: the quantification of cannabidiol will be determined at intervals of 0, 15, 30, 60, 120, 240, 360, 480 minutes and 24 hours.

Chronic: the quantification of cannabidiol will be determined at intervals of 0, 7, 14, 21 and 28 days.

A. Acute exposure

Each Sprague-Dawley rat will be intraperitoneally administered a single dose of 10mg/kg cannabidiol. Three rats will be terminated from the acute group at the respective time interval (table 1), to determine the concentration of cannabinoids in the brain tissue and its effect on the neurotransmitters.

Table 4: Acute exposure group and the number of animals to be terminated at the time points of euthanasia.

Group	**Time points after administration (minutes)**									**Number of rats**
	0	15	30	60	120	240	360	480	24hr	
Acute	3	3	3	3	3	3	3	3	3	27

B. Chronic exposure

Each Sprague-Dawley rat will be intraperitoneally administered a daily dose of 10mg/kg cannabinoid, 6 days of the week. Three rats will be terminated from the

chronic group at the respective time interval (table 2), to determine the concentration of cannabinoids in the brain tissue and its effect on the neurotransmitters.

Table 5: Chronic exposure group and the number of animals to be terminated at the time points of euthanasia.

Group	**Time points after administration (days)**						**Number of rats**
	0	7	14	21	28	28 (control)	
Chronic	3	3	3	3	3	3	18

7.8. Euthanasia

The animals will be euthanized by isoflurane overdose followed by cardiac puncture to collect blood. The blood will be collected in heparin tubes. The carcass will be disposed of in an appropriate bag, which will be handed over to the BRU for appropriate disposal.

7.9. Anaesthetic

Isoflurane will be utilized as an anaesthetic, which will be administered to the animals prior to intraperitoneal injection to reduce any discomfort and pain caused to the animals.

7.10. Tissue Harvest

Following euthanasia of the animal, the brain tissues will be removed from the animal. The brain tissue will be cooled on ice for 15 minutes, before gradually freezing using liquid nitrogen vapour. Tissues will be stored at -80ºC until analysis. The carcass will be disposed of in an appropriate bag, which will be disposed by the BRU according to suitable disposal procedures.

7.11. Analysis of tissue samples

The analysis will be performed using Liquid chromatography-Mass Spectrometry, in combination with Bruker QTOF-II (Bruker Daltonics, Bremen, Germany), to quantify the concentration of cannabidiol in the brain, as well as the neurotransmitters, in both the acute and chronic groups.

References

Deiana, S., Wantanabe, A., Yamasaki, Y., Amada, N., Arthur, M., et al., 2012. Plasma and brain pharmacokinetic profile of cannabidiol (CBD), cannabidivarine (CBDV), Δ9-tetrahydrocannabivarin (THCV) and cannabigerol (CBG) in rats and mice following oral and intraperitoneal administration and CBD action on obsessive–compulsive behaviour. *Psychopharmacology,* 219: 859 – 873.

Hložek, T., Uttl, L., Kadeřábek, L., Balíková, M., Lhotková, E., Horsley, R.R., Nováková, P., Šíchová, K., Štefková, K., Tylš, F., Kuchař, M., Páleníček, T. 2017. Pharmacokinetic and behavioural profile of THC, CBD, and THC+CBD combination after pulmonary, oral, and subcutaneous administration in rats and confirmation of conversion in vivo of CBD to THC. *European Neuropsychopharmacology*, 27: 1223 – 1237.

Long, L.E., Chesworth, R., Huang, X., McGregor, I. S., Arnold, J. C., Karl, T. 2010. A behavioural comparison of acute and chronic Δ9-tetrahydrocannabinol and cannabidiol in C57BL/6JArc mice. *The International Journal of Neuropsychopharmacology,* 13: 861 – 876.

Taffe, M.A., Creehan, K. M., Vandewater, S. A. 201. Cannabidiol fails to reverse hypothermia or locomotor suppression induced by Δ^9-tetrahydrocannabinol in Sprague-Dawley rats. *British Journal of Pharmacology,* 172(7): 1783- 1791.

Wiley, J.L., O'Connell, M.M., Tokarz, M.E., Wright, M. J. Jr. 2007. Pharmacological Effects of Acute and Repeated Administration of Δ^9-Tetrahydrocannabinol in Adolescent and Adult Rats. *The Journal of Pharmacology and Experimental Therapeutics,* 320: 1097 – 1105.

8. INDEX OF PROCEDURES

Consult the Approved Standard Protocols Booklet (available from the Research office website under "Research Ethics" at http://research.ukzn.ac.za/Libraries/Notices2011/Animal_Ethics-Approved_standard_protocols_booklet_and_appendices_2007_sflb.sflb.ashx)

8.1 Experimental procedures (other than antibody production-see Table 8.2) included in the *Approved Standard Protocols Booklet*: Using the *Approved Standard Protocols Booklet*, note by title and code the protocols to be used for each of the experimental procedures other than antibody production in your proposed studies.

Species[1]	Rat				
Strain	Sprague-Dawley				
Age/weight	110-120g				
Sex	Male				
Number of animals	45				
Handling (code)	RH				

Code(s) for procedure(s)	RIIP				
Pain level	Moderate				
Euthanasia (code)	REOD				
Name of anaesthetic/ analgesic	Isoflurane				

[1] In the case of amphibians and reptiles, indicate genus and fish genus or other convenient grouping.

8.2 Antibody production: Antibody production follows the general format of animal handling, immunisation, bleeding and eventually euthanasia, with each researcher using a number of unique schedules. To expedite review, use the *Approved Standard Protocols Booklet* code numbers and simply indicate the species, route of injection, total number of injections, type of adjuvant, method of bleeding, including volume and frequency, and method of euthanasia.

