INVESTIGATING THE EFFECTS OF CORTICOSTERONE AND CANNABINOIDS ON HIPPOCAMPAL NEUROPLASTICITY AND MITOCHONDRIA

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LAY ABSTRACT

Neurogenesis is a process that describes the production of new nerve cells in the brain. It mainly occurs during early life, but persists in a central brain structure responsible for learning and memory, known as the hippocampus, throughout our lives. This active brain structure relies on the function of certain organelles called mitochondria, which are the primary cellular energy producers and promote nerve cell production. Mood disorders, such as anxiety and depression, may result as a consequence of impaired hippocampal neurogenesis. Evidently, people suffering from anxiety and depression turn to cannabis use for management and treatment of their mood disorders. Considering cannabis has been shown to affect neurogenesis and mitochondrial function, our primary objective was to explore its effects on hippocampal neurogenesis by focusing on mitochondrial function, in the context of stress. We demonstrate that components found in cannabis, delta-9-tetrahydrocannabinol (THC) and cannabidiol (CBD), alter the stress-induced changes in mitochondrial functions related to neurogenesis, suggesting that cannabis may play a role in protecting nerve cells.

ABSTRACT

Hippocampal neurogenesis is linked to the onset, progression and remission of major mood disorder such as anxiety and depression. Neurogenesis is the process by which new neurons are formed in the brain. Mitochondria mediate cellular adaption and provide energy to support growth of new neurons. Chronic stress and mood disorders have been associated with impairments in mitochondrial function and neuronal growth. Individuals experiencing stress and mood disorders reportedly use cannabis as a means to self-medicate. The impacts of cannabis on stress-related effects on hippocampal neurogenesis and mitochondria are vastly unexplored. To investigate these effects we generated an in vitro model of hippocampal neuron stress by treating HT22 cells with corticosterone, the major effector molecule of stress in rodents. We first characterized the impacts of corticosterone on markers of neurogenesis and mitochondrial function in HT22 hippocampal cells. We found that corticosterone decreased gene markers of neurogenesis, mitochondrial biogenesis, content, dynamics and decreased mitochondrial membrane potential. Corticosterone also decreased levels of antioxidant enzymes but did not alter levels of reactive oxygen species (ROS) or elicit lipid peroxidation. We then investigated with potential impacts of cannabis components, delta-9-tetrahydrocannabinol (THC) and cannabidiol (CBD), on corticosterone-induced stress. Individually, THC and CBD decreased markers of neurogenesis, dysregulated mitochondrial dynamics and decreased mitochondrial membrane potential. Interestingly, both THC and CBD increased a marker of mitochondrial biogenesis. Finally, we co-treated HT22 cells with corticosterone and THC or CBD to interrogate the impacts of THC and CBD on corticosterone-induced alterations. Our results indicated THC and CBD had no effect on corticosterone-related reductions in neurogenesis markers or mitochondrial membrane potential. However, THC demonstrated a rescuing effect on a marker of mitochondrial biogenesis and CBD

normalized a marker of mitochondrial fission; both of which were decreased with individual corticosterone treatments. This thesis ultimately identifies some of the pathways THC and CBD may impact stress response in relation to neurogenesis and mitochondria.

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LIST OF ABBREVIATIONS

ACTH - adrenocorticotropin

AEA - anandamide

ATP - adenosine triphosphate

BCA - bicinchoninic assay

BDNF - brain-derived neurotrophic factor

BSA - bovine serum albumin

Ca^{2+} - calcium

CBD - cannabidiol

CB1 - cannabinoid receptor 1

CB2 - cannabinoid receptor 2

CRH - corticotropin-releasing hormone

CS - citrate synthase

DCFDA- 2',7'-dichlorodihydrofluorescein diacetate

Drp1 - dynamin-1-like protein

ECS - endocannabinoid system

FAAH - fatty acid amide hydrolase

FCCP - carbonyl cyanide-p-trifluoromethoxyphenylhydrazone

Fis1 - mitochondrial fission 1 protein

GR - glucocorticoid receptor

HPA - hypothalamic-pituitary-adrenal

MAGL - monoacylglycerol lipase

MAPK/EKR - mitogen-activated protein kinase/extracellular signal-regulated kinase

MFN1 - mitofusin 1

MFN2 - mitofusin 2

MR - mineralocorticoid receptor

mRNA - messenger ribonucleic acid

NGF - nerve growth factor

NMDA - N-methyl-D-aspartate

Nrf2 - Nuclear factor erythroid 2-related factor 2

Opa1 - optic atrophy type 1

PI3K - phosphoinositide-3kinase

PLC - phospholipase C

PPARγ - peroxisome proliferator-activated receptor-γ

RIPA - radioimmunoprecipitation assay

ROS - reactive oxygen species

RT - room temperature

SAM - sympathetic-adreno-medullar

SEM - standard error of the mean

SOD1 - superoxide dismutase 1

SOD2 - superoxide dismutase 2

SYPIII - synapsin III

TBHP - tert-butyl hydrogen peroxide

TBST - tween

TFAM - mitochondrial transcription factor A

THC - Δ9-tetrahydrocannbinol

TRPV1 - transient receptor vanilloid potential 1

UD - undifferentiated

4-HNE - 4-hydroxy-2-nonenal

ΔΨm - mitochondrial membrane potential

↓ - decrease

DECLARATION OF ACADEMIC ACHIEVEMENT

Andie MacAndrew contributed to the writing, experimental design, conducting of experiments, all aspects associated with data analyses, and figure/table generation in this thesis with the assistance of Dr. Sandeep Raha.

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CHAPTER 1. INTRODUCTION

Neurocircuitry of stress and impacts on the brain

Stress is a healthy response to normal pressures and problems but there is a thin line between stress responses that are adaptive and maladaptive [1]. Stress aids organisms in satisfying everyday demands in the short-term but can also impair functioning and have long term, plastic effects on the brain following repeated or extended conditions. Neuroplastic changes in the brain refer to adaptive morphological and functional reorganization, regeneration/degeneration of neuronal networks [2]. Physiological activation of the neurocircuitry of stress response is mediated by both slow and fast responses. The fast response involves the sympathetic-adreno-medullar (SAM) axis. The activation of the SAM axis mobilizes release of catecholamines, epinephrine and norepinephrine which interact with α-adrenergic and β-adrenergic receptors. Activation of specific 7-transmembrane receptors by these biological molecules facilitates several downstream events that result in physiological manifestations often associated with stress and anxiety. These events include increased heart rate, blood pressure, oxygen consumption and cardiac output. The slow response is related to the hypothalamic-pituitary-adrenal (HPA) axis. The activation of this axis results in the release of corticotropin-releasing hormone (CRH) from the hypothalamus. CRH facilitates the anterior pituitary release of adrenocorticotropin (ACTH) hormone into circulation which stimulates the adrenal cortex to secrete the glucocorticoid, cortisol, into the blood stream [3,4]. In humans, cortisol is the major stress effector molecule that acts as a primary messenger by passing through plasma membranes and eliciting it's effects mainly via glucocorticoid receptors (GR) [5]. The downstream biological events associated with GR binding include increased metabolism, cardiovascular output and gluconeogenesis, all of which are characteristic of fight or flight response [4].

The hippocampus is a limbic brain structure that has been implicated in the regulation of stress response. Steroid receptor binding has been shown to be more prevalent in the hippocampus relative to other brain structures making it a prominent location for the downstream effects of cortisol [6,7]. Studies have associated hippocampal neurons with negative feedback on HPA axis activation, terminating stress response when it is no longer adaptive, indicating the hippocampus as a prominent site for the regulation of stress response via glucocorticoid mediated feedback [8–10] (Figure 1).

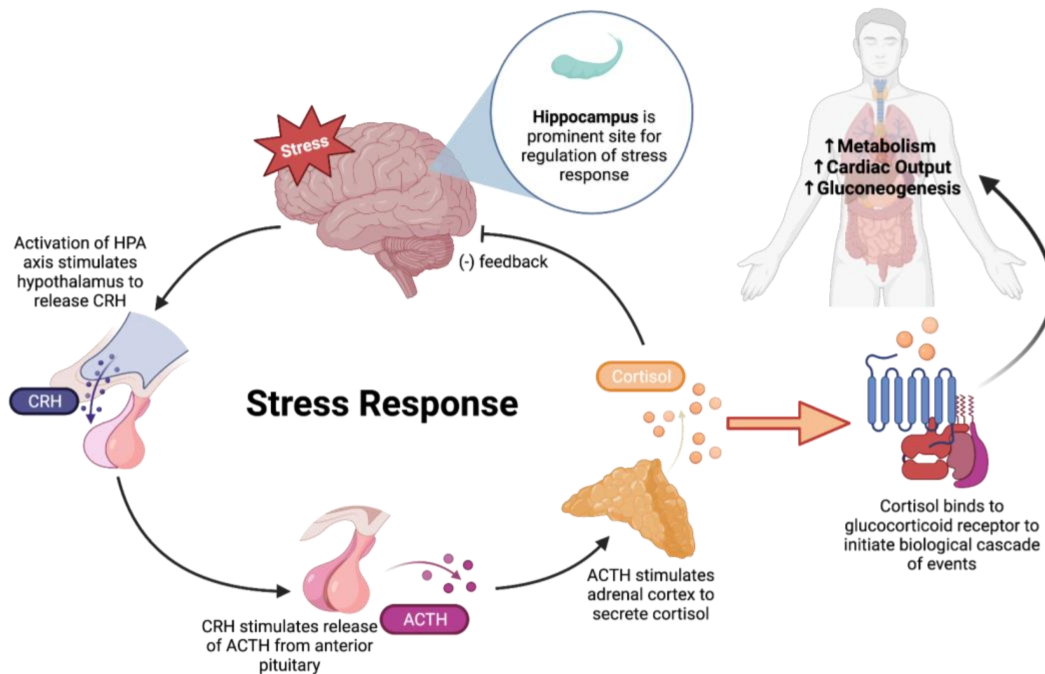

Figure 1. Schematic summary of the steps involved in HPA axis activated stress response. Following a stressor, the hypothalamic-pituitary-axis (HPA axis) is activated as part of the slow response of stress. Activation of this axis stimulates the hypothalamus to release corticotrophin-releasing hormone (CRH) which then stimulates anterior pituitary release of adrenocorticotrophin hormone (ACTH). This hormone is secreted into circulation and facilitates the release of the major steroid effector of stress response from the adrenal cortex. This molecule is referred to as cortisol in humans or corticosterone in rodents. Cortisol binds to mineralocorticoid receptors (MR) and glucocorticoid receptors (GR) to initiate cascades of downstream signalling mediating stress response. Hippocampal neurons exhibit an inhibitory undertone on the HPA axis and are also densely populated with GRs making it not only a regulatory site for cortisol secretion, but also a target for an overactive stress response. This figure was created using Biorender.

A stressor is an aversive stimuli that provides a real or perceived threat to homeostasis [11]. These stimuli can range from psychological (i.e. anxiety) to chemical (i.e. drugs) to environmental (i.e. pain) [12–14]. Chronic stress results in chronic HPA axis activation which promotes increased cortisol secretion. Increased glucocorticoid levels have been shown to impair hippocampal neurons morphologically and functionally [15–17]. Structural alterations mainly comprise dendritic loss and spine retraction in the CA3 of the hippocampus and to a lesser extent pyramidal neurons in the CA1 region [18–23]. Corticosterone, the murine form of cortisol, has been associated with a decrease in hippocampal neurogenesis [24]. Neurogenesis is a process that encompasses coordinated proliferation, differentiation and migration of neural precursor cells by way to form new neurons in the brain [25]. It occurs predominantly throughout development, but also continues in certain brain structures, such as the hippocampus, through the duration of adulthood [26]. Damage and/or atrophy to the hippocampus is associated with impairments in the feed-forward hippocampal inhibition of the HPA axis when the stress response should be terminated. This compromises the inhibition of the biological stress response resulting in prolonged HPA axis activation, enhanced cortisol release and further neuronal damage [27].

Impaired hippocampal neurogenesis has been linked to the onset, progression, and remission of major mood disorders, such as anxiety and depression [28,29]. The hippocampus is a brain structure primarily involved in memory and mood regulation and decreased hippocampal volume is associated with major depressive disorder manifestations in humans [30]; decreased neurogenesis could lead to a smaller hippocampus [31]. The role of hippocampal neurogenesis in determining affective states has been validated in vivo. A 2009 study reported an association between decreased hippocampal adult-born neurons (decreased neurogenesis) and increased anxiety-related behaviors [32]. In vivo models of depression also demonstrate decreases in

hippocampal neurogenesis [33] and standard pharmacological therapies for anxiety and depression demonstrate promotional and reversal effects [34].

A family of molecules referred to as neurotrophic factors play major roles in mediating neuroplasticity in the brain. The two prominent neurotrophic factors implicated in proper neurogenesis are brain-derived neurotrophic factor (BDNF) and nerve-growth factor (NGF). BDNF and NGF mainly elicit their effects through tyrosine kinase receptors (Trk), Trkβ and Trkα respectively. The pro-forms of these neurotrophic factors also evoke effects through p75 neurotrophin receptors [35]. BDNF and NGF regulate neuronal proliferation, differentiation and dendritic growth mainly via mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK), phosphoinositide-3kinase (PI3K), and phospholipase C (PLC)-gamma pathways downstream Trk receptor activation [36,37]. Reductions in both BDNF and NGF signaling are implicated in impaired neurogenesis [38,39]. The most representative neurotrophin linked to depression is BDNF [37,40]. Chronic corticosterone exposure is associated with decreased BDNF levels. Jacobsen and colleagues demonstrated that chronic corticosterone treatment (21 days) decreased BDNF mRNA expression and protein levels in the murine hippocampus [41]. In vitro analysis has demonstrated that low doses (1μM) of corticosterone acutely (24 hours) increases hippocampal neurotrophic factor (BDNF, NGF) mRNA levels. Contrastingly, longer exposure times (72 hours) result in decreased neurotrophic factor (BDNF, NGF) mRNA expression [42]. These results are in alignment with previously described evidence illustrating that an acute stress response is adaptive and neuroprotective whereas an uncontrolled, chronic stress response can be maladaptive and neurotoxic. Standard antidepressants reportedly increase neurotrophic factor, BDNF, in the hippocampus [43]. Unfortunately, although standard antidepressants demonstrate various neuroprotective aspects and aid in the treatment of many individuals suffering from

affective disorders, two-thirds of patients receiving standard pharmacological treatment are refractory, and half of patients relapse after an initial response [44] illustrating the need for novel avenues of therapy.

Cannabis use in the treatment of stress-related disorders

Cannabis has widely been used for both recreational and medicinal purposes ascribed to its anti-inflammatory, anti-nociception, anti-emetic, anticonvulsant and anxiolytic properties [45,46]. In recent years, the prevalence of cannabis use has dramatically surged due to legalizations, decreased social stigma and novel medicinal discoveries, making cannabis one of the most popularly used substances in Canada [47]. At the end of 2020, 6.2 million Canadians aged 15 or older reported using cannabis in the past 3 months with 7.9% using almost daily to daily [48]. This trend was noted in 2020 when there was a 25% increase in Canadians reporting cannabis use compared to the previous cycle [49]. Overall cannabis consumption has increased throughout the COVID-19 pandemic, with one of the most commonly reported reason for use being stress [50]. Further, a significant proportion of adults with affective disorders, such as anxiety and depression, turn to cannabis as a form of treatment, with approximately 25% of Canadian adults diagnosed with mood and anxiety disorders using cannabis to self-medicate [49]. These trends demonstrate the exponential increase in cannabis use, as well as the relationship between cannabis use, stress and affective disorders.

THC and CBD: neuroprotective or neuroprogressive?

Cannabis is a complex plant that comprises over 400 chemical entities, with approximately 70 identified bioactive components called cannabinoids [46]. The two phytocannabinoids of major interest are Δ9-tetrahydrocannbinol (THC), the primary psychoactive compound in cannabis, and cannabidiol (CBD), a non-psychoactive constituent. THC and CBD interact with the

endocannabinoid system (ECS) along with other biological systems to initiate a cascade of biochemical events [51–53]. There is a range of conflicting data investigating the impact of THC and CBD on neuronal morphology and functionality. Currently available research is under much debate as to if these exogenous bioactive molecules may, in the future, pose as novel compounds for the treatment and prevention of neuroprogressive and neurodegenerative disorders or if they serve as neurotoxic elements, impairing development and regulation of homeostatic brain functioning and morphology. The biochemical and physiological outcomes related to THC and CBD exposure may be strikingly variable depending on factors such as concentration, developmental time-periods of exposure, durations of exposure and tissue specific variations. Based on current clinical survey evidence claiming a substantial proportion of individuals use cannabis as a self-prescribed therapy to treat anxiety and depression [49], one may initially postulate the impacts of major cannabinoids on neuronal integrity are likely to be positive and/or neuroprotective. Various research has been conducted which supports this hypothesis.

THC, the psychoactive component of cannabis, has been found to improve components of neurodegeneration in in vivo models of multiple sclerosis [54], Parkinson's disease [55,56], Huntington's disease [57], amyotrophic lateral sclerosis [58] and Alzheimer's disease [59,60]. Recent research also illustrates how THC exposure can alter BDNF levels in circulation and in the brain. Butovsky and colleagues reported intraperitoneal injection of THC (1.5mg/kg) over 7 days increased BDNF mRNA in specific brain areas involved in reward and addiction (nucleus accumbens, ventral tegmental area, medial prefrontal cortex and paraventricular nucleus) [61]. Interestingly, a clinical study reported increased BDNF serum levels in healthy controls exposed to THC but no change in chronic users exposed to THC [62], implying that exclusively acute THC exposure may enhance BDNF production. This is supported by a murine study that found that 1

hour after THC injection (1mg/kg), THC increased BDNF mRNA levels in the CA1 and CA3 of the hippocampus [63]. Although Butovsky and colleagues detected no significant changes in hippocampal BDNF levels and both studies utilized the same breed of rat (adult male Sprague-Dawley rats); the different durations of treatment and slight variance in concentrations may account for these variabilities. It is also unclear if Butovsky analyzed mRNA levels in specific hippocampal subregions (CA1, CA2, CA3) as the latter study described. Another in vivo study linked THC exposure (1.5/3mg/kg) to enhanced hippocampal neurogenesis and improved cognitive function [64]. Much of THC's neuro-promoting and neuroprotective effects have been ascribed to THC's regulation of oxidative stress. Oxidative stress occurs when there is an imbalance between reactive oxygen species (ROS) and antioxidants. When ROS levels exceed antioxidant capacity this can have a destructive effect on cellular components [65]. ROS that contribute to neurodegeneration include hydrogen peroxide (H_2O_2), superoxide anion (O_2^-), and highly reactive hydroxyl radical ($HO^{\bullet}$) [66]. These species are highly reactive biomolecules that can damage DNA, proteins and lipids within the cell, impairing membrane integrity and impeding cellular energy production resulting in neuronal death and degeneration [65]. Reportedly, THC was able to reduce ROS by ~80% in an in vitro model of oxidative stress induced by hydrogen peroxide [67]. Similarly, a plethora of evidence has implicated CBD as a potentially pro-neurogenic compound. CBD (1nM-0.1μM;10mg/kg) exhibits anti-inflammatory and neuroprotective effects, increasing hippocampal neurogenesis, in in vitro and in vivo models of Alzheimer's disease. These effects were dependent on peroxisome proliferator-activated receptor-γ (PPAR-γ) activity [68]. CBD has also been reported to illicit anti-inflammatory effects in the hippocampus via serotonergic 5-HT1A receptor. In this study CBD (5mg/kg) was also shown to have a rescuing effect on hippocampal BDNF gene expression in a model of hepatic

encephalopathy, although this effect was independent of 5-HT1A receptor activity [69]. A 2013 study further exemplifies CBD's ability to induce hippocampal neurogenesis in a mechanism related to increased anandamide (AEA) levels through CBD-mediated inhibition of fatty acid amide hydrolase (FAAH) [70]. Similar to THC, CBD has been shown to increase BDNF mRNA in multiple brain regions (hippocampus, medial prefrontal cortex) in in vivo models of depression [71]. The main mechanisms proposed to facilitate much of CBD's neuroprotective effects involve CBD's regulation of oxidative stress and inflammation (reviewed in [72]).

Contrastingly, in an early development environment, studies have identified aspects of THC and CBD exposure related to impairments in proper brain development and function. Prenatal cannabis exposures have been linked with fetal growth restriction, learning disabilities and memory impairment in offspring [73]. Prenatal THC exposure has been shown to alter hippocampal oscillations, brain hyperexcitability and spatial memory impairment in adult mice prenatally exposed. This study also reports prenatal THC exposure to affect development of pyramidal neurons and GABAergic interneurons, although the contribution of this to behavioral alterations remains unknown [74]. Miranda et al., assessed an in vitro model of fetal neuronal brain development and reported neurotoxic effects attributable to CBD exposure and precocious neuronal/glial differentiation and impairments on normal voltage-gated calcium signalling facilitated by THC [75] exemplifying the impacts of cannabinoids on developing neurons and neuronal differentiation. In contrast to previously described evidence, a study reported adolescent mice injected with THC (3mg/kg) for 21 days were found to have decreased hippocampal BDNF mRNA transcripts levels [76]. Further, chronic cannabis users are reported to have reduced serum nerve growth factor (NGF) levels compared to non-users [77]. NGF plays an important role in the development and maintenance of cholinergic neurons [77,78]. THC exposure has further been

related to increased oxidative stress in the brain and reduced brain metabolism and uncoupled respiration from oxidative respiration illustrating potential mechanisms of neurotoxicity related to metabolic markers of neuronal damage (i.e. low energy production) [79,80].

Mitochondria as major regulators of neuroplasticity

The mitochondria are integral organelles involved in cellular energy production via the metabolism of lipids, steroids and proteins and in the regulation of Ca^{2+}, ROS levels and apoptosis [81,82]. Neurogenesis comprises an increased rate of mitochondrial biogenesis, requiring increased mitochondrial genome and protein for proper neuronal growth and differentiation [82–84]. Through the generation of energy, regulation of homeostatic calcium and redox signalling, mitochondria play integral roles in regulating cellular processes comprising neuroplasticity such as neural differentiation, neurite outgrowth, apoptosis and dendritic remodelling [85]. Neuronal differentiation is associated with increased mitochondrial mass per cell. This increased mitochondrial content is linked with elevated ATP production, providing cells with the energetic support needed to achieve fundamental processes coordinating neurite outgrowth [85,86]. Following differentiation, neurons extend projections called neurites. Mitochondria are localized at the base of neurites and as axons form, mitochondria congregate at the growth cone. Reductions in mitochondrial content before or during this stage of axonogenesis prevents axon formation [87,88]. NGF has been reported to promote mitochondrial membrane potential and thus, ATP production, along with the transportation of mitochondria to sites of stimulation required for neurite outgrowth [89].

Mitochondria dynamically regulate through fusion and fission of inner and outer membranes. Three dynamin-related proteins mediate mitochondrial fusion, mitofusin 1 (MFN1), mitofusin 2 (MFN2) and optic atrophy 1 (Opa1). MFN1 and MFN2 tether and fuse outer

mitochondrial membranes while Opa1 mediates fusion of inner mitochondrial membranes. Mitochondrial fusion is mediated by Fis1 and Drp1. Fis1 recruits Drp1 to the surface of the mitochondria and Drp1 forms a ring-like structure around the mitochondria constricting the mitochondria leading to fission [90]. This dynamic balance plays an essential role in bioenergetic outputs and cellular adaption to environmental inputs [91–93]. Therefore, chronic dysregulation of mitochondrial dynamics may impact neuronal health through impaired mitochondrial bioenergetics. Mitochondrial dynamics impact autophagias regulation of dysfunctional mitochondria and mediate mitochondrial quality control. Mitophagy, mitochondrial autophagy, is the process by which damaged mitochondria are removed from the cell, balancing mitochondrial content with mitochondrial biogenesis. Dysregulation of dynamic fusion and fission of mitochondria can mark impairments in mitochondrial function promoting mitophagy [94]. Mitochondrial dysfunction is associated with impaired energy supply, Ca^{2+} buffering, increased ROS production and apoptosis, all of which are characteristic of neurodegeneration [95].

Impacts of stress and cannabinoids on the mitochondria

As previously mentioned, stress results in enhanced glucocorticoid levels in the CNS; this has illustrated a biphasic effect on mitochondrial regulation. Cultured cortical neurons exposed to low doses of corticosterone (100nM-1μM) demonstrated increased mitochondrial membrane potential and Ca^{2+} holding capacity whereas higher doses (50 μM) demonstrated decreased membrane potential and Ca^{2+} holding capacity. These effects were also correlated with effects on neuronal viability with lower doses of corticosterone implementing a neuroprotective effect on subsequent kainic acid toxicity and higher doses enhancing apoptotic effects of kainic acid [96]. Chronic stress also induces inhibition of the mitochondrial respiratory chain [97] which is associated with impaired differentiation [98]. Furthermore, chronic, mild stress and corticosterone

injections (20mg/kg for 40 days) have been found to impair mitochondrial function, alter mitochondrial dynamic (↓ MFN1/2) and oxidative stress markers (↓ SOD1/2) in the rodent brain. These mitochondrial impacts were correlated to characteristics of depressive-like states [99]. Mitochondrial regulation is distinctly involved in neuroplastic modulations and the capacity for stress to alter mitochondrial function is apparent. An increasing proportion of evidence has begun to suggest mitochondrial dysfunction plays an integral role in the etiology of mood disorders. Modulations in mitochondrial biogenesis, energy metabolism, oxidative stress and apoptosis have associated with the pathophysiology of mood disorders. In mice, reductions in mitochondrial biogenesis marker, PGC1a, was associated with anxiety-like behaviors and reduced expression of genes associated with proper brain functioning [100]. Further, in vivo models of chronic stress report reduced brain antioxidant enzymes, superoxide dismutase (SOD), glutathione peroxidase, glutathione reductase, and catalase [101]. In cases of anxiety and depression, dysfunctional mitochondrial electron transport chains tend to produce an enhanced level of ROS and less ATP making mitochondrial function toxic and inefficient [82,102].

Phytocannabinoids, THC and CBD, have also been shown to influence mitochondrial function. CBD has been found to target the mitochondria to promote regulation of intracellular Ca^{2+} levels [103], which are dysregulated in neurodegenerative disorders such as Alzheimer's disease [104]. CBD has exhibited an ability to protect hippocampal neurons from oxygen-glucose deprivation/reperfusion-induced oxidative stress. In this study CBD was able to reverse increases in malondialdehyde, ROS, gluthionine, among other oxidative stress markers [105]. This is an example of CBD's antioxidant capacity and ability to rescue neurotoxic conditions related to redox homeostasis. CBD also demonstrated a rescuing effect on markers of mitochondrial dynamics (Drp1) and apoptosis (Cas3) in hippocampal cells exposed to iron toxicity [106]. Similar to CBD,

THC impacts mitochondrial parameters. Previous research from our lab illustrated attenuation of mitochondrial respiration and decreased mitochondrial membrane potential and induced oxidative stress in placental cells (BeWo) in response to THC exposure [107]. These findings are supported by Wolff and colleagues' findings stating that THC impairs complexes I, II and III of the mitochondrial respiratory chain and mitochondrial coupling and enhances oxidative stress in the brain [108]. Previous research supports the capacity of major cannabinoids to impact mitochondria function but the implication and mechanisms behind these effects in the context of stress response and neuroplasticity remains obscure.

Neurobiological overlap between stress and the endocannabinoid system

The ECS is an integral neuromodulatory system with roles in numerous physiological systems including brain, immune and endocrine regulation [51–53]. This system is composed of enzymes, endocannabinoids and cannabinoid receptors localized throughout a vast number of tissues. The two major endogenous molecules involved in the ECS are endocannabinoids, anandamide (AEA) and 2-arachidonyl glycerol (2-AG). These ligands bind to receptors; cannabinoid receptor type 1 (CB1) and cannabinoid receptor type 2 (CB2). CB1 receptors are most abundant in the central nervous system (CNS), while CB2 receptors are more highly concentrated in peripheral tissues with immune function. CB2 receptors have recently been located in the brain in subsets of neurons and microglia [109,110] and therefore, like CB1 receptors, are likely implicated in neurotransmission and neurodevelopment. Exogenous cannabinoid, THC, interacts with CB1 and CB2 receptors while CBD has little affinity for CB1/CB2 receptors and binds more potently with transient receptor vanilloid potential 1 (TRPV1) and transient receptor potential melastatin 8 (TRPM8) channels [111].

The ECS is involved in linking the perception of external or internal stimuli to neuropsychological or behavioral outcomes (anxiety, stress coping, fear response) [112]. Activation of the HPA axis, a well-characterized component of response to stress, causes a cascade of biological events that result in release of glucocorticoid hormones (corticosterone in rodents and cortisol in humans). Glucocorticoids enlist regulatory mechanisms, such as mobilizing energy stores, to help the organism adapt and function during stressful stimuli or experiences. These glucocorticoids exert negative feedback inhibition on the HPA axis to prevent overproduction of glucocorticoids. Excessive glucocorticoid secretion can result in a variety of health issues ranging from increased susceptibility to mood disorders to type II diabetes [113]. Endocannabinoid signalling is widely present throughout the CNS, specifically in corticolimbic circuits integral to behavioral and emotional homeostasis. CB1 receptors are densely populated in these corticolimbic circuits and blockage of these receptors has been associated with an increase in HPA activity under baseline conditions after acute stress [114], indicating the ECS provides an inhibitory undertone on the HPA axis and results in activation when disrupted [113]. Chronic stress decreases hippocampal expression of CB1 receptors but increases CB1 receptor activity in the prefrontal cortex demonstrating tissue-specific effects of stress on the ECS [115]. Stress has been shown to result in a bidirectional effect on endocannabinoids, decreasing AEA levels and increasing 2-AG levels in multiple brain structures (reviewed in [116]), including the hippocampus [117,118]. Decreases in AEA levels contribute to activation of the HPA axis and increased anxious-manifestations whereas increased 2-AG levels correspond to termination of the HPA axis and also plays roles in memory and synaptic plasticity [116]. Contrastingly to homotypic stress utilized in the above mentioned studies, chronic and heterotypic stress has demonstrated variable results on endocannabinoid levels. This may be related to differences in time after cessation of stress

exposure when AEA and 2-AG were measured or may be described by biochemical differences in chronic homotypic and heterotypic stress on HPA signalling [116]. Studies have reported increased FAAH with chronic and acute homotypic stress in various brain structures (reviewed in [95]), including the hippocampus [119,120], which may explain decreases in AEA since FAAH breaks down AEA resulting in its cellular uptake. Similarly, reductions in MAGL, the enzyme that degrades 2-AG, have been reported in the amygdala following chronic, homotypic stress further relating changes in the activity of enzymes linked with endocannabinoid hydrolysis to stress response and subsequent variations in endocannabinoid levels [121]. Overall, the ECS may have an integral role in mediating stress response but the cellular mechanisms responsible for this regulation require further investigation.

CHAPTER 2. PROJECT PROPOSAL

Rationale

Considering hyper-induced stress and depressive disorders are associated with increased cortisol/corticosterone levels and many individuals experiencing these effects reportedly use cannabis, we aimed to investigate the potential neuroprotective effects of cannabinoids, THC and CBD, on corticosterone-induced stress in undifferentiated HT22 hippocampal cells and the role of the mitochondria in potentiating these effects. *The overarching goal of our research is to determine the impact of THC and CBD on markers of neurogenesis and mitochondrial function in the context of corticosterone-induced stress in hippocampal HT22 cells.*

Research Hypotheses

1. We hypothesized that corticosterone would impair neurogenesis and mitochondrial function in HT22 hippocampal neurons.
2. We hypothesized that CBD would exhibit protective effects on neurogenesis and mitochondrial function at low concentrations in stress-induced HT22 hippocampal neurons.
3. We hypothesized that THC would promote neurotoxic effects on neurogenesis and mitochondrial function in stress-induced HT22 hippocampal neurons.

Project Objectives

1. **(a)** To develop and characterize a model of corticosterone-induced stress in HT22 hippocampal neurons

   (b) To interrogate the impacts of corticosterone on neurogenesis and mitochondria in undifferentiated HT22 cells

2. To investigate the potential for cannabinoids, THC and CBD, to mitigate the impacts of corticosterone-induced stress on neurogenesis and mitochondrial function in undifferentiated HT22 cells

CHAPTER 3. MATERIALS AND METHODS

Cell Culture

HT22 cells were utilized to generate our in vitro model of hippocampal neuron stress. HT22 cells are an immortalized, mouse hippocampal cell line subcloned from HT4 cells [122–124]. They are popularly used in studies investigating oxidative stress and neuroprotection [125–127]. The hippocampal HT22 cell line was cultured in 1X DMEM (containing 4.5 g/L glucose, L-glutamine and sodium pyruvate) supplemented with 10% heat-inactivated Fetal Bovine Serum (HI-FBS) and 1% penicillin-streptomycin (10,000 units/mL) (pen-strep), incubated at 37ºC with 5% CO_2. HT22 cells were differentiated using Neurobasal™ media supplemented with 1% pen-strep, 10% HI-FBS and 1X N2 supplement. Undifferentiated HT22 cells were seeded at a density of ~$2.6x10^3$ cells/cm^2 to achieve 70-80% confluency. After 24 hours, cells were exposed to treatments. Treatments are comprised of 100µM and 200µM corticosterone, 1µM, 5µM and 25µM CBD and 1µM, 3µM and 10µM THC individually and combination. Combinatorial treatments included simultaneous co-treatment with corticosterone 200µM and 1µM or 5µM CBD and simultaneous co-treatment with corticosterone 200µM and 1µM or 3µM THC (detailed in Figure 2). Vehicle controls include 100µM of methanol (MeOH) for THC/CBD and corticosterone.

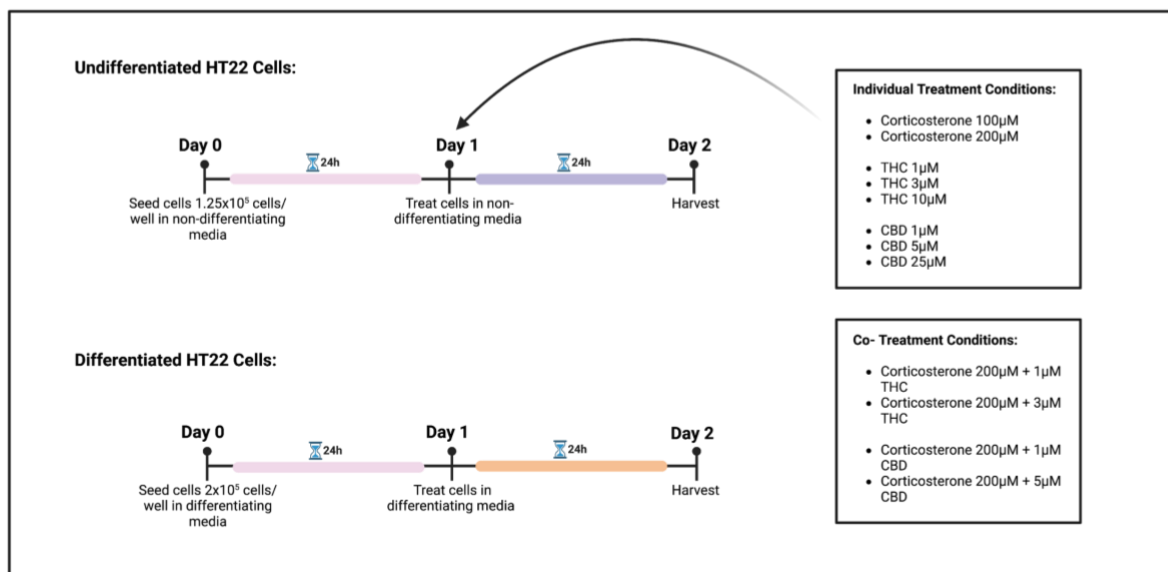

Figure 2. Experimental plan depicting in vitro methodology and treatment conditions for differentiated and undifferentiated HT22 cells. Differentiated HT22 cells are seeded $2x10^5$ cells/well in Neurobasal™ media supplemented with 1X pen-strep, 10% HI-FBS and 1X N2 supplement. Undifferentiated HT22 cells are seeded in 6-well plates at $1.25x10^5$ cells/well in DMEM containing 4.5 g/L glucose, L-glutamine and sodium pyruvate supplemented 5% HI- FBS and 1X pen-strep. 24 hours after seeding, HT22 cells are treated with corticosterone and/or THC or CBD at a range of concentrations in differentiating or non-differentiating media. Cells are harvested following a 24 hour treatment period. Figure made using Biorender.

Cell Proliferation and Cell Viability Assay

In order to assess mitochondrial activity and cell proliferation, undifferentiated HT22 cells were seeded at $1.5x10^2$ cells/well in a 96-well plate with 100µL of culturing media. 24 hours after plating, cells were treated with a range of corticosterone, CBD and THC concentrations along with the vehicle control. Background absorbance was determined via absorbance readings of blank wells containing media without cells. Following 24 hours of treatment exposure, the cells were treated with 20 µL of CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (Promega, G5421) and incubated for 45 minutes in a humidified chamber at 37°C with 5% CO_2. The absorbance was measured at 490nm using the Tecan Spark® Multimode Microplate Reader. Blank absorbance readings were averaged and subtracted from treated wells to correct for background noise. Additionally, to measure treatment-induced cytotoxicity, plasma membrane integrity was

assessed using the CyQUANT™ LDH Cytotoxicity Assay (C20300), to quantify the levels of lactate dehydrogenase released with a range of treatment concentrations. Lactate dehydrogenase release correlates to necrotic cell death. HT22 cells were cultured as described above and included two sets of untreated triplicates allocated for either spontaneous or 10X lysis buffer controls. Following 24 hours of treatment exposure, 50 µL of the Substrate Mix was added to 50 µL of media from each well for 30 minutes at RT after which the absorbance was measured at 490nm and 680nm using the Tecan Spark® Multimode Microplate Reader.

Neuritic Cell Analysis

HT22 cells were seeded $2.5x10^4$ cells/well in 6-well plates in differentiated media comprised of Neurobasal™ media supplemented with 1% pen-strep, 10% HI-FBS and 1X N2 supplement. Undifferentiated HT22 cells are seeded at a density $8x10^3$ cells/well in DMEM containing 4.5 g/L glucose, L-glutamine and sodium pyruvate supplemented 5% HI- FBS and 1% pen-strep constituting non-differentiating (proliferating) media. Microscope images of non-treated, undifferentiated and differentiated HT22 cells, were taken at 3-5 fields of view (technical replicates) in 4 wells (n=4 biological replicates) over 3 days (72 hours). Undifferentiated HT22 cells were treated with 100/200µM corticosterone and vehicle control, 100µM MeOH. Images of undifferentiated cells treated with corticosterone and vehicle were taken over 3 days (72 hours). Media was changed every other day and all images were taken at 20X magnification. The total number of cells and the total number of neuritic cells were manually counted for each image respectively. A neuritic cell was defined as a cell possessing a projection greater than one-half the length of the cell body, as previously described [128]. The cell diameter was identified as the longest diameter extending through the center of the cell (detailed in Figure 3). A cell that did not possess any projections, or possessed a projection that was less than one-half the length of the cell

body, was considered a non-neuritic cell. Any cells that extended outside of the image frame, or that were occluded by other cells, were excluded from both cell counts. Unadhered and/or dead cells were also excluded from both cell counts. Neuritic cell counts were normalized to total cell counts.

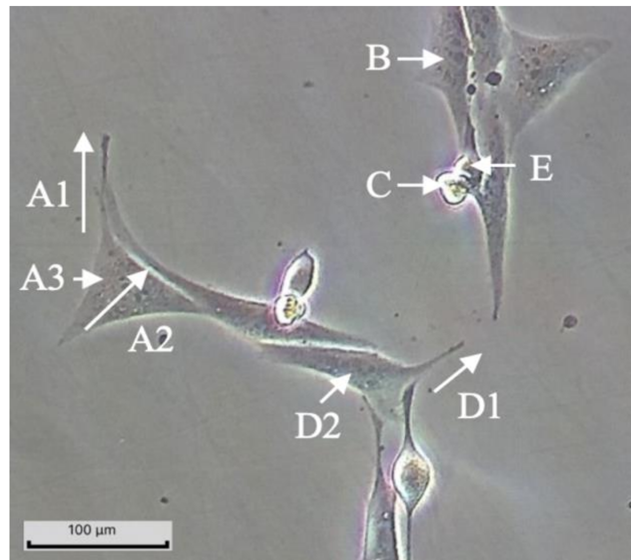

Figure 3. Inclusion and exclusion criteria for total and neuritic cell counts. (A1) An example of a neurite, a projection that is greater than one-half the length of the cell body. (A2) An example of the length of the cell body. (A3) An example of a neuritic cell. (B) An example of a cell excluded from both cell counts as it extends out of the frame of the image. (C) An example of a dead cell excluded from both cell counts. (D1) An example of a projection that is less than one-half the length of the cell body. (D2) An example of a non-neuronal cell. (E) An example of a cell excluded from both cell counts as it is occluded by another neuron. This image was taken at 20X magnification and the scale bar represents 100µm.

RT-qPCR

HT22 cells were seeded 1.25×10^5 cells/ well in 6 well-plates in non-differentiating media and were treated 24 hours after plating. After 24 hours of treatment, $\sim 4 \times 10^5$ cells were lysed with 500µL of TRIzol™ reagent (Thermofisher). Cellular components in TRIzol™ were scraped from the plate and transferred into Eppendorf tubes and either stored at -80°C or RNA was directly extracted. RNA was isolated and purified using Direct-zol™ RNA extraction kit (Zymo) following the designated manufacturers protocol. Nanodrop™ spectrophotometry instrument was used to

determine sample RNA concentrations (ng/µL) and purity. Purity was assessed via $A_{260/280}$ and $A_{260/230}$ absorbance values revealing protein or salt contaminations, respectively. cDNA Reverse Transcriptase Kit (Applied Biosystems™) was used to convert 1µg of RNA to cDNA using the designated manufacturers protocol. RT-qPCR was carried out using CFX384 Touch^{FM} Real-Time PCR Detection System (Bio-Rad). PCR results were normalized to housekeeping genes, RPLO and RPl37a, and analyzed using the ΔΔCT method ($2^{-\Delta\Delta CT}$).

Protein Extraction & Western Blot

Grown and/or treated HT22 cells were harvested as described above and dissolved in ice-cold radioimmunoprecipitation assay (RIPA) and sonicated for 5 pulses, 3 times at 7 Hz to homogenize samples. Bicinchoninic assay (BCA) was used to quantify total protein concentrations standardized to Bovine Serum Albumin (BSA). 35-100µg of protein were separated on a 10% polyacrylamide gel and then transferred onto a PVDF membrane. After protein transfer, membranes were blocked in 5% BSA or skim milk for 2 hours at room temperature (RT) and incubated with corresponding primary antibody at 4°C. Primary antibody was washed with tween (TBS-T) and membrane was incubated in secondary horseradish peroxidase-linked donkey anti-rabbit or sheep anti-mouse antibody for 1 hour at RT. Enzyme-linked chemiluminescence detection reagent was applied (BioRad #1705062S) and the blot was visualized using ChemiDoc imaging system (Biorad). Intensity of bands were analyzed using Image Lab and all blots will be normalized to β-actin or the stain-free image.

DCFDA (2’, 7’-dichlorofluorescin diacetate) Assay

HT22 cells were seeded 1.5×10^3 cells/well in a black-walled, clear bottom 96-well microplate in 100µl of culturing media. 24 hours after plating, cells were treated with

corticosterone (100µM, 200µM), CBD (1µM, 5µM, 25µM) and THC (1µM, 3µM, 10µM) concentrations along with the vehicle control, methanol (100µM). Levels of ROS were detected using Abcam DCFDA Cellular ROS Detection Assay Kit (ab113851) following the manufacturers protocol. 200µM of Tert-Butyl Hydrogen Peroxide (TBHP) in 1X Supplemented Buffer was incubated in a humidified chamber at 37˚C with 5% CO2 for 3 hours on every plate in triplicate as a positive control. After 3 hours of TBHP treatment, all wells were treated with 2 µM DCFDA solution in 100µl of Supplemented Buffer for 45 minutes and analyzed on the Tecan Spark® Multimode Microplate Reader at an excitation/emission of 485/535nm. Fluorescence readings were normalized to total protein content (BCA).