Species[1]					
Strain					
Age/weight					
Sex					
Number of animals/immunogen					
Handling (code)					
Injection (code)					

Total number of immunisations					
Adjuvant(s)					
Bleeding route (code)					
Blood volume					
Bleeding frequency					
Euthanasia (code)					
List of immunogens to be used: Will non-physiological, unusually painful, or harmful material be injected? If so, explain and justify (see pain categories).					

8.3 Experimental procedures **NOT** included in the *Approved Standard Protocols Booklet.* Please give details of all procedures using the same format as that in the *Booklet*. Please use additional sheets if necessary.

Yes, all procedures have been accounted for under section 7.9 Tissue Harvest.

9. What is your assessment of the overall severity of this project?

Please tick in one box only to indicate your assessment.

- ☐ **Substantial**
- ☑ **Moderate**
- ☐ **Mild**
- ☐ **Unclassified**

9.1 Have all surgical and non-surgical procedures been clearly and completely described, consistent with the experimental design outline? – Explain briefly.
Yes, all the procedures have been described in accordance with the Animal Ethics Approved Standard Protocols.

9.2 Has pain, discomfort and distress to the animal(s) been minimized or avoided to the fullest extent possible? – Explain briefly.
Yes, the experimental design has been developed with the 3R's in consideration.
Replacement: This study will investigate the effect of cannabinoids on the neurotransmitters in the brain, as well as the related epigenetic changes. Therefore, the use of animals in this project cannot be replaced as the changes in neurotransmitters cannot be observed using cell cultures or cell lines. There were efforts made to reduce the number of animals and refine the protocol whilst ensuring the number of animals per group can be used for statistical analysis

Reduction: To reduce the number of animals used, the LC-MS will be conducted by utilizing half of the cerebral hemisphere, and the other half will be used to conduct epigenetic tests, instead of sacrificing a single animal for each experimental analysis.

Refinement: The rats will be injected with a 27-gauge needle to reduce pain and discomfort experienced by the animal. The chronic treatment will be administered 6 days a week, allowing one day for the recovery and rest of the animal During the chronic treatment, there will be swapping of the peritoneum sides during intraperitoneal injection to minimize discomfort to the animal. Environmental enrichment, in the form of shredding paper and/or plastic tunnels, will be added to the cages to minimize stress caused to the animal. Upon termination, the animals will be euthanized by an isoflurane overdose, before collecting tissue samples, thereby preventing pain and discomfort to the animal.

9.3 Is there any appropriate plan for monitoring animals for pain, discomfort and distress, including criteria for determining early euthanasia (humane endpoint)? – Explain briefly.

Yes, the humane endpoint form is attached to the application to record the well-being of the animal and will be kept on file.

SUBMISSION CHECK LIST (Click on the appropriate boxes to complete)

		YES	NO	N/A
1	Is your supervisor's (if not self-supervised) progress reports for all applications / renewals approved since January 2017 up to date (if applicable)?	☒	☐	☐
2	Are the contact details of the applicant and main supervisor supplied?	☒	☐	☐
3	Are the members of the research team qualified and experienced in the procedures to be performed?	☒	☐	☐
4	Are the PROOFs of animal training for main applicant (mandatory) and other members in the application enclosed as mentioned in section 3?	☒	☐	☐
5	Is a clear and succinct statement of the background, objectives and potential benefits of the project given under section 6?	☒	☐	☐
6	Are all housing, feeding, surgical and non-surgical procedures clearly and completely described, and are they consistent with the experimental design?	☒	☐	☐
7	Is there adequate statistical or technical justification provided for the number of requested animals?	☒	☐	☐
8	Are the following mandatory items attached to the application form? a) a complete flow diagram of experimental design; b) a humane endpoint form / table specific to you project.	☒	☐	☐
9	Have you discussed your experimental design with an expert animal facility staff/technician?	☒	☐	☐
10	Have you obtained all necessary permits for your experimental procedures?	☐	☐	☒
11	Do you need a provisional approval letter from AREC to apply for any permits/site approval?	☐	☒	☐
12	Has scientific content of the application been thoroughly reviewed by your supervisors (if applicable)? (mandatory for all application submitted by postgraduate students and postdoctoral / research fellows)	☒	☐	☐
13	Are all sections of the applications completed and signed by all parties?	☒	☐	☐

10. DECLARATION BY THE APPLICANT AND HEAD OF SCHOOL

I have considered the feasibility of achieving the purpose of the project by means other than those using animals and, in my opinion, no such alternatives would achieve the objectives of this project. I agree to follow the Approved Standard Protocols Booklet and any delineated modifications as approved by the Animal Ethics Sub-committee. I will also supervise and assure compliance and training by my co-workers and students as listed above.

23 August 2019

SIGNATURE OF APPLICANT **DATE**

S. Baijnath

INITIALS & SURNAME OF SUPERVISOR*

(Please complete – required for mailing copy of approval letter.)

SIGNATURE OF SUPERVISOR* **DATE**

INITIALS & SURNAME OF D&HoS / ALR

(Please complete – required for mailing copy of approval letter.)

SIGNATURE OF D&HoS / ALR **DATE**

*Name and signature of supervisor required if application submitted by postgraduate & postdoctoral students

FOR ANIMAL RESEARCH ETHICS COMMITTEE USE

AREC REVIEW/APPROVAL DATE _____

APPROVAL AREC _____

SIGNATURE

REFERRED BACK TO APPLICANT
FOR REASONS SHOWN _____

_____