JC-1 Mitochondrial Membrane Potential Assay

HT22 cells were seeded 1.5×10^3 cells/well in a black-walled, clear bottom 96-well microplate in 100µl of culturing media. 24 hours after plating, cells were treated with corticosterone (100µM, 200µM), CBD (1µM, 5µM, 25µM) and THC (1µM, 3µM, 10µM) concentrations individually or in combination along with the vehicle control, methanol (100µM) for 24 hours. Treatment-stimulated changes in mitochondrial membrane potential (ΔΨm) were assessed using the fluorescent reagent tetraethylbenzimidazolylcarbocyanine iodide (JC-1) with the JC-1-Mitochondrial Membrane Potential Assay kit (Abcam, Cat. No. ab113850) following the manufacturer's protocol as previously described by our lab [129]. 100µM of carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) was used as a positive control. Following 24-hour treatment, cells were washed once with PBS and then incubated with 20µM JC-1 dye in 1X supplemented buffer for 10 minutes at 37 °C, protected from light. JC-1 dye was then removed, cells were washed once with PBS, 100 µL of fresh supplemented buffer was added to each well. Tecan Spark® Multimode Microplate Reader was used to measure red fluorescence

excitation/emission (535nm/590 nm) and green fluorescence excitation/emission (475 nm/530 nm). Background fluorescence was subtracted from the fluorescence of treated cells, then the ratio of red (polarized) fluorescence divided by that of green (depolarized) fluorescence was obtained.

Statistical analysis

Statistical analysis of results was conducted using Graphpad Prism Software V6.0. All in vitro portions of experimentation comprise 3-6 biological replicates ($n \geq 3$) and technical duplicates or triplicates. Data sets with two-groups will be compared using two-tailed Student's t-test. Data sets with two or more groups will be analyzed using one-way or two-way analysis of variance (ANOVA) and corrected using Bonferroni post hoc test. Experimental results will be presented as means of independent data points ± standard error of the mean (SEM). Post hoc significance will be expressed as: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.00001$ (*****).

CHAPTER 4. RESULTS

Objective 1a. The development of an in vitro model of corticosterone-induced stress in HT22 cells

Differentiation of HT22 cells and corticosterone treatment does not significantly impact percentage of neuritic cells and neurite length in undifferentiated HT22 cells.

To explore the impact of differentiation on HT22 cell phenotype we compared morphological changes and neuritic cell count variations over 72 hours in non-differentiating media and differentiating media. The percent of neuritic cells was not different with differentiating media after 24 and 48 hours of differentiation compared to cells in non-differentiating media at these timepoints (Figure 4A). At 72 hours of differentiation, a significant increase in neuritic cells was detected (Figure 4A, $p < .01$). Assessment of cell morphology does not exhibit significant variations in neurite outgrowth with differentiating and non-differentiating media compositions (Figure 4C). Although, cell proliferation is enhanced with non-differentiating media compared to differentiating media (Figure 4C). There were no statistically significant variations in average neurite length with differentiation over 72 hours (Figure 4B). Corticosterone treatments at 100µM and 200µM also did not alter percentage of neuritic cells or average neurite lengths in undifferentiated HT22 cells with 24-72 hours of treatment (Figure 5A/B). Microscope images of HT22 cells did not reveal any significant phenotypic changes with the corticosterone treatments, although cell density appeared to decrease with corticosterone treatments compared to control (Figure 5C).

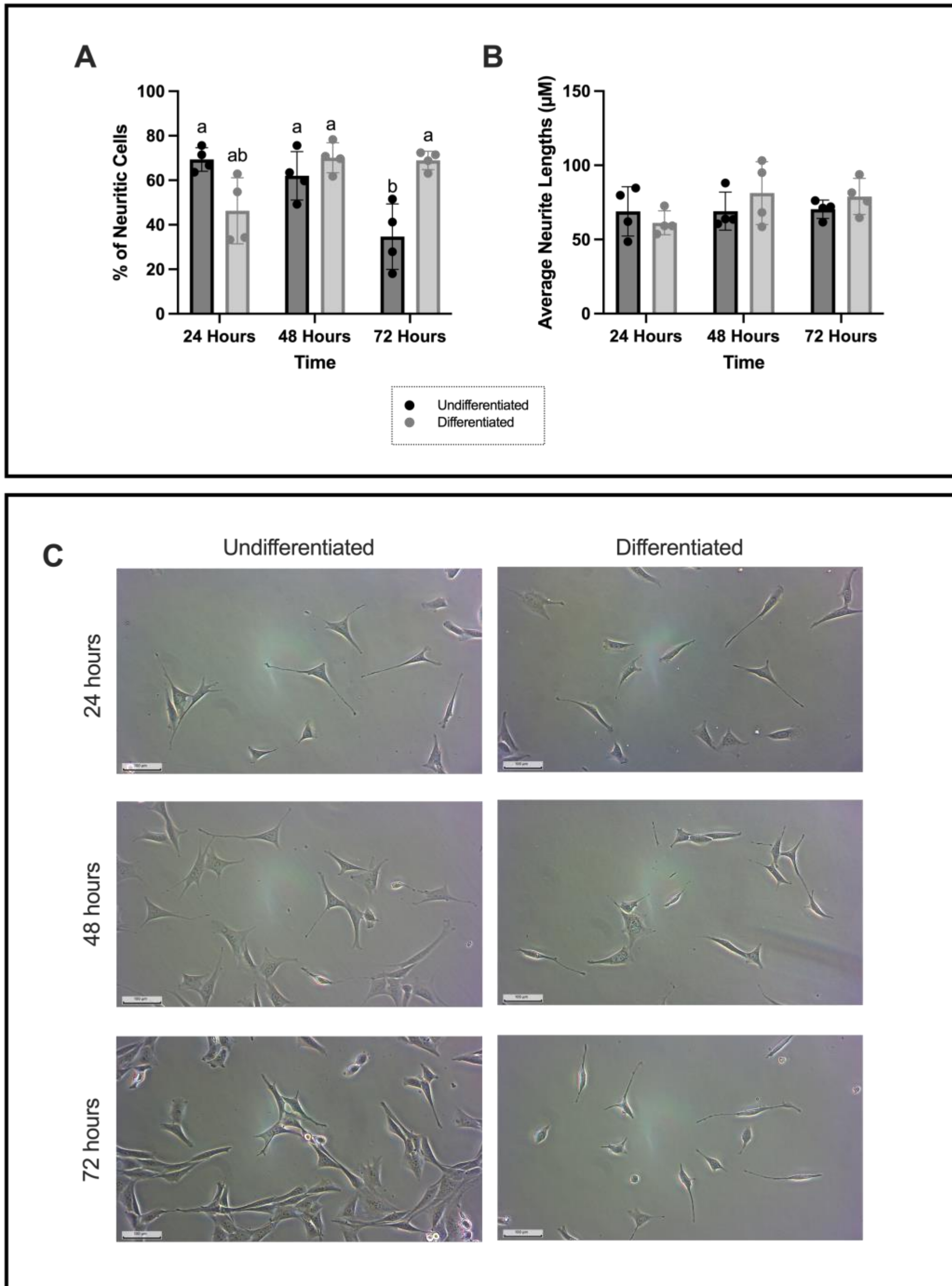

Figure 4. Differentiation does not significantly impact HT22 cell phenotype. HT22 cells were cultured in differentially supplemented media compositions every other day and (A) percentage of neuritic cells and (B) average neurite length were compared for undifferentiated and differentiated HT22 cells at 20X magnification over 72 hours. Undifferentiated cells were seeded 8×10^3 cells/well in DMEM containing 4.5 g/L glucose, L-glutamine and sodium pyruvate supplemented 5% HI- FBS and 1X pen-strep and differentiated cells were seeded 2.5×10^4 cells/well in differentiating Neurobasal™ media supplemented with 1X pen-strep, 10% HI-FBS and 1X N2 supplement and media was changed every other day for both media compositions. Data points represents mean ± SEM of 3-5 technical replicates from n=4 biological replicates. Significant

differences were determined using two way ANOVA followed by a Bonferroni post hoc test. Data points with different letters signify *P < 0.05; ****P < 0.0001. (C) Visual depictions of cells plated with DMEM containing 4.5 g/L glucose, L-glutamine and sodium pyruvate supplemented 5% HI-FBS and 1X pen-strep and Neurobasal™ media supplemented with 1X pen-strep, 10% HI-FBS and 1X N2 supplement at 20X magnification over 72 hours. The scale bar represents 100µM.

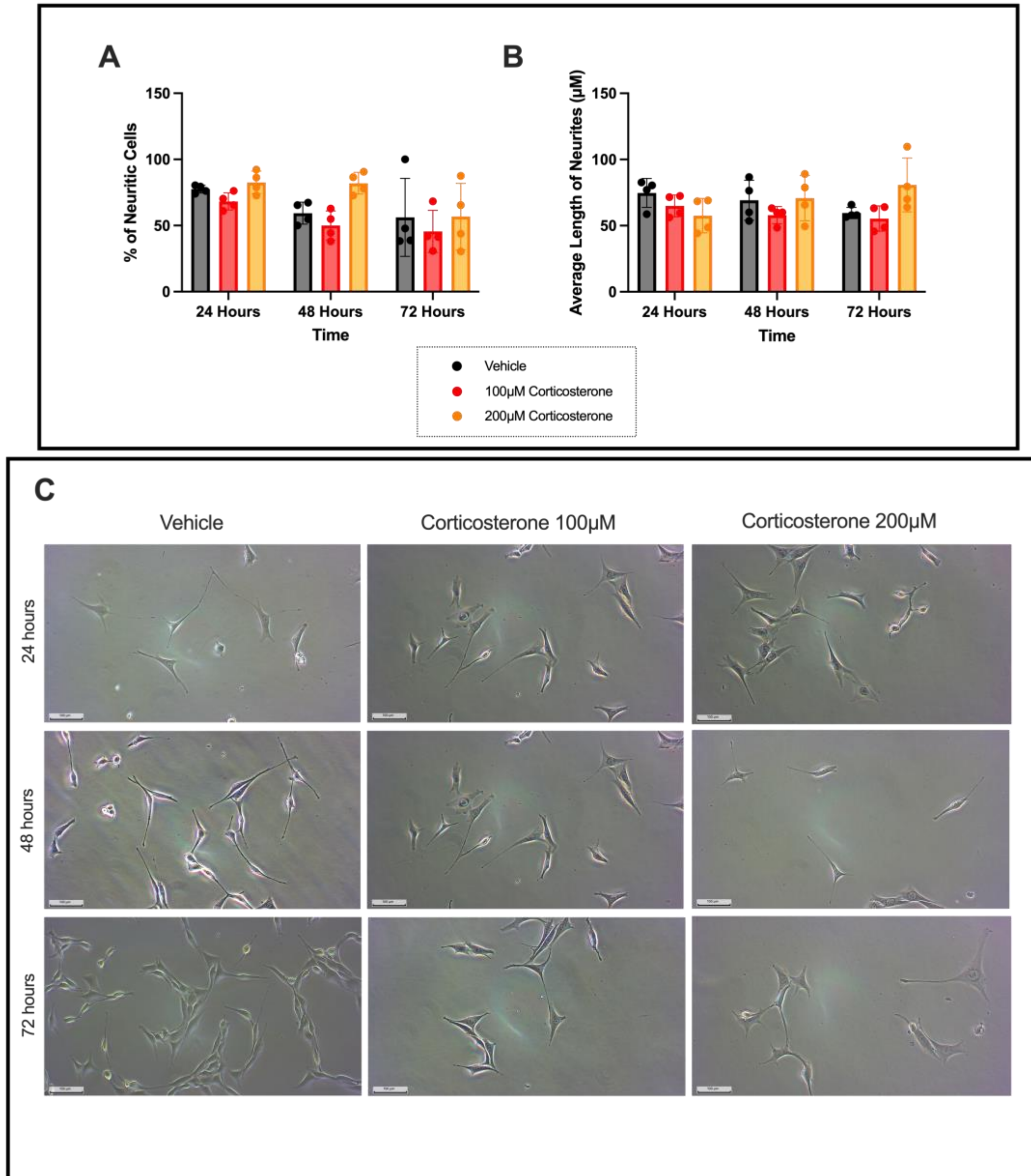

Figure 5. Corticosterone does not affect percentage of neuritic cells or neurite length in undifferentiated HT22 cells. HT22 cells seeded in 6-well plate $8x10^3$ cells/well in DMEM containing 4.5 g/L glucose, L-glutamine and sodium pyruvate supplemented 5% HI- FBS and 1X pen-strep and after 24 hours were treated with vehicle control, 100µM corticosterone and 200µM corticosterone every other day for 3 days (72 hours). HT22 cells were assessed each day for (A) percentage of neuritic cells and (B) average neurite length for all treatment groups over 3 days.

Data points represents mean ± SEM of 3-5 technical replicates from n=4 biological replicates. Significant differences were determined using two way ANOVA followed by a Bonferroni post hoc test. Data points with different letters signify $*P < 0.05$; $****P < 0.0001$. (C) Visual depictions of undifferentiated HT22 cells treated with vehicle control, 100µM MeOH, 100µM corticosterone and 200µM corticosterone are depicted over 3 days. All images were taken at 20X magnification and the scale bar represents 100µM.

Undifferentiated HT22 cells do not express detectable levels of CB1 receptor but do express other relevant receptors which are impacted by corticosterone treatments.

Undifferentiated HT22 cells respond to cannabinoid and corticosterone treatments (Appendix Figure 1-3). We aimed to investigate receptors though which our treatments may be eliciting effects. Western blot analysis of protein expression of cannabinoid receptors reveals that undifferentiated HT22 cells do not express detectable amounts of CB1 receptor (Figure 6A). Conversely, significant CB2 receptor protein expression was detected (Figure 6B). Transcript levels of GR, TRPV1, CB2 and peroxisome PPARγ were detected in undifferentiated HT22 cells via RT-qPCR (Figure 6C-F). Corticosterone decreased gene expression of GR (Figure 6C, $p <.001$) and PPARγ (Figure 6F, $p <.0001$) at 100µM and 200µM. Interestingly, 200µM of corticosterone increased TRPV1 expression (Figure 6D, $p <.05$) but no alteration in TRPV1 transcript expression was detected with 100µM corticosterone treatment. Both concentrations of corticosterone did not impact CB2 receptor expression (Figure 6E).

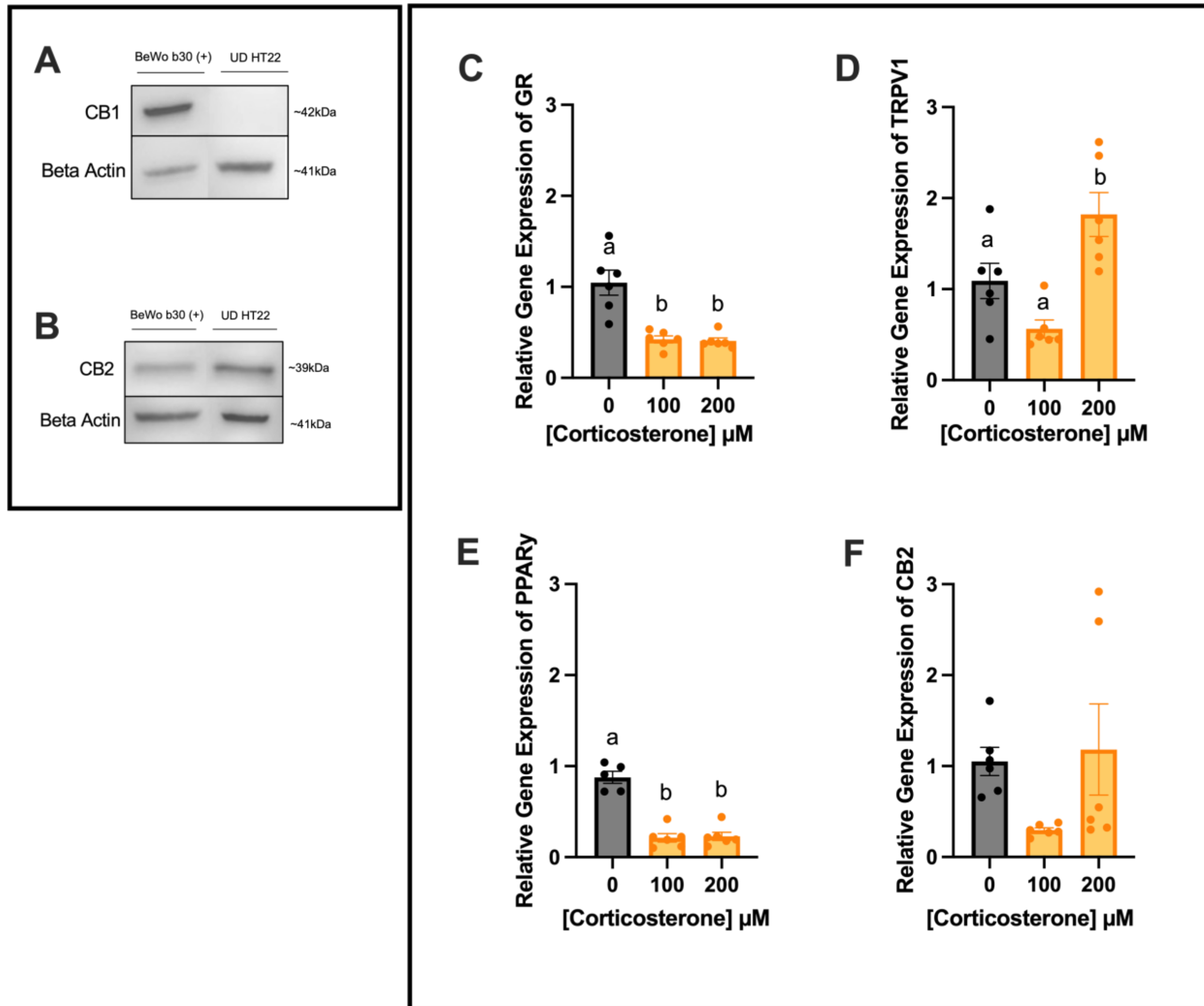

Figure 6. Undifferentiated HT22 cells do not express detectable levels of CB1 receptor but do express other relevant receptor which are impacted by corticosterone. Undifferentiated HT22 cells were plated 1.25x10^{5}cells/well and protein was extracted 48 hours later. Protein levels of (A) CB1 (B) CB2 and beta actin were assessed in BeWo B30 cells (positive control), HT22 cells. 60µg BeWo b30 protein/lane and 100µg HT22 protein/lane was loaded on SDS-PAGE and quantified using a polyclonal anti-CB1 receptor (Cayman, #101500) and anti-CB2 antibody (Cayman, #101550) normalized to beta actin (BD Transduction Laboratories, #612657). Undifferentiated HT22 cells were seeded in 6-well plates at 1.25x10^5 cells/well in DMEM containing 4.5 g/L glucose, L-glutamine and sodium pyruvate supplemented 5% HI- FBS and 1X pen-strep. Cells were harvested after 24 hours of 100µM and 200µM corticosterone treatments. RT-qPCR was used to assess changes in mRNA levels (A) GR, (B) TRPV1 (C) CB2 and (D) PPARγ relative to vehicle control. Changes in gene expression were normalized to RPLO and RPl37a. Data points represent the mean ± SEM ≥ 5 biological replicates. Significant differences were determined using one way ANOVA followed by a Bonferroni post hoc test. Data points with different letters signify *P < 0.05; ****P < 0.0001 relative to vehicle control.

Objective 1b. Corticosterone impairs neurogenesis and mitochondrial function in undifferentiated HT22 cells

Corticosterone decreases mRNA levels of markers of neurogenesis and neuronal plasticity.

In order to validate our model of corticosterone-induced stress and assess the effects of corticosterone on neurogenesis in our in vitro model, RT-qPCR was used to assess alterations in gene expression of neurotrophic factors, BDNF and NGF, and synaptic protein, SYPIII (Figure 7). Corticosterone decreased transcript levels of BDNF at 100μM and 200μM by ~27% and ~37% respectively (Figure 7A, $p < .01$; $< .001$). Similarly, there were also reductions in expression of SYPIII (~33% and ~49% respectively) at both corticosterone concentrations (Figure 7C, $p < .05$; $< .001$). In addition, NGF gene expression demonstrated a ~54% decrease but only with 200μM corticosterone treatment (Figure 7B, $p < .01$); no change in expression was detected with 100μM of corticosterone.

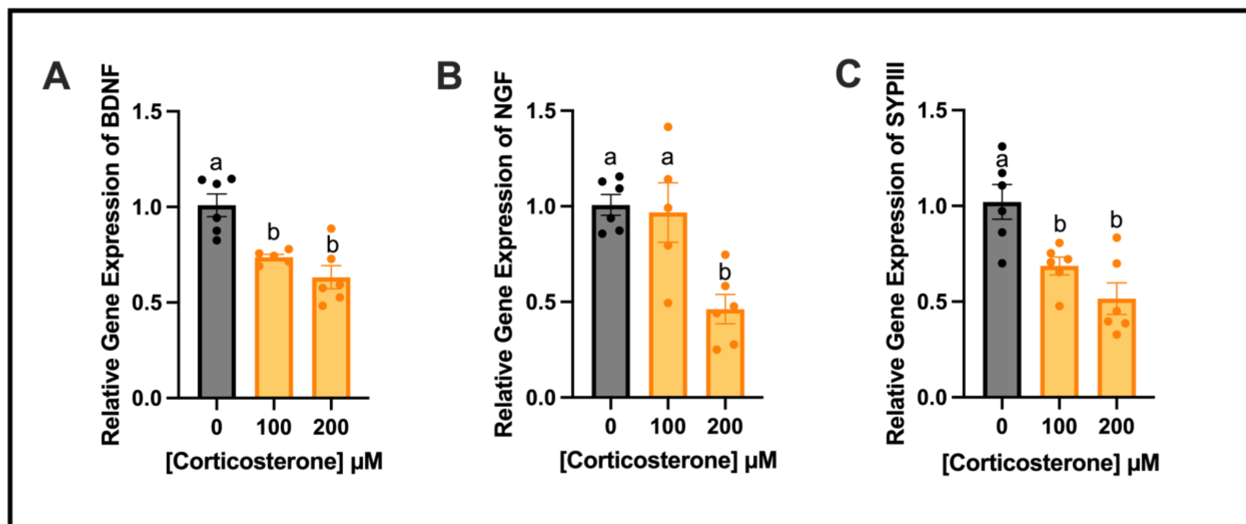

Figure 7. Corticosterone treatment of undifferentiated HT22 cells results in decreased mRNA expression of key indicators of neurogenesis and neurite outgrowth. Undifferentiated HT22 cells were seeded in 6-well plates at $1.25x10^5$ cells/well in DMEM containing 4.5 g/L glucose, L-glutamine and sodium pyruvate supplemented 5% HI- FBS and 1X pen-strep. Cells were harvested after 24 hours of 100μM and 200μM corticosterone treatments. Changes in mRNA expression of

(A) BDNF, (B) NGF and (C) SYPIII relative to vehicle control. Changes in gene expression were normalized to RPLO and RPl37a and assessed via RT-qPCR. Data points represent the data ± SEM of n≥5 biological replicates. Significant differences were determined using one way ANOVA followed by a Bonferroni post hoc test. Data points with different letters signify *P < 0.05; ****P < 0.0001 relative to vehicle control.

Corticosterone decreases mitochondrial biogenesis, mitochondrial membrane potential and mitochondrial fusion/fission in undifferentiated HT22 cells.

To characterize the impact of corticosterone-induced stress on HT22 mitochondria, we used RT-qPCR to detect genomic changes in levels of the master regulator marker of mitochondrial biogenesis, PGC1α, and its downstream effector or target genes, Nrf2 and TFAM. 200μM of corticosterone was found to decrease mRNA levels of PGC1α (~63% reduction) indicating a potential decrease in mitochondrial biogenesis (Figure 8A, p <.001); this decrease was not seen with 100μM treatment. Both downstream genes, Nrf2 and TFAM, were significantly downregulated with corticosterone treatments. Nrf2 expression was reduced by ~60% and ~76% by 100μM and 200μM of corticosterone respectively (Figure 8B, p <.0001). Similarly, TFAM mRNA levels were reduced ~59% by 100μM corticosterone and ~66% by 200μM corticosterone (Figure 8C, p <.0001). To further investigate the impact of corticosterone on mitochondrial content we looked at transcript levels of citrate synthase (CS), which is a validated marker of mitochondrial mass [130-134]. Corticosterone significantly decreased transcript levels of CS (~21% reduction) at 100μM and 200μM (Figure 8D, p <.01; <.05), which coincides with the decreases we detected in the mitochondrial biogenesis gene markers. We also found that corticosterone decreased mitochondrial membrane potential with 100μM and 200μM treatments (~6-10% reduction) further demonstrating potential bioenergetic impairments (Figure 8E, p <.05). Characterization of HT22 mitochondrial fusion and fission following corticosterone exposure was examined via alterations in transcriptional levels of mitochondrial fusion markers, MFN1, MFN2, Opa1 (Figure 9A-C) and

fission markers, Drp1 and Fis1 (Figure 9D-E). Corticosterone treatments caused significant decreases in gene markers of inner mitochondrial membrane fusion, MFN1 and MFN2. MFN1 transcripts were reduced ~34% compared to control at 200µM corticosterone (Figure XA, $p < .0001$) and MFN2 was decreased with 100µM (~29% reduction) and 200µM (~55% reduction) treatments (Figure XB, $p < .01$; $< .0001$). Next, we assessed a marker indicative of outer mitochondrial membrane fusion, Opa1. Corticosterone decreased Opa1 expression at both 100µM (~30% reduction) and 200µM (~49% reduction) (Figure 9C, $p < .01$; $< .0001$). Similarly, significant decreases in both fission markers were seen with 100µM and 200µM of corticosterone, Fis1 (~51% and ~60% reduction respectively) (Figure 9D, $p < .0001$) and Drp1 (~17% and ~50% reduction respectively) (Figure 9D, $p < .05$; $< .0001$).

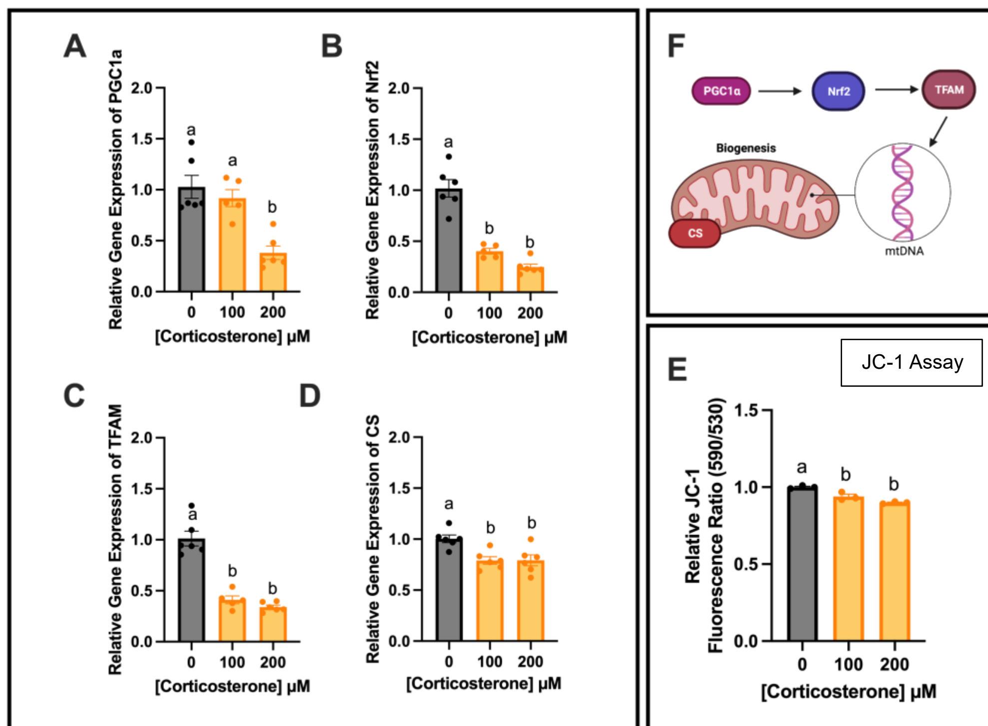

Figure 8. Corticosterone treatment of undifferentiated HT22 cells decreases mRNA expression of key markers of mitochondrial biogenesis and reduces mitochondrial membrane potential. Undifferentiated HT22 cells were seeded in 6-well plates at $1.25x10^5$ cells/well in DMEM containing 4.5 g/L glucose, L-glutamine and sodium pyruvate supplemented 5% HI- FBS and 1X pen-strep. Cells were harvested after 24 hours of 100µM and 200µM corticosterone treatments. Gene expression, relative to vehicle control, of major factors involved

in mitochondrial biogenesis (A-C) PGC1α, Nrf2 and TFAM, and (D) marker of mitochondrial content, CS, were normalized to RPLO and RPl37a levels using RT-qPCR. Data points represent the mean ± SEM ≥ 5 biological replicates. JC-1 was performed 24 hours after treatment to assess changes in mitochondrial membrane potential (ΔΨm) (E). HT22 cells seeded in 96-well plate $1.5x10^3$ cells/well in DMEM containing 4.5 g/L glucose, L-glutamine and sodium pyruvate supplemented 5% HI- FBS and 1X pen-strep and treated with range of corticosterone concentrations for 24 hours. The red fluorescence in excitation/emission (535nm/590nm) and green fluorescence excitation/emission (475nm/530nm) was measured using a Spark multimode microplate reader (Tecan Group Ltd.). Background fluorescence was subtracted from fluorescence of treated cells and then the ratio of red (polarized) fluorescence was divided by the green (depolarized) fluorescence. Data points represent the mean ± SEM of technical duplicates from n=3 biological replicates. Significant differences were determined using one way ANOVA followed by a Bonferroni post hoc test. Data points with different letters signify *P < 0.05; ****P < 0.0001 relative to vehicle control. Schematic representation of some of the key genes involved in mitochondrial biogenesis (F). PGC1α is designated as the master regulator of mitochondrial biogenesis. Nrf2 is a coactivator that binds to specific promoter sites that regulate the expression of effector proteins related to mitochondrial production such as, TFAM. TFAM is an enhancer protein that ensures mtRNA unwinding, promoting mtDNA transcription and maintenance. CS is an enzyme in the citric acid cycle that catalyzes the condensation of acetyl CoA and oxaloacetate to form citrate and CoA and is a marker of mitochondrial content. Figure made using Biorender.

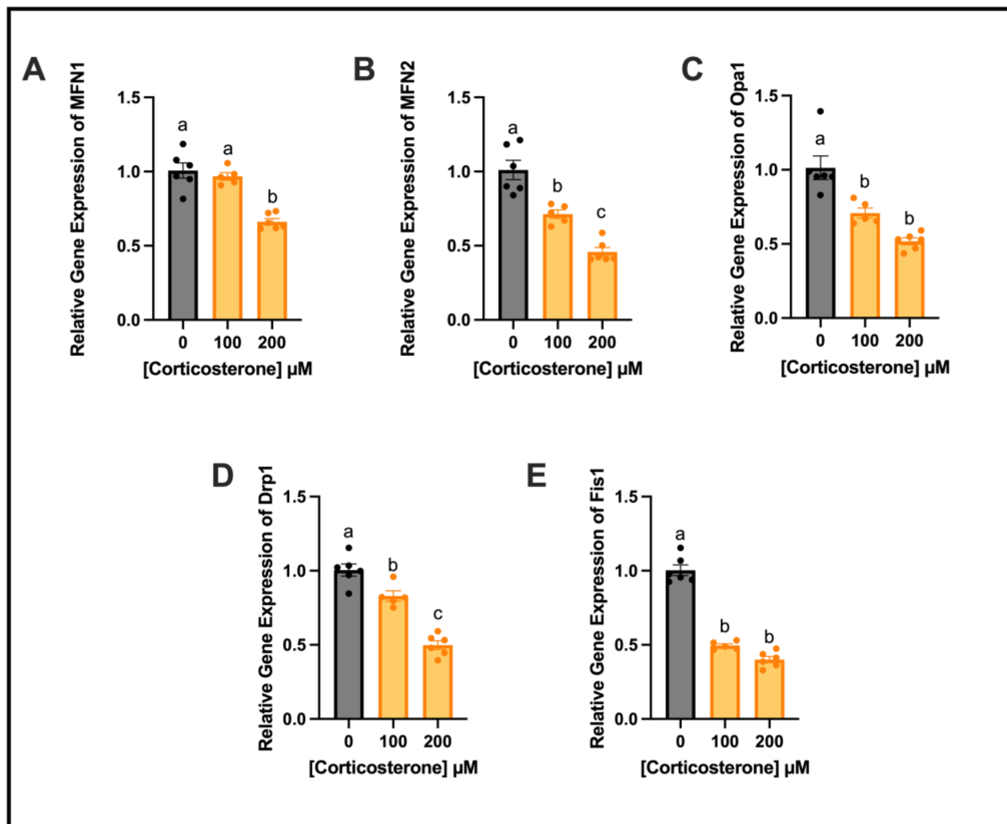

Figure 9. Corticosterone treatment of undifferentiated HT22 cells decreases transcript levels of mitochondrial fusion and fission markers. Undifferentiated HT22 cells were seeded in 6-well plates at $1.25x10^5$ cells/well in DMEM containing 4.5 g/L glucose, L-glutamine and sodium

pyruvate supplemented 5% HI- FBS and 1X pen-strep. Cells were harvested after 24 hours of 100µM and 200µM corticosterone treatments. RT-qPCR was used to assess changes in mRNA levels of biomarkers of (A-C) mitochondrial fusion and (D-E) mitochondrial fission relative to vehicle control. Changes in gene expression were normalized to RPLO and RPl37a. Data points represent the mean ± SEM ≥ 5 biological replicates. Significant differences were determined using one way ANOVA followed by a Bonferroni post hoc test. Data points with different letters signify *$P < 0.05$; ****$P < 0.0001$ relative to vehicle control.

Corticosterone decreases expression of antioxidant enzymes but does not impact ROS levels or lipid peroxidation.

Gene expression of antioxidant enzymes, SOD1 and SOD2, was assessed to explore the effects of corticosterone on redox homeostasis and oxidative stress (Figure 10). At 100µM, corticosterone decreased SOD1 and SOD2 expression by ~32% and 38% respectively (Figure 10A, $p <.01$; Figure 10C, $p <.0001$). At 200µM, corticosterone decreased SOD1 gene expression by ~53% (Figure 10A, $p <.0001$) and SOD2 expression by ~57% (Figure 10C $p <.0001$). Similarly, SOD1 protein expression was decreased by ~20% (Figure 10B, $p <.01$) and SOD2 protein expression was decreased by ~36% with 200µM of corticosterone (Figure 10D, $p <.05$). The DCFDA assay was utilized to further investigate the impact of these reductions on HT22 cell free radical production. However, we found no significant alterations in ROS levels with corticosterone treatments (Figure 10G). We also detected no significant changes in 4-HNE protein expression, a marker of lipid peroxidation, using western blot analysis (Figure 10E/F).

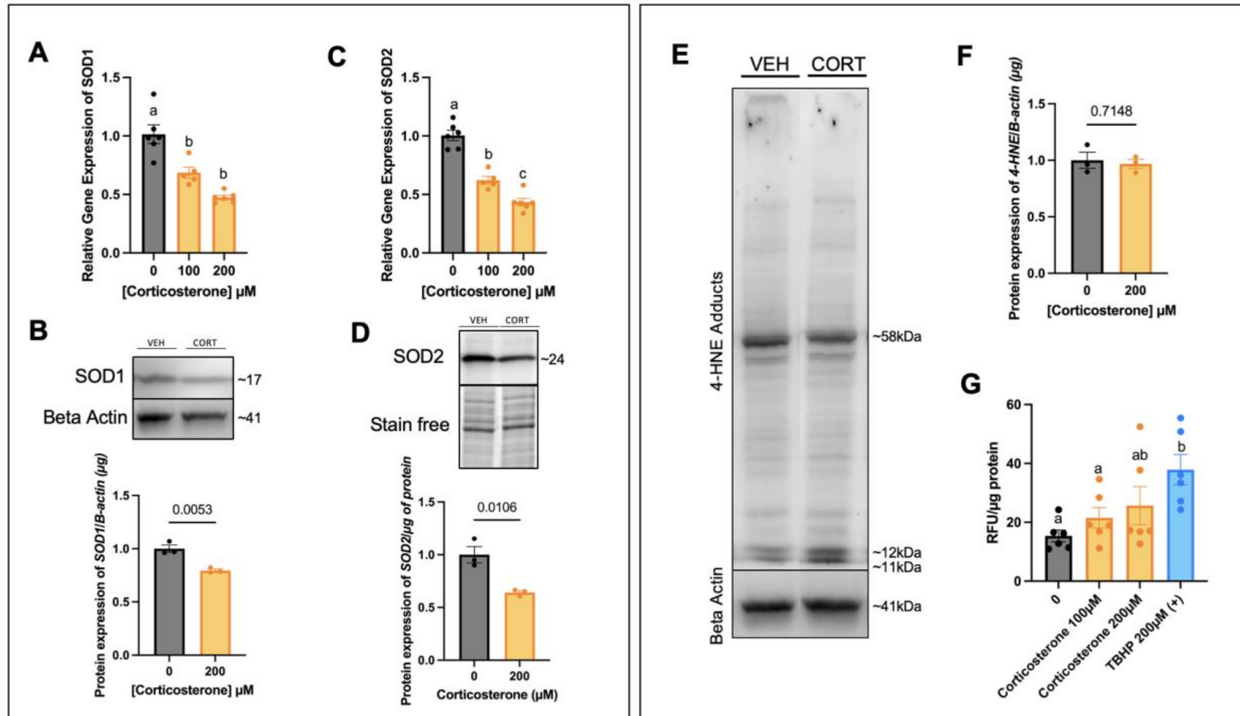

Figure 10. Corticosterone decreases antioxidant enzyme expression but does not impact redox homeostasis levels in undifferentiated HT22 cells. Undifferentiated HT22 cells were seeded in 6-well plates at 1.25×10^5 cells/well in DMEM containing 4.5 g/L glucose, L-glutamine and sodium pyruvate supplemented 5% HI- FBS and 1X pen-strep. Cells were harvested after 24 hours of 100µM and 200µM corticosterone treatments. RT-qPCR and western blotting were used to investigate changes in gene expression and protein levels of SOD1 (A/B) and SOD2 (C/D) normalized to β-actin or total protein content relative to control. A western blot demonstrating 3 prominent protein bands were quantified with molecular weights of approximately 58 kDa, 12 kDa and 11 kDa show immunoreactivity for 4-HNE modifications in HT22 cells treated with 200µM corticosterone compared to vehicle control treated cells (E). The intensity of the 3 prominent bands were normalized to β-actin (F). HT22 cells seeded in 96-well plate 1.5×10^3 cells/well in DMEM containing 4.5 g/L glucose, L-glutamine and sodium pyruvate supplemented 5% HI- FBS and 1X pen-strep and treated with range of corticosterone concentrations for 24 hours. DCFDA assay was performed 24 hours after treatment to assess alterations in ROS levels (G). 200 µM of Tert-Butyl Hydrogen Peroxide (TBHP) was used as a positive control and results were normalized to total protein content determined through the BCA assay. Data points represent the mean ± SEM from n=3-6 biological replicates. Significant differences were determined by a two-tailed Student's t-test or using one way ANOVA followed by a Bonferroni post hoc test. Bars with different letters signify $*P < 0.05$; $****P < 0.0001$ relative to vehicle control.

DCFDA Assay

Objective 2. THC and CBD impact corticosterone-induced effects on some pathways related to neurogenesis in undifferentiated HT22 cells

THC and CBD significantly decrease mRNA levels of markers of neurogenesis in undifferentiated HT22 cells.

To characterize the impacts of THC and CBD on HT22 cell neurogenesis we assessed the transcriptional levels of neurotrophic factors essential to proper neurogenesis, BDNF and NGF, and synaptic protein, SYPIII at a range of concentrations. THC decreased gene expression of neurotrophic factor BDNF by ~42% and ~47% at 1µM and 3µM respectively (Figure 11A, $p < .05$; $< .01$). Similarly, CBD decreased BDNF gene expression at 25µM by ~47% (Figure 11D, $p < .01$). No significant alterations in BDNF mRNA were detected with 10µM of THC or with 1µM and 5µM of CBD (Figure 11A/D). NGF mRNA was decreased by 1µM of THC by ~83% (Figure 11B, $p < .05$) but no differences were seen with 3µM or 10µM THC or CBD at any concentration (Figure 11B/E). THC and CBD both decreased expression of SYPIII at our highest concentrations 10µM (Figure 11C, $p < .001$) and 25µM (Figure 11E, $p < .0001$) respectively, but did not impact gene expression at lower concentrations (Figure 11C/E).

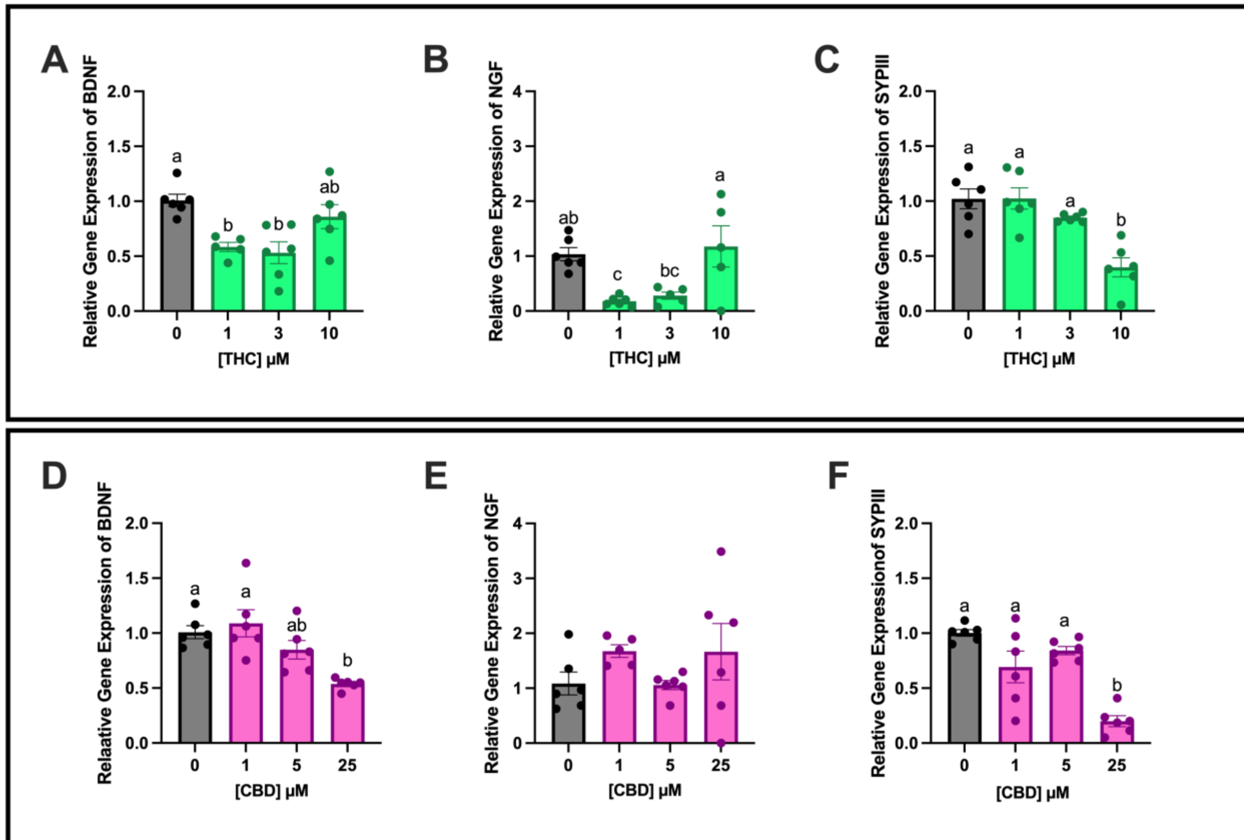

Figure 11. THC and CBD treatment of undifferentiated HT22 cells decreases mRNA levels of key regulators of neurogenesis and neurite outgrowth. Undifferentiated HT22 cells were seeded in 6-well plates at 1.25×10^5 cells/well in DMEM containing 4.5 g/L glucose, L-glutamine and sodium pyruvate supplemented 5% HI- FBS and 1X pen-strep. Cells were harvested after 24 hours of treatment with (A-C) 1µM, 3µM and 10µM of THC and (D-F) 1µM, 5µM and 25 µM of CBD. RT-qPCR was used to assess altered gene expression of markers of neurogenesis, BDNF (A/D) ,NGF (B/E) and SYPIII (C,F) normalized to RPLO and RPl37a relative to vehicle control. Data points represent the mean ± SEM ≥ 5 biological replicates. Significant differences were determined using one way ANOVA followed by a Bonferroni post hoc test. Data points with different letters signify $*P < 0.05$; $****P < 0.0001$ relative to vehicle control.

THC and CBD alter gene expression of mitochondrial biogenesis, mitochondrial fusion/fission markers and decrease mitochondrial membrane potential in undifferentiated HT22 cells.

RT-qPCR analysis of specific genes was used to explore the impacts of THC and CBD on mitochondrial biogenesis and content. THC and CBD increased mRNA levels of the major regulator of mitochondrial biogenesis, PGC1α at various concentrations (Figure 12A/B).
1µM of THC facilitated a sharp rise in PGC1α, increasing expression ~3.5 fold (Figure 12A, $p < .0001$). Likewise, CBD promoted ~2.5 fold increase PGC1α expression at 1µM (Figure 12B, $p < .01$). PGC1α was also increased at with 3µM and 5µM of THC and CBD respectively (Figure 12A/B, $p < .001$; $< .05$). However, these increases were not detected for downstream regulator, Nrf2, or its target gene, TFAM with THC (Figure 12A) or CBD (Figure 12B) at any of concentrations explored. Surprisingly, a significant decrease in gene expression of Nrf2 was seen with 3µM of THC (Figure 12A, $p < .05$). We further explored the potential for THC and CBD to impact mitochondrial production by assessing the expression of CS, a marker of mitochondrial content. Our results demonstrated no significant alterations in CS expression with THC treatments (Figure 12C, $p > .05$). Interestingly, we detected a significant decrease in CS expression with 5µM and 25µM CBD treatments by ~31% and ~59% respectively (Figure 12D, $p < .05$; $< .001$). Additional modulations in mitochondrial bioenergetic regulation attributable to THC and CBD were analyzed via assessment of mitochondrial membrane potential using the JC-1 assay. These results revealed 12-15% reductions in mitochondrial membrane potential with THC treatments (Figure 12F, $p < .01$; $< .001$) and 13-22% reductions with CBD treatments (Figure 12F, $p < .05$; $< .01$; $< .001$). Considering mitochondrial bioenergetic outputs are not only regulated by mitochondrial content and transmembrane potential but also through dynamic homeostasis, we investigated the

impacts of THC and CBD on mitochondrial fusion and fission though RT-qPCR analysis. We assessed outer mitochondrial membrane fusion markers, MFN1 and MFN2 with our range of THC and CBD concentrations. We found that THC decreased markers of outer mitochondrial fusion. mRNA levels of MFN1 (Figure 13A, $p < .05$; $< .01$) and MFN2 (Figure 13A, $p < .01$; $< .0001$) were both reduced with 1µM and 10µM of THC; no significant differences were seen with 3µM of THC. Transcript levels of inner mitochondrial membrane fusion, Opa1, were decreased with 1µM THC (Figure 13A, $p < .05$). No significant changes were seen in fission markers, Drp1 and Fis1, with THC (Figure 13B). CBD decreased markers of outer mitochondrial fusion, decreasing MFN1, MFN2 at 25µM (Figure 13C, $p < .001$; $< .0001$). CBD did not impact inner mitochondrial fusion marker Opa1 (Figure 13C). CBD marginally increased both mitochondrial fission markers, Drp1 and Fis1, at 1µM (Figure 13D, $p < .05$) but decreased Fis1 at 25µM (Figure 13D, $p < .0001$) illustrating a potential bi-phasic impact of CBD on mitochondrial fission. No changes in fission markers were detected with 5µM of CBD (Figure 13F).

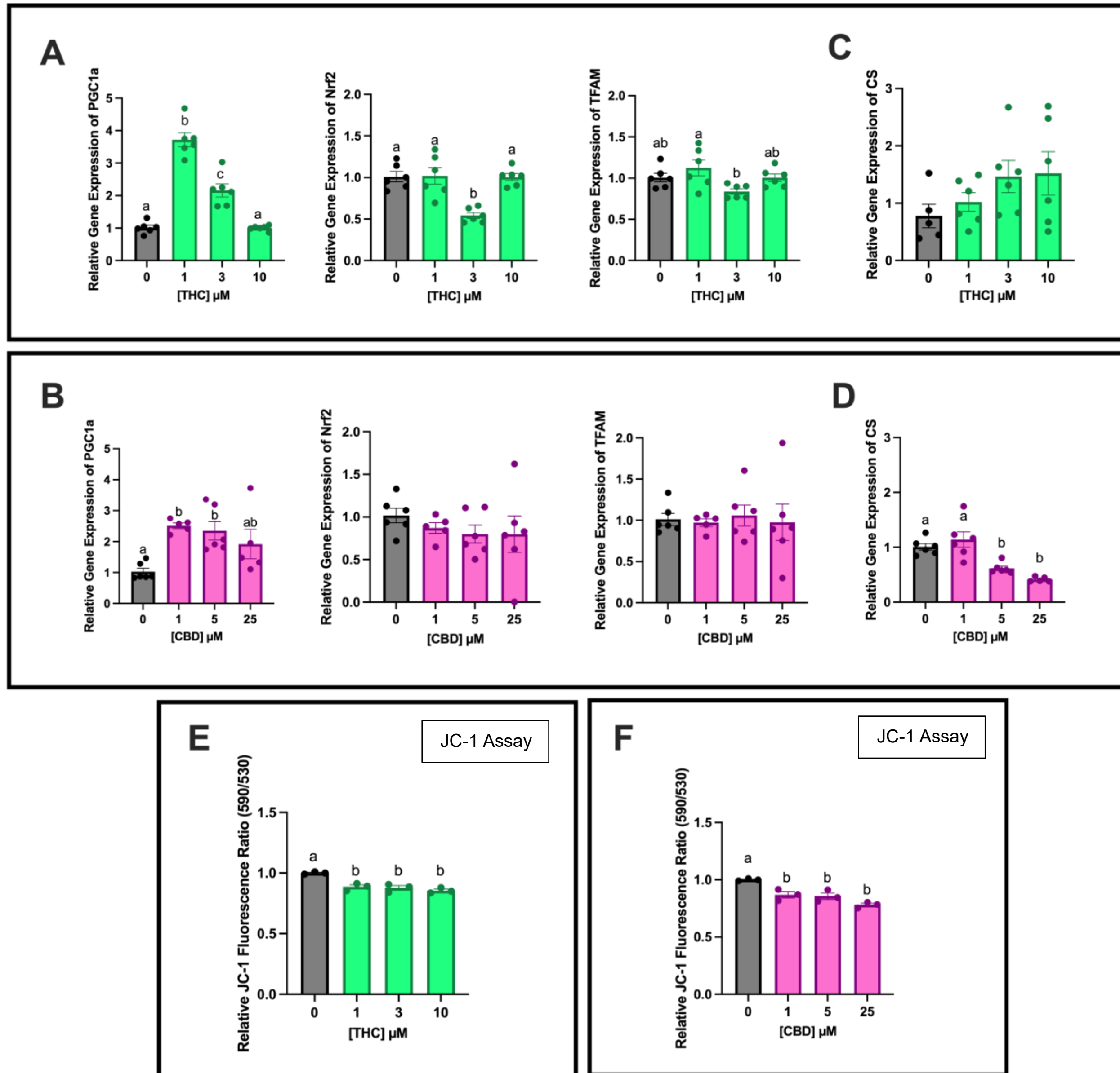

Figure 12. THC and CBD alter gene markers of mitochondrial biogenesis and decreases mitochondrial membrane potential at various concentrations in undifferentiated HT22 cells. Undifferentiated HT22 cells were seeded in 6-well plates at 1.25x10^5 cells/well in DMEM containing 4.5 g/L glucose, L-glutamine and sodium pyruvate supplemented 5% HI- FBS and 1X pen-strep. Cells were harvested after 24 hours of treatment with (A/C) 1μM and 3μM of THC and (B/D) 1μM, 5μM and 25 μM of CBD. RT-qPCR was used to assess altered gene expression of (A-B) PGC1α, Nrf2 and TFAM and CS (C/D) normalized to RPLO and RPl37a relative to vehicle control. Data points represent the mean ± SEM ≥ 5 biological replicates. JC-1 was performed 24 hours after treatment to assess changes in mitochondrial membrane potential (E/F). HT22 cells seeded in 96-well plate 1.5x10^3 cells/well in DMEM containing 4.5 g/L glucose, L-glutamine and sodium pyruvate supplemented 5% HI- FBS and 1X pen-strep and treated with range of corticosterone concentrations for 24 hours. The red fluorescence in excitation/emission (535nm/590nm) and green fluorescence excitation/emission (475nm/530nm) was measured using a Spark multimode microplate reader (Tecan Group Ltd.). Background fluorescence was

subtracted from fluorescence of treated cells and then the ratio of red (polarized) fluorescence was divided by the green (depolarized) fluorescence and normalized to average control fluorescence. Data points represent the mean ± SEM of technical duplicates from n=3 biological replicates relative to control. Significant differences were determined using one way ANOVA followed by a Bonferroni post hoc test. Data points with different letters signify *P < 0.05; ****P < 0.0001 relative to vehicle control.

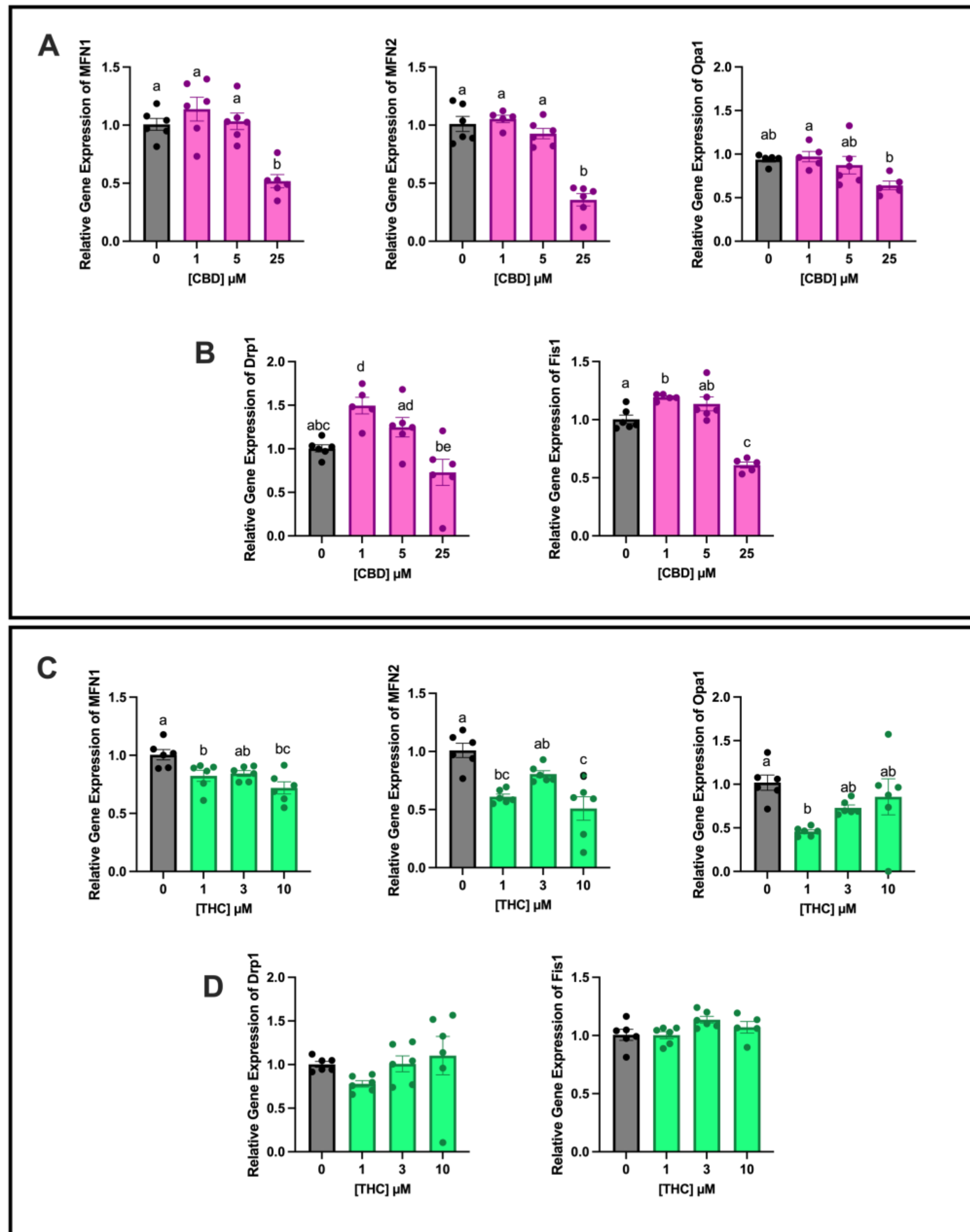

Figure 13. THC and CBD treatment of undifferentiated HT22 cells dysregulated mitochondrial dynamics. Undifferentiated HT22 cells were seeded in 6-well plates at $1.25x10^5$ cells/well in DMEM containing 4.5 g/L glucose, L-glutamine and sodium pyruvate supplemented 5% HI- FBS and 1X pen-strep. Cells were harvested after 24 hours of treatment with (A) 1µM,

3μM and 10μM of THC and (B) 1μM, 5μM and 25 μM of CBD. RT-qPCR was used to assess altered gene expression of (A/C) mitochondrial fusion markers, MFN1, MFN2, Opa1 and (B/D) mitochondrial fission markers, Drp1 and Fis1, normalized to RPLO and RPl37a. Data points represent the mean ± SEM ≥ 5 biological replicates relative to control. Significant differences were determined using one way ANOVA followed by a Bonferroni post hoc test. Data points with different letters signify *P < 0.05; ****P < 0.0001 relative to vehicle control.

THC and CBD decrease expression of antioxidants enzymes but do not impact ROS levels in undifferentiated HT22 cells.

THC and CBD have previously been related to various neuroprotective effects related to their mediation of oxidative stress [135]. For this reason, we examined the expression of gene markers related to mediation oxidative stress. Surprisingly, we found that THC and CBD decreased transcript expression of enzymes that directly scavenge ROS, SODs. THC decreased SOD1 expression by ~24% at 10μM and SOD2 expression by ~34% at 1μM (Figure 14A, p <.05). CBD decreased SOD1 levels at 25μM by ~31% and SOD2 expression at 25μM by ~29% (Figure 14C, p <.05). Considering these reductions in antioxidant enzymes, we assessed the effects of THC and CBD on ROS levels with cannabinoid treatments using the DCFDA assay. Interestingly, both THC and CBD did not significantly impact ROS levels at these concentrations (Figure 14B/D).

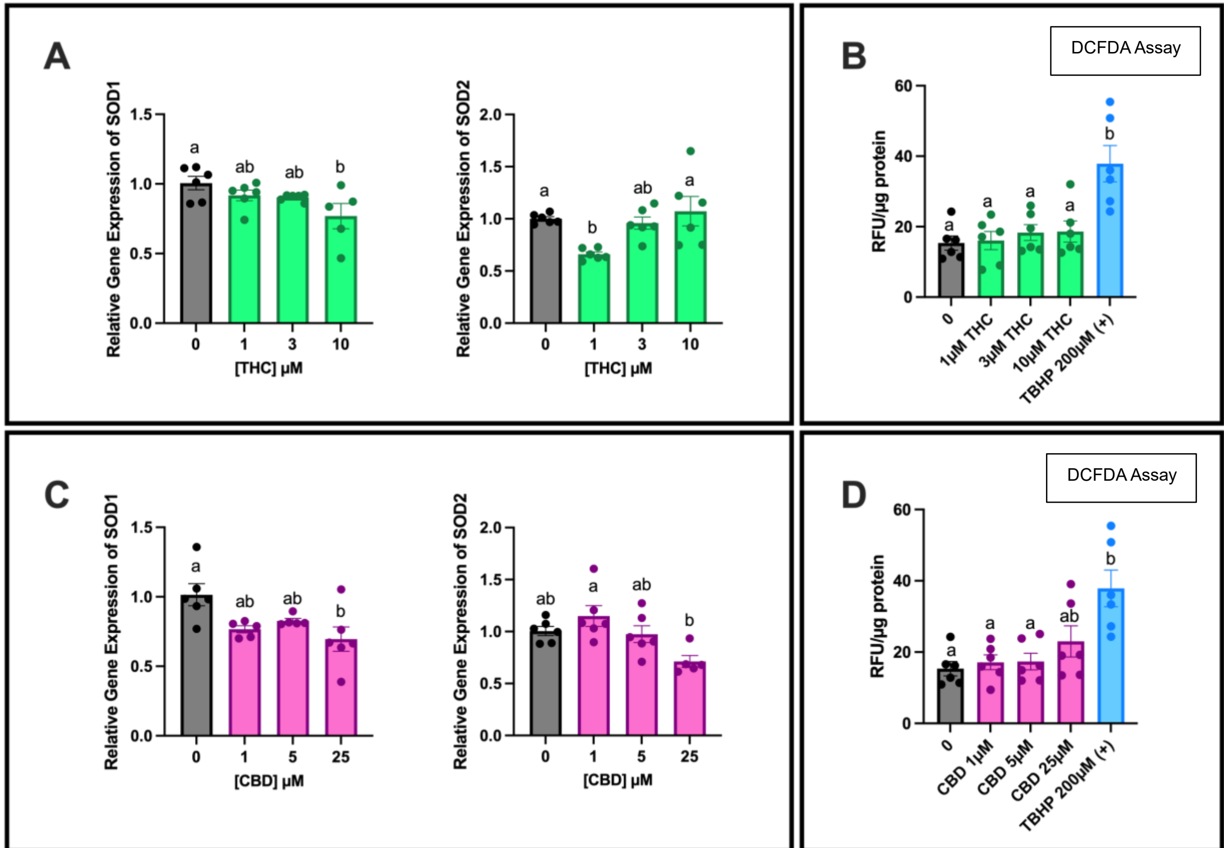

Figure 14. THC and CBD decrease expression of antioxidant enzymes but do not significantly impact ROS levels in undifferentiated HT22 cells. Undifferentiated HT22 cells were seeded in 6-well plates at 1.25×10^5 cells/well in DMEM containing 4.5 g/L glucose, L-glutamine and sodium pyruvate supplemented 5% HI- FBS and 1X pen-strep. Cells were harvested after 24 hours of treatment with (A-B) 1µM, 3µM and 10µM of THC and (C-D) 1µM, 5µM and 25 µM of CBD. RT-qPCR was used to assess altered gene expression of enzymes, SOD1 (A) and SOD2 (C) normalized to RPLO and RPl37a relative to control. Data points represent the mean ± SEM ≥ 5 biological replicates. Intracellular ROS levels were quantified using the DCFDA assay in undifferentiated HT22 cells (B/D). HT22 cells seeded in 96-well plate 1.5×10^3 cells/well in DMEM containing 4.5 g/L glucose, L-glutamine and sodium pyruvate supplemented 5% HI- FBS and 1X pen-strep and treated with THC at 1µM, 3µM and 10 µM (B) or CBD at 1µM, 5µM and 25µM (D) for 24 hours compared to vehicle control. 200 µM Tert-Butyl Hydrogen Peroxide (TBHP) was used as a positive control and results were normalized to total protein content determined through the BCA assay. Data points represent the mean ± SEM technical triplicates from n=6 biological replicates. Significant differences were determined using one way ANOVA followed by a Bonferroni post hoc test. Data points with different letters signify $^*P < 0.05$; $^{****}P < 0.0001$ relative to vehicle control.

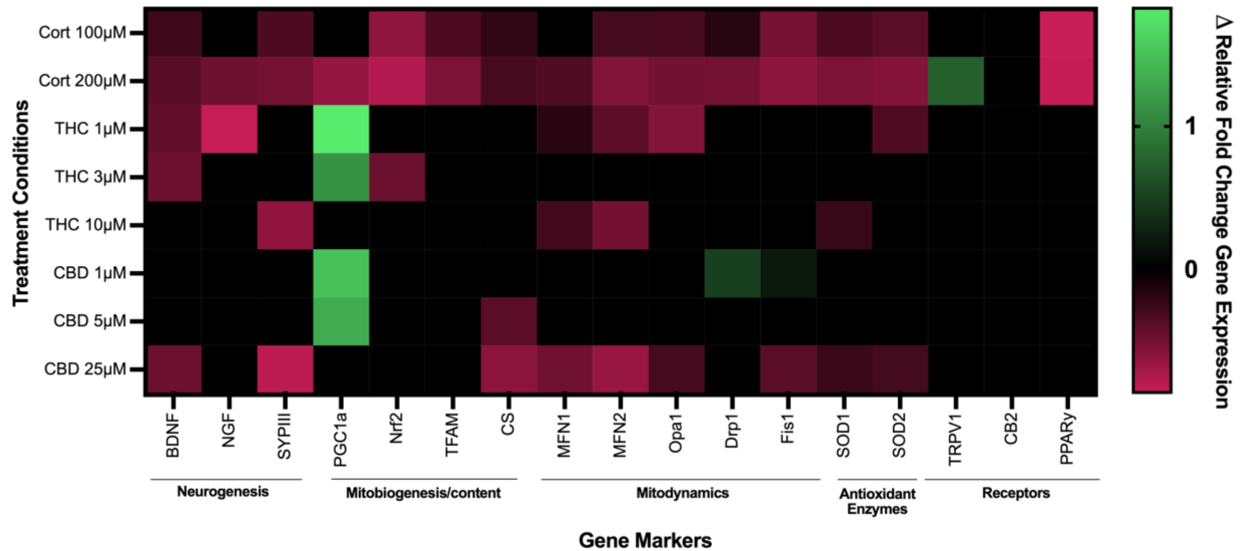

Figure 15. Heat map depicting alterations in transcript levels of neurogenesis, mitochondrial biogenesis, dynamic regulation, oxidative stress and receptor markers with individual corticosterone, THC and CBD treatments <u>compared to control</u> in undifferentiated HT22 hippocampal neurons. Undifferentiated HT22 cells were seeded in 6-well plates at 1.25×10^5 cells/well in DMEM containing 4.5 g/L glucose, L-glutamine and sodium pyruvate supplemented 5% HI-FBS and 1X pen-strep. Cells were harvested after 24 hours of treatment. Individual treatment conditions include 100µM and 200µM corticosterone (Cort), 1µM, 5µM and 25µM CBD and 1µM, 3µM and 10µM THC. Green panels indicate fold-change increases in gene expression and red panels represent fold-change decreases in gene expression compared to vehicle control. Black panels indicate no significant changes in marker expression compared to vehicle control. Gene expression of all markers were normalized to RPLO and RPl37a and include analysis of technical triplicates from ≥ 5 biological replicates. Significant differences were determined using one-way ANOVA followed by a Bonferroni post hoc test.

THC and CBD impact corticosterone-induced alterations in receptor expression and markers of mitochondrial biogenesis and fission but do not influence modulations in neurogenesis markers or mitochondrial membrane potential.

To explore the effects of THC and CBD on hippocampal neuron stress, HT22 cells were co-treated with combinations of 200µM of corticosterone with THC (1µM or 3µM) or CBD (1µM or 5µM) concentrations for 24 hours and RT-qPCR was used to explore variations in gene expression compared to control and individual cannabinoid and corticosterone treatments. Cannabinoids, THC and CBD, did not impact corticosterone-induced transcriptional changes in

markers of neurogenesis, BDNF, NGF or SYPIII (Figure 16). 200μM corticosterone treatment exhibited a decrease in these markers (Figure 7A-C, $p < .001$; $< .01$) but this reduction was not altered by co-treatments with THC or CBD. Similarly, CBD did not impact corticosterone-induced gene alterations in mitochondrial biogenesis markers (Figure 18; Mitobiogenesis/content) but THC did normalize gene expression of mitochondrial biogenesis marker, PGC1α, at 1μM and 3μM in co-treatments with corticosterone 200μM, significantly increasing this marker compared to decreased expression seen with individual corticosterone 200μM treatment (Figure 17D, $p < .001$; $< .01$). However, this regulatory impact was not seen with PGC1α downstream effectors, Nrf2 and TFAM or our marker of mitochondrial content, CS (Figure 18; Mitobiogenesis/content). Co-treatment with CBD did not impact any stress-induced alterations in mitochondrial fusion markers (Figure 18; Mitodynamics) but 1μM of CBD did increased expression of mitochondrial fission marker, Drp1, rescuing corticosterone-induced reductions (Figure 17F, $p < .0001$).

Next, we investigated the impacts of THC and CBD on corticosterone-induced alterations in antioxidant enzymes. We found that co-cannabinoid-treatments did not impact stress-related alterations antioxidant enzymes, SOD1 and SOD2 (Figure 18; Oxidative stress). THC and CBD also did not impact stress-related reductions in mitochondrial membrane potential (Figure 19A/B).

As corticosterone impacted transcript levels of various receptors known to demonstrate crosstalk with the endocannabinoid system, we aimed to investigate the potential for THC and CBD to affect stress-related alterations in receptor expression. CBD treatment resulted in increased TRPV1 expression following corticosterone treatment at all concentrations investigated (Figure 17B, $p < .05$; $< .01$). Similarly, THC normalized TRPV1 expression with 3μM co-treatment (Figure 17A, $p < .05$). THC and CBD also both normalized reduced PPARy expression (Figure 17C/D, $p < .0001$) but did not impact stress-related reductions in GR expression (Figure 18; Receptors).

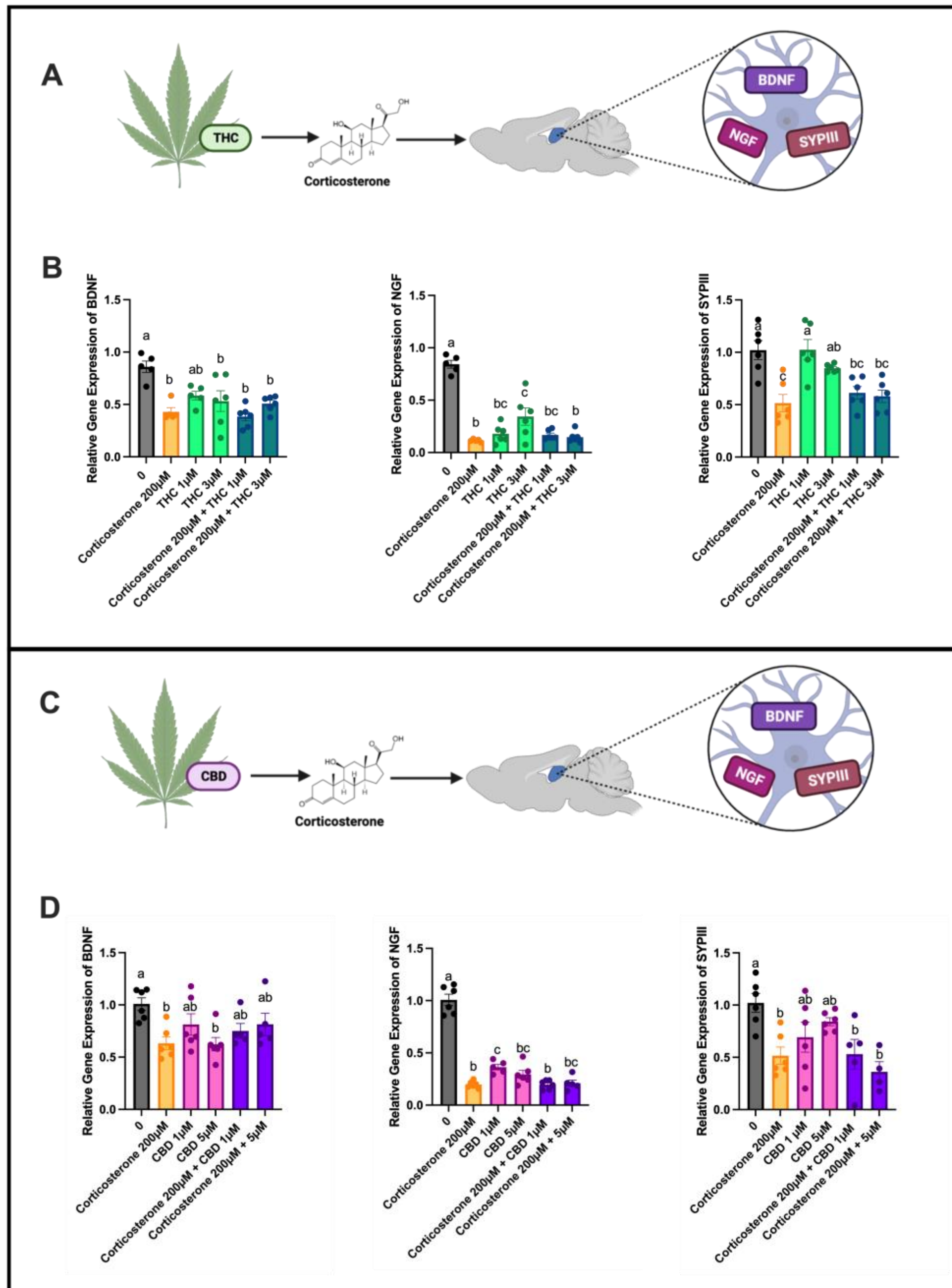

Figure 16. THC and CBD do not impact corticosterone-induced modulations in markers of neurogenesis. Schematic representation of combinatorial drug treatments with THC or CBD and corticosterone to assess markers of mitochondrial neurogenesis and neurite outgrowth. Figure made using Biorender (A/C). Undifferentiated HT22 cells were seeded in 6-well plates at 1.25×10^5 cells/well in DMEM containing 4.5 g/L glucose, L-glutamine and sodium pyruvate supplemented 5% HI- FBS and 1X pen-strep. Cells were harvested after 24 hours of treatment with 1μM and 3μM of THC and 200μM of corticosterone individually and in combination (B) or 1μM and 5μM of CBD and 200μM of corticosterone individually and in combination and (D). RT-qPCR was used to assess altered gene expression markers of neurogenesis, BDNF, NGF and SYPIII (B/D) normalized to RPLO and RPl37a relative to vehicle control. Data points represent the mean ± SEM

≥ 5 biological replicates. Significant differences were determined using one way ANOVA followed by a Bonferroni post hoc test. Data points with different letters signify *P < 0.05; ****P < 0.0001 relative to vehicle control.

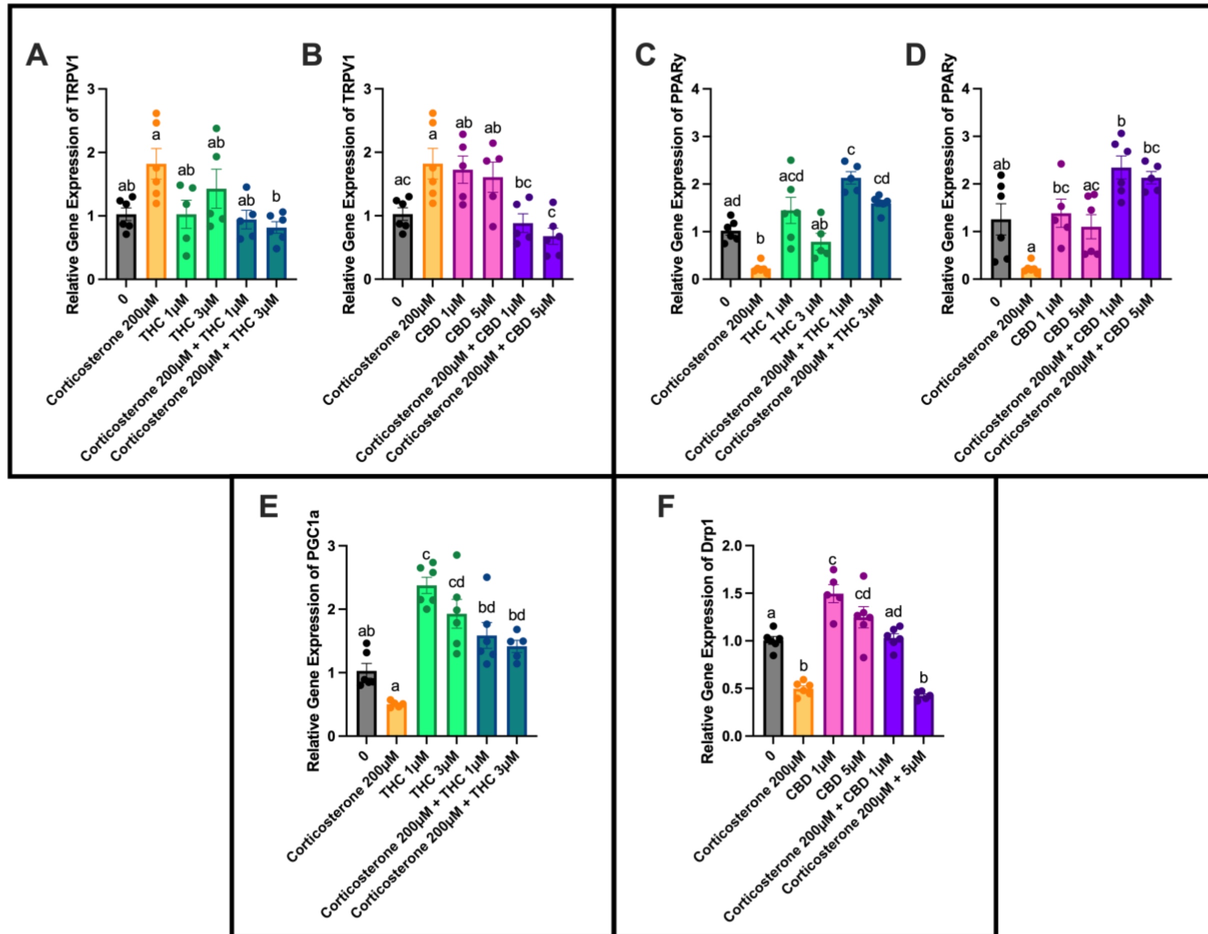

Figure 17. THC and CBD exhibit rescuing effects on corticosterone-induced alterations on markers mitochondrial biogenesis, mitochondrial fission and receptor expression in HT22 hippocampal neurons. Undifferentiated HT22 cells were seeded in 6-well plates at 1.25x10^5 cells/well in DMEM containing 4.5 g/L glucose, L-glutamine and sodium pyruvate supplemented 5% HI- FBS and 1X pen-strep. Cells were harvested after 24 hours of treatment with 1µM and 3µM THC and 200µM corticosterone individually and in combination (A/C/E/G) or 1µM and 5µM CBD and 200µM corticosterone individually and in combination and (B/D/F/H). RT-qPCR was used to assess altered gene expression of receptors, TRPV1 (A-B) and PPARγ (C-D), mitochondrial biogenesis marker, PGC1α (E) and mitochondrial fission marker, DRP1 (F), normalized to RPLO and RPl37a. Data points represent the mean ± SEM ≥ 5 biological replicates relative to control. Significant differences were determined using one way ANOVA followed by a Bonferroni post hoc test. Data points with different letters signify *P < 0.05; ****P < 0.0001 relative to vehicle control.

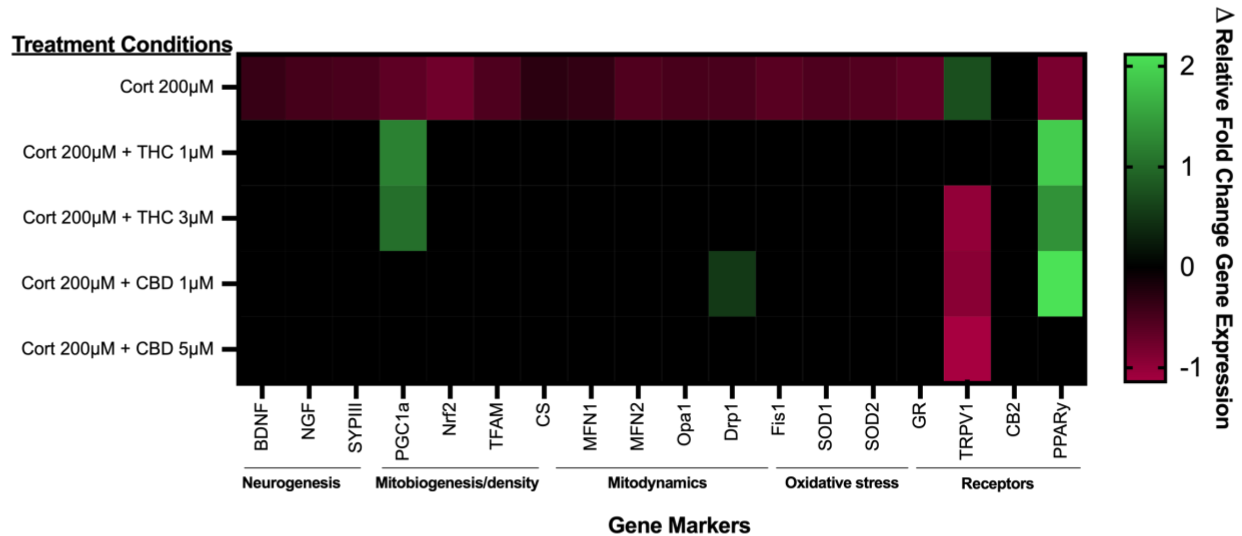

Figure 18. Heat map depicting alterations in transcript levels of neurogenesis, mitochondrial biogenesis, dynamic regulation, oxidative stress and receptor markers with combinatorial corticosterone and THC or CBD treatments compared to individual corticosterone treatments in hippocampal neurons. Undifferentiated HT22 cells were seeded in 6-well plates at 1.25×10^5 cells/well in DMEM containing 4.5 g/L glucose, L-glutamine and sodium pyruvate supplemented 5% HI-FBS and 1X pen-strep. Cells were treated with 200µM corticosterone (Cort) individually and co-treated with corticosterone at 200µM and THC at 1µM and 3µM or CBD at 1µM and 5µM for 24-hours. For individual corticosterone treatment, green panels indicate fold-change increases in gene expression and red panels represent fold-change decreases in gene expression and black panels indicate no significant changes in marker expression compared to vehicle control (MeOH). For co-treatments, green panels indicate fold-change increases in gene expression and red panels represent fold-change decreases in gene expression compared to gene expression with individual corticosterone 200µM treatments. Black panels indicate no significant changes in marker expression compared to individual corticosterone 200µM treatments. Gene expression of all markers were normalized to RPLO and RPl37a and include analysis of technical triplicates from ≥ 5 biological replicates. Significant differences were determined using one-way ANOVA followed by a Bonferroni post hoc test.

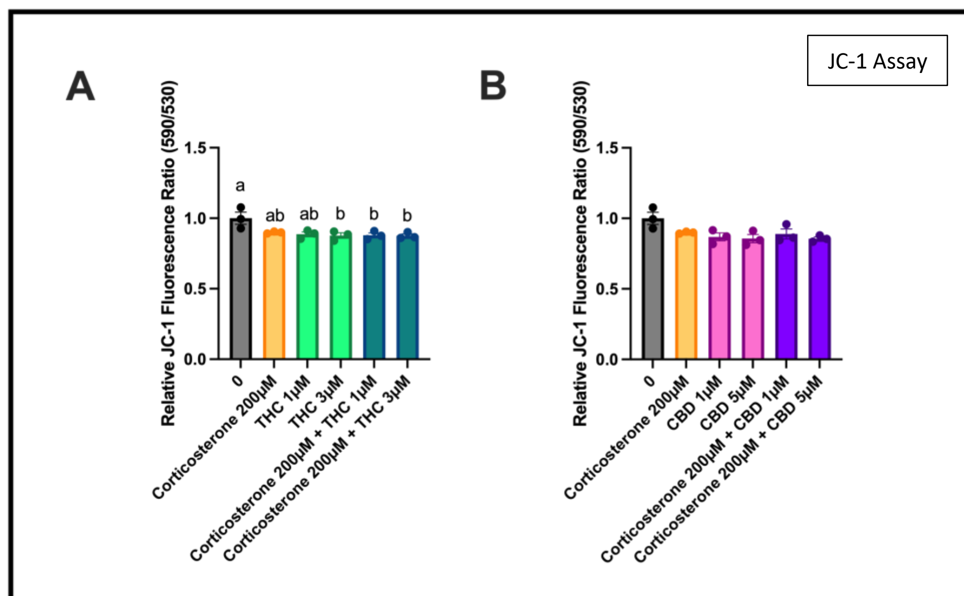

Figure 19. THC and CBD do not alter corticosterone-induced reductions in mitochondrial membrane potential. HT22 cells seeded in 96-well plate 1.5x10^3 cells/well in DMEM containing 4.5 g/L glucose, L-glutamine and sodium pyruvate supplemented 5% HI- FBS and 1X pen-strep and treated with1µM and 3µM of THC and 200µM of corticosterone individually and in combination (A) or 1µM and 5µM of CBD and 200µM of corticosterone individually and in combination and (B). JC-1 was performed 24 hours after treatment to assess changes in mitochondrial membrane potential. The red fluorescence in excitation/emission (535nm/590nm) and green fluorescence excitation/emission (475nm/530nm) was measured using a Spark multimode microplate reader (Tecan Group Ltd.). Background fluorescence was subtracted from fluorescence of treated cells and then the ratio of red (polarized) fluorescence was divided by the green (depolarized) fluorescence and normalized to average control fluorescence. Data points represent the mean ± SEM of technical duplicates from n=3 biological replicates relative to vehicle control. Significant differences were determined using one way ANOVA followed by a Bonferroni post hoc test. Data points with different letters signify *P < 0.05; ****P < 0.0001 relative to vehicle control.

CHAPTER 4. DISCUSSION

HT22 cells as an in vitro model of hippocampal neuron stress

Hippocampal neurons regulate stress response by providing negative feedback on the HPA axis when stress is no longer adaptive. Further, enhanced glucocorticoid secretion, an event characteristic of prolonged HPA axis activation, impairs hippocampal neurogenesis which potentiates the etiology of mood disorders [28,29]. To better understand the relationship cannabinoid signaling and corticosterone-induced signalling in mouse hippocampal neurons (HT22 cells) we first generated an in vitro model of hippocampal neuron stress. HT22 cells are a murine, immortalized, hippocampal cell line subcloned from the HT4 cell line. In contrast to mature, in vivo hippocampal neurons, undifferentiated HT22 cells express minimal levels of cholinergic markers and glutamate receptors [122–124]. Undifferentiated HT22 cells also reportedly have low or undetectable expression of other receptors that are seen in in vivo, mature neurons such as, mineralocorticoid receptors (MR) [136], Trkß [137] and CB1 receptors [138]. Differentiation of HT22 cells has been proposed to enhance expression of receptors seen in in vivo neurons such as NMDA receptors [139], muscarinic acetylcholine receptors [123], 5-HT1a receptors [124]. Therefore, undifferentiated HT22 cells are proposed to be more representative of neuro-progenitor precursor cells, while differentiated HT22 cells derive more features from post-mitotic neurons [140,141]. While the general consensus in the literature relates undifferentiated HT22 cells to immature neurons, low expression of mRNA and proteins distinctive to post-mitotic hippocampal neurons has been reported in undifferentiated HT22 cells. Zhao and colleagues propose this to be attributable to small portions of undifferentiated HT22 cells that spontaneously differentiate resulting in marginally detectable levels of markers usually seen with differentiated cells [141].

We initially examined the impact of differentiation on phenotypic attributes in HT22 cells by comparing levels of neuritic cells and average neurite lengths with differentiating and non-differentiating media over 72 hours. No significant changes in percentage of neuritic cells were seen with 24 hour to 48 hour differentiation. At 72 hours of differentiation we detected a significant increase in neuritic cells compared to undifferentiated HT22 cells. Although, this is likely attributable to the propensity of HT22 cells to proliferate at a much faster rate in non-differentiating media; meaning by the later time points the cells begin to grow in clumps and form aggregates (as seen in Figure 4C, 72 hours), where the cells are unable to effectively project neurite buds for axonal elongation. It is unclear if the percentage of neuritic cells is significantly increased with 72 hour differentiation or if by this later time point, undifferentiated cells are overly confluent impairing neurite outgrowth compared to the differentiated cells. Differentiation did not impact average neurite lengths over 72 hours. Therefore, differentiation of HT22 cells does not seem to illustrate obvious phenotypic changes related to neurite outgrowth with 72 hour differentiation.

Considering undifferentiated HT22 cells reportedly have differential receptor expression compared to primary hippocampal neuron cultures or in vivo hippocampal neurons, we then aimed to investigate the expression of relevant receptors in the undifferentiated cells and to determine how corticosterone treatments impact expression of these receptors. There is conflicting evidence in the literature pertaining to HT22 cell CB1 receptor expression; some studies reporting receptor expression [142,143] and other studies reporting no detectable expression [138]. We determined that there is no detectable expression of CB1 receptors in undifferentiated HT22 cells but that there is detectable expression of CB2, GR, TRPV1 and PPARγ receptors.

The above-mentioned receptors have previously been related to cross talk with stress response pathways associated with the ECS [144–148]. We investigated the mechanisms by which

corticosterone may be eliciting its effects in HT22 cells by exploring the resulting effects on CB2, TRPV1 and PPARγ receptor gene expression. Previous research examining the impacts of corticosterone or stress on the endocannabinoid system mainly focuses on the interactions with CB1 receptors. Since this receptor is not expressed in our cell line, we investigated the impacts of corticosterone on the other major cannabinoid receptor, CB2 receptor. Corticosterone did not impact gene expression of CB2 receptor. However, we did find that corticosterone treatments impacted GR expression. Corticosterone decreased mRNA levels of GR at 100μM and 200μM by about 40%. Cortisol/corticosterone signaling is mainly mediated through the GR [149]. The decrease seen in GR expression may indicate corticosterone receptor binding, desensitization and downregulation in GR mRNA expression. Conversely, corticosterone treatment increased TRPV1 expression at 200μM by ~67% compared to control. Corticosterone has previously been reported to increase TRPV1 expression in dorsal root ganglion neurons [150]. This impact was related to enhanced AEA production attributable to corticosterone exposure. AEA binds TRPV1 and increased levels have been shown to enhance TRPV1 expression [151]. This study proposed a CB1-dependent mechanism for enhanced TRPV1 activity under stress conditions, our results illustrate stress-induced increases in TRPV1 independent of CB1 [150]. Acute restraint stress has also been found to increase TRPV1 expression in the hippocampus and inhibition of TRPV1 receptor has been shown to rescue behavioral deficits induced by stress in rats [152]. Interestingly, TRPV1 knockout models exhibit anxiolytic and antidepressant behavioral effects [153] and stimulatory effect on hippocampal neurogenesis [154]. Therefore, increased receptor expression and potentially enhanced activity mediated by corticosterone may potentiate neuroprogressive effects in our model. Finally, we investigated the effect of corticosterone on PPARγ expression. PPARγ activity has been linked with neuroprotective effects in hippocampal neurons [155,156].

Major mechanisms by which PPARγ agonism has been related to neuroprotection is through its promotion of neurogenesis [157], mediation of oxidative stress and enhancement of mitochondrial biogenesis and energy production [158]. Our results indicated ~20-21% decrease in transcript levels of PPARγ receptor with both 100µM and 200µM corticosterone treatments. Contrary to these results, previous literature suggests stress-related increases in expression of PPARγ receptors in cortical neurons [159]. Hence, corticosterone may differentially affect PPARγ expression in a tissue-dependent manner, but further exploration is paramount to characterize this postulation.

In conclusion, HT22 cells are a more representative model of immature neurons when undifferentiated. As the hippocampus is one of the only brain structures in which neurogenesis is continuous throughout adulthood, varying degrees of differentiated cells from neural stem cells to immature neurons to post-mitotic, mature neurons may be seen in the hippocampus at one time [160] making semi-differentiated HT22 cells an appropriate model for assessing biochemical mechanisms related to early-stage neurogenesis in the hippocampus. Despite this fact, primary hippocampal neurons may be a more advantageous model to investigate later-stage neurogenesis and phenotypic alterations in mature neurons.

CB1 expression has been detected in the mouse hippocampus as early as E13.5 [161] but undifferentiated HT22 cells do not express detectable levels of CB1 receptors and therefore, obtained results can be postulated to be mediated by a CB1-independent mechanism. Protein and/or gene expression of CB2, GR, TRPV1 and PPARγ was detected in undifferentiated HT22 cells using western blot or RT-qPCR analysis. Corticosterone decreases mRNA levels of GR and PPARy at 100µM and 200µM and increases TRPV1 expression at 200µM. The relation of these alterations in receptor gene expression to the downstream impacts of corticosterone or stress in HT22 hippocampal neurons requires further investigation. These results address the effects of

differentiation on phenotypic alterations in HT22 cells and demonstrate our characterization of an in vitro model of hippocampal neuron stress using HT22 cells detailed in objective 1a.

Corticosterone impairs markers of neurogenesis and mitochondrial function in undifferentiated HT22 cells

Mood disorders, like anxiety and depression, have been associated with various neurotoxic effects in various brain structures [33,162]. Specifically, much interest has been focused on neuroprogressive effects in the hippocampus related to mood disorders and maladaptive stress-response. The hippocampus is a limbic structure in the brain that has major roles in the regulation of mood, behavior, and memory [163,164]. It has also been determined to be a brain structure particularly vulnerable to glucocorticoids and maladaptive stress response [165]. Prior research has designated cognitive deficits, morphological and biochemical changes and even neuronal death in the hippocampus attributable to excess glucocorticoid exposure [162]. Moreover, cognitive impairments and decreased hippocampal volumes have been reported in individuals with mood disorders [162]. Corticosterone, the major murine glucocorticoid effector of stress response, alters hippocampal neurotrophic factor expression in vitro and in vivo [41,42]. Neurotrophic factors are integral endogenous soluble proteins that regulate the survival, growth, morphological plasticity and differentiation of neurons [166]. We generated an in vitro model of hippocampal neuron stress by exposing HT22 cells to 100µM and 200µM corticosterone and assessing the impacts of depressive-like stress response on HT22 cell morphology, neurite outgrowth, neurotrophic factor expression, bioenergetic regulation and oxidative stress. 100µM and 200µM corticosterone concentrations have been utilized previously to produce neuronal in vitro models of depression; notably, 200µM of corticosterone is utilized more frequently [167–169]. We first validated our cellular model of stress-induced depression by assessing the gene expression of BDNF. RT-qPCR

analysis of transcriptomic variations revealed significant decreases in BDNF mRNA in undifferentiated HT22 cells with 24 hour treatment of 100µM and 200µM corticosterone. We also discerned marked decreases in gene expression of another neurotrophic factor, NGF, with 200µM of 24 hour corticosterone treatment but not with the lower concentration. Enhanced BDNF and NGF levels are associated with neuroprotective effects such as increased hippocampal neurogenesis [170,171], a process that is implicated in aspects of hippocampal-dependent cognition and antidepressant effects. Alternatively, decreases in BDNF expression are associated with reduced hippocampal neurogenesis [172] along with neurogenerative diseases such as Parkinson's disease, Alzheimer's disease, multiple sclerosis and Huntington's disease [173]. Similarly, deficits in hippocampal NGF levels have been linked with decreased hippocampal volume and neurogenesis [174] in addition to impairments in hippocampal long-term potentiation [175]. We also investigated the impacts of corticosterone on expression of synaptic protein, synapsin III (SYPIII). SYPIII is one of 3 neuron-specific genes called synapsins. Although there are structural and functional similarities between the 3 synapsins, SYPIII has roles distinct from synapsin I (SYPI) and synapsin II (SYPII) [176]. SYPIII has been shown to be mainly expressed in neurons during development and has much lower expression in mature neurons than SYPI and SYPII [176–178]. While SYPI and II are almost exclusively localized at synaptic sites and have more integrated roles in synaptogenesis, SYPIII protein is densely localized at extrasynaptic sites; mainly around the cell body and growth cones exemplifying its prominent role in axon outgrowth [179]. Neuronal precursor cells in the hippocampus are known to be enriched in SYPIII protein [180] and SYPIII knockout models demonstrate impairments in hippocampal neurogenesis and neurite outgrowth [181,182]. Deletion of SYPIII is shown to compromise morphology of growth cones and axon bodies indicating its role in axongenesis [176,179]. SYPIII has also been found to

play a role in mediating dopaminergic neurotransmission [183], which plays a significant role in various neuropsychiatric disorders [176]. We found that corticosterone decreased transcript levels of SYPIII at both 100μM and 200μM. There is limited evidence exploring the impacts of stress on SYPIII but, contrary to our work, one study did report no change in hippocampal gene expression of SYPIII following chronic restraint stress [184]. SYPIII expression has been linked to mediation of BDNF activity and expression. Faustini and colleagues reported hindered BDNF production following SYPIII deletion [185]. Therefore, the reductions we see in BDNF may in-part be related to SYPIII reductions. Altogether, reductions in gene expression neurotrophic factors, BDNF and NGF, and synaptic protein, SYPIII, all indicate impairments in neurogenesis and axonal outgrowth.

Emerging evidence has indicated the regulation of mitochondrial dynamics, bioenergetics and mediation of oxidative stress contribute to proper neuronal growth, neurogenesis and neurite outgrowth [186]. Mitochondrial dysfunction has also been linked with impairments in hippocampal neurogenesis relating to manifestation of depressive disorders [82]. Neuronal differentiation is associated with increased mitochondrial biogenesis and thereby increased mitochondrial mass per cell. Further this increased mitochondrial biogenesis is linked with enhanced ATP production, providing cells with energetic support needed to achieve fundamental processes coordinating neurite outgrowth [85,86]. Reduced hippocampal mitochondrial biogenesis is associated with neurodegenerative disease, Alzheimer's disease [187]. In addition, in vivo models of depression illustrate decreased mitochondrial respiratory rates and reduced mitochondrial membrane potential in the hippocampus, cortex, and hypothalamus [188]. RT-qPCR analysis of undifferentiated HT22 cells treated with corticosterone exhibited decreased expression of mitochondrial biogenesis gene markers. 24-hour treatment of HT22 cells with 200μM of corticosterone decreased transcript levels of PGC1α, Nrf2 and TFAM. PGC1α is a transcriptional

coactivator that is considered a master regulator of mitochondrial biogenesis [189,190]. Nrf2 is a transcriptional factor that guides the action of PGC1α to specific effector genes which mediates the expression of mitochondrial proteins including TFAM. TFAM is essential for the initiation of mitochondrial DNA (mtDNA) transcription and regulation [189,191,192]. In summary, PGC1α activates Nrf2 which binds to specific promoter sites that regulate the expression of TFAM [193]. TFAM is an enhancer protein that ensures mtRNA unwinding, regulating mtDNA transcription and maintenance [194]. Significant decreases in PGC1α, Nrf2 and TFAM mRNA levels were indicated with 200μM of corticosterone. 100μM of corticosterone also decreased Nrf2 and but no change was seen in PGC1α . In support of decreased biogenesis, we established a reduction in transcript levels of CS at both 100μM and 200μM. CS is an enzyme in the citric acid cycle that catalyzes the condensation of acetyl CoA and oxaloacetate to form citrate and CoA [195]. It is a validated marker of mitochondrial content [130‑134] and reductions in its expression coincide with reductions we see in mitochondrial biogenesis markers. Considering increased mitochondrial content is required for proper neurite outgrowth, decreases in mitochondrial biogenesis in HT22 cells may be related to reductions in markers of neurogenesis and neurite outgrowth. Mitochondrial parameters have also been connected with BDNF activity. BDNF has been shown to increase enhance mitochondrial respiration through upregulation of mitochondrial biogenesis and coupled respiratory efficiency [196,197]. Therefore, reductions in BDNF expression may be linked to decreased mitochondrial biogenesis and impaired bioenergetics. We also detected reductions in mitochondrial membrane potential with corticosterone treatments. The mitochondrial membrane potential is a major indicator of mitochondrial function as, together with the proton gradient, it comprises the transmembrane potential required to facilitate ATP production. Reductions in mitochondrial membrane production are associated with mitochondrial dysfunction and impaired

ATP production resulting in bioenergetic stress and apoptosis [198–200]. Since neurogenesis comprises enhanced ATP production to support bioenergetic demands, impairments in mitochondrial membrane potential may also in part give rise to the reductions we see in neurogenesis markers. Decreased mitochondrial membrane potential has also been implicated in neurodegenerative diseases like Alzheimer's disease [201] and Friedreich's ataxia [202] further exemplifying the importance of mitochondrial function in proper neuronal growth and cellular health.

Mitochondrial dynamic regulation mediates bioenergetic outputs [91–93] and have been found to play a profound role in adult and embryonic neurogenesis [203]. To regulate proper mitochondrial function and meet bioenergetic demands, mitochondria uphold a dynamic equilibrium balancing mitochondrial fusion and fission. Dysfunction in this balance has been implicated in a multitude of neurodegenerative disorders [204–207]. Transcriptional analysis of mitochondrial dynamic gene makers in undifferentiated HT22 cells demonstrated decreased fusion and fission with 100µM and 200µM corticosterone. 200µM of corticosterone decreased expression of all fusion markers: MFN1, MFN2 and Opa1 and decreased expression of both fission markers: Drp1 and Fis1. 100µM corticosterone significantly reduced all the same markers except MFN1. The decreases in both mitochondrial fusion and fission may in part be attributable to the overall decrease in mitochondrial biogenesis and thus, mitochondrial content.

Dysregulation of mitochondrial dynamics and impaired mitochondrial membrane potential are indicative of mitochondrial dysfunction and enhanced oxidative stress [208,209]. Oxidative stress occurs when there is an imbalance between ROS and antioxidants. When ROS levels exceed antioxidant capacity this can have a destructive effect on cellular components including neurons, resulting in neuronal death and degeneration [65]. Mitochondria are the main source of ROS

production but are also a major target for ROS-induced damage. Compromised ROS production from dysfunctional mitochondria can alter proteins required for mitochondrial biogenesis and fusion and fission further implementing mitochondrial impairments creating a vicious cycle [210,211]. We found that corticosterone 200µM decreased mRNA and protein levels of enzymes that directly scavenge ROS, SOD1 and SOD2, in undifferentiated HT22 cells. However, significant alterations in ROS levels with corticosterone treatments were not detected, nor changes in 4-HNE protein expression, a biomarker of lipid peroxidation and oxidative damage. Enhanced ROS levels increase lipid peroxidation of the cell membrane which produces 4-HNE as a by-product [212]. We detected about 30-50% reductions in gene expression of SOD enzyme and only about a 20-36% reduction in SOD enzyme protein expression. Therefore, there may not be a significant enough reduction in these antioxidant enzymes to facilitate a disbalance in redox state. SODs are not a cells sole antioxidant defense, there are various other cellular defense systems such as glutathione/thioredoxin peroxidases, glutathione, peroxiredoxins and many others which also mediate oxidative stress [213].

In conclusion, corticosterone treatment of undifferentiated HT22 decreases gene markers of neurotrophic factors that play essential roles in neuronal regulation and growth. In addition, mitochondrial biogenesis, membrane potential and dynamic regulation displays impairments in corticosterone treated HT22 cells; all of which are characteristic of neurodegeneration [214–216] relating mitochondrial dysfunction with impairments in neurogenesis. Corticosterone treatment decreases expression of antioxidant enzymes but this does not seem to impact overall redox state and oxidative stress in the cell. Overall, corticosterone reduces expression of markers of neurogenesis, mitochondrial biogenesis and content and mitochondrial dynamics in our in vitro model of hippocampal neuron stress, but further investigation is required to characterize the

impacts of corticosterone on antioxidant capacity in relation to oxidative stress mediation in HT22 hippocampal neurons. These results address the effects of corticosterone on neurogenesis in relation to mitochondrial function in undifferentiated HT22 cells as proposed in objective 1b.

Phytocannabinoids, THC and CBD, impact some pathways related to neurogenesis but the translation of these effects to neuroprotection are unknown

Many individuals experiencing stress reportedly turn to cannabis as a form of treatment [50]. Similarly, individuals with mood disorders also self-medicate with cannabis [49]. As previously discussed, the bioactive molecules comprising cannabis, THC and CBD, have been associated with various neuroprotective [54–56,61,67–70,72] and neuroprogressive effects [74–77,79,80]. Considering chronic stress and depressive disorders are associated with hypercortisolemia (increased cortisol levels) and many individuals experiencing these effects reportedly use cannabis, we aimed to investigate the potential neuroprotective effects of cannabinoids, THC and CBD, on corticosterone-induced stress in hippocampal HT22 cells. In order to investigate the impacts of THC and CBD on corticosterone-induced stress, we first characterized the effects of these cannabinoids individually on undifferentiated HT22 cells. We evaluated the impact of THC and CBD at a range of concentrations on gene markers related to neurogenesis and neurite outgrowth in undifferentiated HT22 cells. We found significant decreases in mRNA levels of neurotrophic factor, BDNF, with 1μM and 3μM THC. Previous studies have found no impact of THC on hippocampal BDNF expression in the hippocampus [61], although THC exposure has been linked with increased serum BDNF levels [62]. Another in vivo study reported increased hippocampal BDNF expression following THC intraperitoneal injection [63]. In contrast to previously described evidence, another study reported adolescent mice injected with

THC for 21 days were found to have decreased hippocampal BDNF mRNA transcripts levels [76]. The impacts of THC on BDNF expression display variable results. All identified studies assessing impacts of THC on BDNF levels encompass clinical or animal models. Therefore, our in vitro model investigating the impacts of THC on BDNF expression may provide more of a segregated biochemical model of THC exposure on direct murine hippocampal cells unaffected by other in vivo variables. Interestingly, CBD also decreased BDNF levels. Majority of studies assessing the impacts of CBD on brain BDNF levels report promotional effects related to CBD [217–219]. Although one study did report no changes in murine hippocampal BDNF in response to CBD injections [220]. The impacts of cannabinoids on BDNF require a more detailed investigation and titration of dose-dependent impacts and tissue-dependent analysis. We also found that THC decreased NGF at 1μM but no impact was found with THC other concentrations. THC has been reported to have neurotoxic impacts and cognitive deficits at low doses that do not manifest at higher concentrations [221]. Cannabis-users have also been reported to have decreased serum NGF levels [77], which is in agreement with the current evaluation of NGF in THC exposed HT22 cells. Reductions in neurotrophic factors, BDNF and NGF, are associated with reductions in hippocampal neurogenesis [172,174], highlighting the propensity of individual cannabinoid treatments to not only fail to illustrate neuro-promoting impacts but to demonstrate neuroprogressive features. We also detected marked reductions in presynaptic protein, SYPIII, with THC and CBD treatments at 10μM and 25μM respectively. SYPIII is a synaptic protein that is important for the establishment of functional and structural hippocampal plasticity [222]. Limited research is currently available demonstrating the impacts of phytocannabinoids on SYPIII but in support of our results, a 2014 rodent study reported attenuated hippocampal plasticity related to reduced SYPIII expression attributed to THC [222]. Overall, individual THC and CBD

treatments do not promote markers of neurogenesis and neuroplasticity and impair gene expression of these markers, potentially illustrating neurotoxic effects in HT22 hippocampal neurons.

Next, we investigated the impact of THC and CBD on gene expression of mitochondrial bioenergetic and fusion/fission markers in HT22 cells. We found that mitochondrial biogenesis marker, PGC1α, was dramatically increased with 1μM THC and increased to a lesser extent with 3μM THC. Albeit, downstream transcription factor and effector protein, Nrf2 and TFAM displayed no difference in expression at 1μM THC and decreased expression at 3μM THC. Previous studies have reported regulation of Nrf2 target genes such as TFAM to occur independently of PGC1α in glia and developing neurons (E18.5-PND3) [223], therefore, this may account for some of the discrepancies in expression of PGC1α and its downstream effectors. THC has previously been associated with decreases in mitochondrial content in placental BeWo cells [224]. PGC1α gene expression was also increased by CBD at 1μM and 5μM but again, no significant differences were detected in expression of Nrf2 and TFAM. There is currently limited evidence available investigating the impact of CBD on mitochondrial biogenesis, but one study reported enhancement of mitochondrial biogenesis with CBD treatments in cardiac tissue [225]. THC did not impact expression of our marker of mitochondrial content, CS. Interestingly, CBD decreased CS expression. Since THC increased PGC1α but not it's downstream effectors or our marker of mitochondrial content, the upregulation of mitochondrial biogenesis may have not yet achieved its downstream impact, upregulating downstream genes and upregulating mitochondrial content. Even though CBD similarly increased PGC1α, considering Nrf2 and TFAM were not enhanced and CS was actually decreased, CBD may not be promoting mitochondrial biogenesis and may even be impairing this process through a different mechanism. It is also possible that CBD may be enhancing mitophagy, essentially reducing mitochondrial content independent of biogenesis.

THC and CBD were both found to impact expression of mitochondrial dynamic markers in undifferentiated HT22 cells. 1µM and 10µM of THC decreased expression of mitochondrial fusion markers MFN1 and MFN2 and 1µM THC decreased fusion marker Opa1. No alterations were seen with fission markers, Drp1 and Fis1. Previous research from our lab has also demonstrated decreases in mitochondrial fusion gene markers with 48 hours of 10µM and 20µM THC treatment in BeWo cells [226]. Contrastingly, we found that mRNA levels of both mitochondrial fusion and fission were decreased with 25µM CBD. Interestingly, decreases in mitochondrial biogenesis markers were not discerned at these concentrations. Bernhardt and colleagues found that simultaneous impairments in mitochondrial fusion and fission decreases cellular replicative lifespans and reduce adaptive abilities in response to cellular stress [227] implicating dysregulated mitochondrial dynamics to impaired stress sensitivity and adaption. Mitochondrial fission markers, Drp1 and Fis1, were increased in HT22 cells treated with 1µM CBD but no changes were seen in fusion markers at this concentration. In addition, we found that THC and CBD decreased mitochondrial membrane potential further demonstrating mitochondrial impairments. In accordance with our work, studies have reported THC and CBD to impair mitochondrial membrane potential [228–231]. Our lab has previously demonstrated reduced mitochondrial potential with THC treatment in placental cells [228]. Dysregulation of mitochondrial biogenesis, mitochondrial dynamics and mitochondrial membrane potential are all parameters associated with cellular dysfunction and hallmarks of neurodegenerative disorders [201,202,207,232].

Impairments in mitochondrial function are indicative of oxidative stress [233], leading us to assess common indicators related oxidative stress. Our results showed that THC and CBD decreased expression antioxidant enzymes, SOD1 and SOD2. But, when we assessed alterations

in ROS levels, we found no significant changes with THC and CBD treatments. Therefore, THC and CBD may be mitigating the expression of these enzymes, but the cells may upregulate other antioxidant cellular mechanisms to account for these effects as an adaptive mechanism to mediate oxidative stress. It is also possible that THC and CBD are impeding antioxidant capacity but not at a rate significant enough to alter redox homeostasis. We detected only ~24-34% reductions in gene expression of SOD enzymes with cannabinoid treatments which may not be enough to significantly alter the cells antioxidant capacity.

Taken together, these findings demonstrate that THC and CBD attenuate markers essential to proper hippocampal neurogenesis and neurite outgrowth, along with dysregulating mitochondrial bioenergetics and mitochondrial dynamic balance in murine hippocampal neurons.

Next, we assessed the same parameters with co-cannabinoid and corticosterone treatments. We found that THC and CBD did not impact corticosterone-induced reductions on markers of neurogenesis. But, co-THC and corticosterone treatment did increase PGC1α expression compared to 200μM corticosterone individually. 1μM and 3μM co-THC treatment increased mRNA levels of PGC1α compared to corticosterone individually, normalizing values relative to vehicle control treatment, illustrating the potential for THC to instigate a rescuing effect on corticosterone-induced impairments mitochondrial biogenesis. Albeit, further investigation is required to investigate the impact of THC on stress-induced modulations in mitochondrial biogenesis, as genes downstream PGC1α and our marker of mitochondrial content were all unchanged with co-treatments. Co-CBD treatment did not impact corticosterone-related modulations in mitochondrial biogenesis markers but combinatorial treatments with 1μM CBD increased mRNA levels of fission marker, Drp1, compared to corticosterone alone. This normalizing impact on mitochondrial fission may suggest a rescuing effect of CBD. CBD has previously been shown to have a regulatory effect on

expression of Drp1 in hippocampus of rats exposed to iron toxicity [234]. THC did not impact corticosterone-induced reductions in mitochondrial dynamic markers.

THC and CBD also normalized alterations in receptor density induced by corticosterone. Both cannabinoids regulated receptor expression of TRPV1 and PPARγ. THC has previously been linked to downregulation of TRPV1 receptors, yet this impact has been related to CB1 receptors [235], which are not expressed in our cells. THC and CBD may be mitigating downstream signalling related to stress response and glucocorticoid receptor activation, reversing the effects of corticosterone on these receptors but further research is required to validate and characterize these effects. These results illustrate the impacts of THC and CBD on neurogenesis and mitochondria individually and in combination with corticosterone, in accordance with our second objective.

CHAPTER 5: CONCLUSIONS

Summary of Findings

In the present thesis, we aimed to develop an in vitro model of hippocampal neuron stress induced by corticosterone and to characterize the impacts of corticosterone on markers of neurogenesis and mitochondrial function in this model. We compared phenotypic alterations in differentiated and undifferentiated HT22 cells. This analysis revealed no significant variations in phenotype, percentage of neuritic cells or average neurite length over 72 hours, facilitating the continuation of research with undifferentiated HT22 cells. We next characterized the expression of relevant receptors in undifferentiated HT22 cells due to variability in current literature pertaining to detectable receptor expression. We concluded undifferentiated HT22 cells express detectable levels of GR, TRPV1, PPARγ and CB2 receptors but do not express CB1 receptors. The second portion of our first objective involved characterizing the impacts of corticosterone on neurogenesis and mitochondrial function in our HT22 cell model. As expected, corticosterone decreased all markers of neurogenesis along with decreasing markers of mitochondrial biogenesis, content and fusion/fission. Corticosterone decreased mitochondrial membrane potential and transcript levels of antioxidant enzymes. Surprisingly, corticosterone did not significantly impact ROS levels or lipid peroxidation in HT22 cells.

For the next objective, the propensity for cannabinoids, THC and CBD, to offer neuroprotective effects from corticosterone-induced stress was investigated. We found that individual THC and CBD treatments reduced expression of neurogenesis markers and decreased antioxidant enzymes but did not impact ROS levels. THC and CBD also dysregulated mitochondrial dynamics and decreased mitochondrial membrane potential. In conjunction, these results demonstrate that individual cannabinoid treatments did not propagate neuro-promoting

effects and dysregulated mitochondrial parameters potentially eliciting neurotoxic impacts in HT22 cells. Interestingly, both THC and CBD increased mRNA levels of a key regulator of mitochondrial biogenesis, which is associated with enhanced neurogenesis, but did not change expression of its downstream effectors.

To explore potential mitigatory or synergistic effects of cannabinoids on corticosterone-induced stress on neurogenesis and mitochondrial function, we assessed the same parameters with co-treatments of THC or CBD and corticosterone. We found the THC and CBD did not impact corticosterone-induced modulations in neurogenesis, antioxidant enzyme expression or mitochondrial membrane potential. However, THC did normalize expression of a mitochondrial biogenesis marker and CBD did display a rescuing effect on a mitochondrial fission marker. THC and CBD also both regulated alterations in PPARγ and TRPV1 receptor mRNA induced by corticosterone.

Overall, although THC and CBD did not impact corticosterone-induced reductions in neurogenesis markers, both cannabinoids impacted pathways related to neurogenesis; but the translation of these effects to neuroprotection requires more investigation.

Limitations

The most prominent limitation of our study pertains to the utilized cell line that comprises our in vitro model of hippocampal neurons stress. While HT22 cells have been used in various other studies to assess neuroprotective effects [236–239], they may not have been the most effective model for the designated objectives. To begin, the differentiation state of these cells remains obscure, and we were unable to ascertain a validated protocol detailing uniform differentiation of these cells. Previous studies have suggested undifferentiated HT22 cells are representative of neural precursor cells or immature neurons while differentiation of the cells

facilitates genotypic changes characteristic of mature or post-mitotic neurons [140,141]. However, the expression of markers of mature neurons have been detected in undifferentiated HT22 cells and this has been proposed to be due to spontaneous differentiation of some cells within a population [141]. This may create variations in ratios of differentiation states of cells within a treatment condition. We did not detect excessive variability with the undifferentiated HT22 cells but when trying to differentiate we experienced substantial variability between our biological replicates making it impossible to establish meaningful changes. This may be due to uneven differentiation of the cells resulting in differential ratios of differentiation and thereby, enhanced variability in marker expression across replicates. Overall, although the undifferentiated HT22 cells may pose as a relevant model of early-stage neurogenesis, the uniform differentiation of this cell line requires further optimization and investigation to characterize the exact neuronal state to explore changes in later-stage differentiation.

Next, multiple relevant receptors that are expressed in hippocampal neurons are not expressed at detectable levels in HT22 cells. Some of these receptors include MRs [136], Trkß [137] and CB1 receptors [138]. This is of extreme relevance especially since our treatment compounds predominantly interact with some of these receptors. Corticosterone mainly elicits its effects through glucocorticoid receptors and mineralocorticoid receptors, therefore, some of the impacts of corticosterone that are seen in vivo may be blunted or altered due to the absence of this receptor. Next, there has been debate in the literature as to the expression of CB1 in undifferentiated HT22 cells. This variance in results across research may in part appertain to the tendency of HT22 cells to spontaneously differentiate as differentiation has been shown to facilitate expression of markers distinctive of mature neurons. We deduced that CB1 receptors were not expressed at detectable levels by western blot analysis. This is important because THC

and CBD both interact with this receptor and the much of the neuroprotective effects of THC in particular have previously been ascribed to its action through CB1 [240,241]. Therefore, lack of pro-neurogenic and neuroprotective effects of THC may be related to the absence of this receptor. Overall, the resemblance of HT22 to in vivo hippocampal neurons presents integral disparities that may not make these cells the most efficient model to investigate neuronal stress or cannabinoid-mediated effects. More research is required to designate these differences and devise protocols detailing uniform differentiation of HT22 cells.

Future Directions

Primary neuronal culture comparison

Primary cultures involve the extraction of neurons from in vivo tissues and the maintenance of these cultures in vitro [242]. Primary cultures are very expensive and difficult to upkeep as mature neurons do not undergo cell division [243]. Immortalized cultures, such as HT22 cells, provide a more convenient and efficient model as they continuously divide. But, as previously described, immortalized neuronal cultures portray many biochemical and phenotypic variations from in vivo neurons [243] potentially making results less extrapolatable. To further investigate these discrepancies and their relevance to our results, future research should repeat our experiments and any other future experiments in both HT22 cells and primary mouse hippocampal cultures. This would not only clarify the relevance and validity of our currently obtained results but also shed light on the suitability of HT22 cells as a model of hippocampal neuron stress.

In-vivo model of hippocampal stress and cannabinoid exposure

An animal model may also be utilized to investigate our objectives and validate our preliminary results. Stress may be induced in mice by methods such as chronic, unpredictable stressors (CUMS) and the intervention of cannabis may be applied to explore potential

neuroprotective or neuroprogressive effects of cannabis on mitochondria, oxidative stress and neurogenesis and the biochemical mechanisms responsible for these effects. This will provide a more extrapolatable model that considers in vivo variables unaccounted for in in vitro models. Previous studies have deduced neuroprotective effects of injected cannabinoids on stress in in vivo models [67,234] but there is a deficiency in studies assessing a smoking route of administration. This is of extreme relevance since smoking is the most common route of administration with cannabis use [244] and this may provide variability in pharmacokinetic and pharmacodynamic outcomes.

Explore the effects of THC and CBD in combination on stress-response

Reportedly, 25% of Canadians with mood disorders associated with maladaptive stress response turn to cannabis as a form of treatment [49]. Cannabis comprises both THC and CBD at differential ratios based on strain. Therefore, many of the individuals using cannabis to treat enhanced stress are exposed to increased cortisol along with THC and CBD in combination. Our work only investigates the impacts of THC or CBD on hippocampal neuron stress. Considering the tendency for both compounds to be ingested concomitantly, future research should explore the potential neuroprotective or neuroprogressive impacts of both cannabinoids with different combinations in a model of hippocampal stress. Online reported ratios of CBD:THC to treat anxiety include 1:1 and 10:1 ratios, thereby these commonly used ratios should be investigated [245,246].

Significance of work

Prolonged, maladaptive stress predisposes the development of anxiety and depressive disorders [158]. Anxiety and depression are debilitating disorders that detrimentally impact an individual's quality of life. Majority of standard pharmacological treatments for these disorders

encompass modulations in monoaminergic transmission however, two-thirds of patients are refractory to treatment and half of patients relapse after initial response [44]. The necessity for novel avenues of therapy is clear. The reported use of cannabis to treat anxiety and depression, along with the described anxiolytic effects ascribed to cannabis, highlight the therapeutic potential of cannabis and it's components in treating mood disorders. The ability of cannabis components, THC and CBD, to illicit promotional effects on neurogenesis and mediatory effects on oxidative stress in previous studies support the use of cannabis in the treatment of not only mood disorders but also in neurodegenerative diseases. Our work contributes to the field by characterizing some of the biochemical effects of cannabinoids, THC and CBD, in stressed neurons and by demonstrating some pathways that are impacted by stress and cannabis components which may contribute to neuroprotection.

APPENDIX

Table 1. Forward and reverse primer sequences used for RT-qPCR.

Gene	Sense Strand Primer Sequence (5´→3´)	Anti-sense Strand Primer Sequence (3´→5´)
RPLO	CCAGCAGGTGTTTGACAACG	CCACCGGCCACTGTTTTCAT
RPl47a	GCTAAACGCACCAAGAAGGTC	TCCAGAAAGCGAGAGTGCAG
GR	TCAGCAGCAGGATCAGAAGC	GCTGTCCTTCCACTGCTCTT
CB2	TGGATACAGAATAGCCAGGACAAG	CTTCTTCTACTGGAGCTGTCCC
TRPV1	GTCTCAGGCAGACATCCCAG	GAGCAATGGTGTCGTTCTGC
PPARγ	GGGGATGTCTCACAATGCCA	GATGGCCACCTCTTTGCTCT
BDNF	GGCTGACACTTTTGAGCACGTC	CTCCAAAGGCACTTGACTGCTG
NGF	CAATAGCTGCCCGAGTGACA	TGAGTGGAGTCTCCGTTTCTTAAA
SYP III	AAATCAGCATCACCCCACCC	GCCTTGGCCTCATCTTCACT
PGC1-α	GCAGTCGCAACATGCTCAAG	GGGAACCCTTGGGGTCATTT
Nrf2	CAGCATAGAGCAGGACATGGAG	GAACAGCGGTAGTATCAGCCAG
TFAM	CCACAGGGCTGCAATTTTCC	CCACAGGGCTGCAATTTTCC
CS	GGACAATTTTCCAACCAATCTGC	TCGGTTCATTCCCTCTGCATA
MFN1	CCACAAGCTGTGTTCGGATTT	AGCACCCTCTGTGCATTTGT
MFN2	TGCACCGCCATATAGAGGAAG	TCTGCAGTGAACTGGCAATG
Opa1	ACCTTGCCAGTTTAGCTCCC	TTGGGACCTGCAGTGAAGAA
Drp1	GCCTCAGATCGTCGTAGTGG	CCATTCTTCTGCTTCAACTCCA
Fis1	CAAAGAGGAACAGCGGGACT	ACAGCCCTCGCACATACTTT
SOD1	CAGGACCTCATTTTAATCCTCAC	CCCAGGTCTCCAACATGC
SOD2	CTGGACAAACCTGAGCCCTA	TGATAGCCTCCAGCAACTCTC

Table 2. Antibodies used for western blot.

Antibody	Manufacturer, catalog #	Host	1º dilution used	Blocking (in TBST)	Antibody dilution (in TBST)
CB1	Cayman, 10006590	Rabbit polyclonal	1:800	5% milk	1.5% milk
CB2	Cayman, 101550	Rabbit polyclonal	1:500	5% milk	1.5% milk
4-HNE	Abcam, ab48506	Mouse monoclonal	1:1000	5% BSA	5% BSA
SOD1	Abcam, ab13533	Rabbit polyclonal	1:2000	5% BSA	5% BSA
SOD2	Proteintech, 66474-1-AP	Mouse Monoclonal	1:2000	5% BSA	5% BSA
β-Actin	BD Transduction Laboratories, 612657	Mouse monoclonal	1:2500	5% BSA/milk	1.5/5% BSA/milk

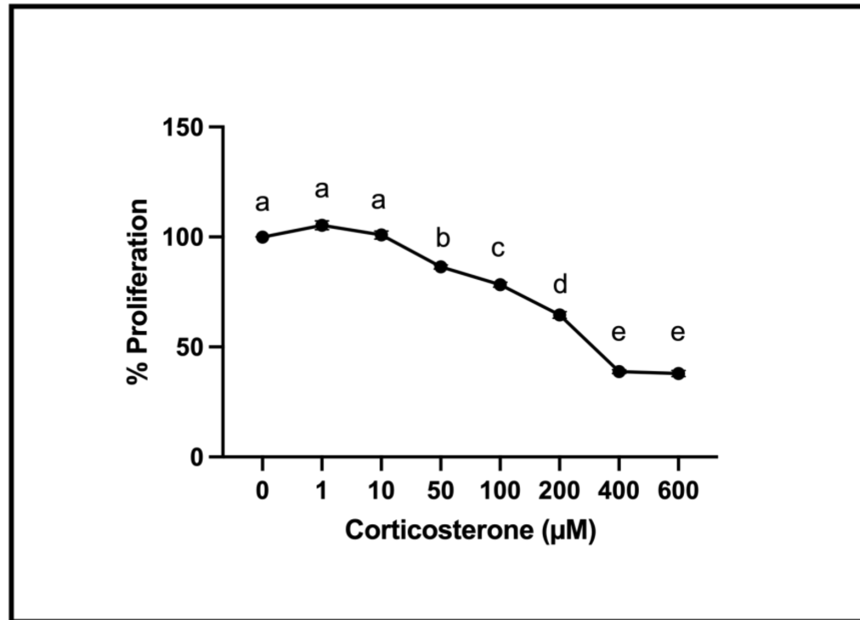

Figure 1. Corticosterone negatively impacts cell proliferation in undifferentiated HT22 cells at high doses. HT22 cells seeded in 96-well plate 1.5x10^3 cells/well in DMEM containing 4.5 g/L glucose, L-glutamine and sodium pyruvate supplemented 5% HI- FBS and 1X pen-strep and treated with range of corticosterone concentrations for 24 hours. (A) MTS assay was performed 24 hours after treatment to assess changes in percent (%) proliferation. Percent proliferation was calculated by subtracting the average blank absorbance and then dividing each value by the average control absorbance and multiplying by 100%. Data points represent the mean ± SEM of technical triplicates from n=4 biological replicates measured at 490 nm. Significant differences were determined using one way ANOVA followed by a Bonferroni post hoc test. Data points with different letters signify *$P < 0.05$; ****$P < 0.0001$ relative to vehicle control.

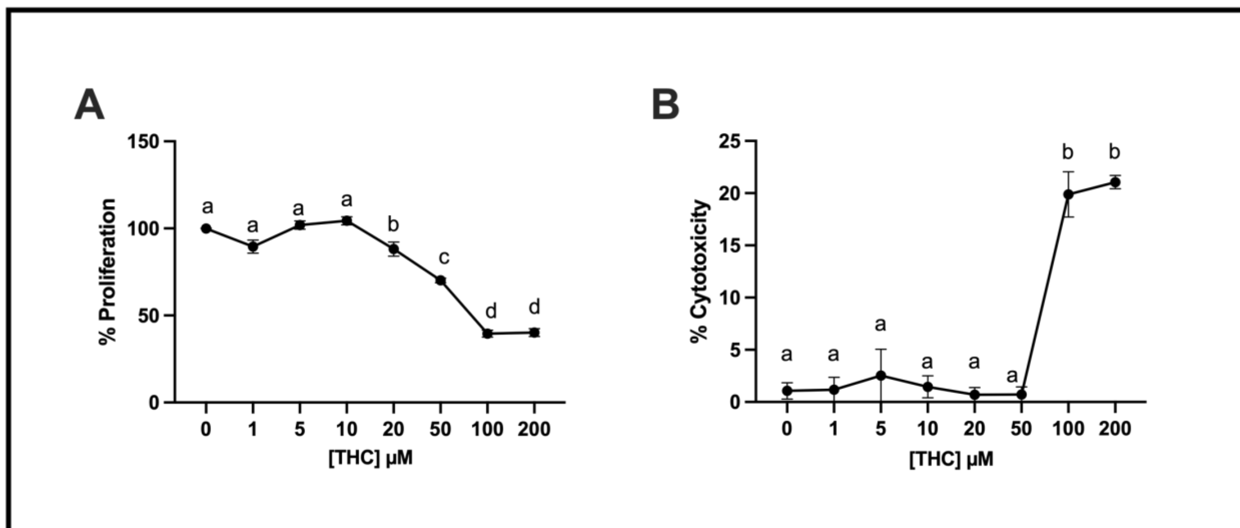

Figure 2. THC alters undifferentiated HT22 cell membrane integrity and proliferation at various concentrations. HT22 cells seeded in 96-well plate 1.5x10^3 cells/well in DMEM containing 4.5 g/L glucose, L-glutamine and sodium pyruvate supplemented 5% HI- FBS and 1X

pen-strep and treated with range of THC concentrations for 24 hours. (A) MTS assay was performed 24 hours after treatment to assess changes in percent (%) proliferation. Percent proliferation was calculated by subtracting the average blank absorbance and then dividing each value by the average control absorbance and multiplying by 100%. Data points represent the mean ± SEM of technical triplicates from technical triplicates of n=4 biological replicates measured at 490 nm. (B)LDH assay was performed 24 hours after treatments to assess lactate dehydrogenase release. Data points represent technical triplicates from n≥ 3 biological replicates ± SEM measured at 490nm and 680nm. Percent (%) cytotoxicity was calculated using the following equation: $\frac{((\text{Abs490}-\text{Abs680})-\text{Spontaneous LDH Release}))}{(LDH\ Max\ Release-Spontaneous\ LDH\ Release)} * 100\%$. Significant differences were determined using one way ANOVA followed by a Bonferroni post hoc test. Data points with different letters signify *P < 0.05; ****P < 0.0001 relative to vehicle control.

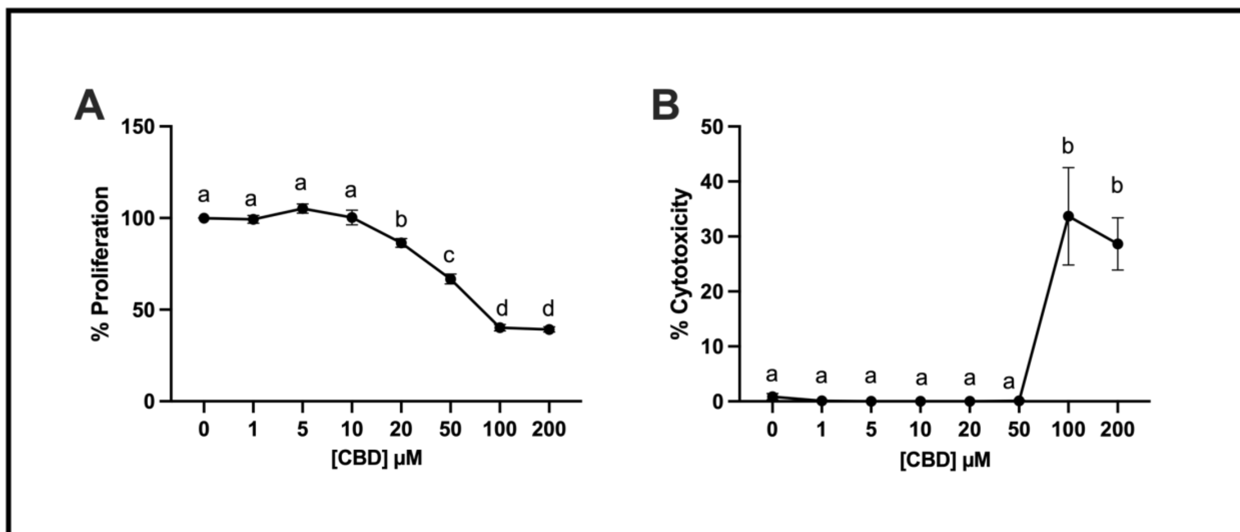

Figure 3. CBD impacts undifferentiated HT22 cell membrane integrity and proliferation at various concentrations. HT22 cells seeded in 96-well plate $1.5x10^3$ cells/well in DMEM containing 4.5 g/L glucose, L-glutamine and sodium pyruvate supplemented 5% HI- FBS and 1X pen-strep and treated with range of CBD concentrations for 24 hours. (A) MTS assay was performed 24 hours after treatment to assess changes in percent (%) proliferation. Percent proliferation was calculated by subtracting the average blank absorbance and then dividing each value by the average control absorbance and multiplying by 100%. Data points represent the mean ± SEM of technical triplicates from technical triplicates of n=4 biological replicates measured at 490 nm. (B)LDH assay was performed 24 hours after treatments to assess lactate dehydrogenase release. Data points represent technical triplicates from n≥ 3 biological replicates ± SEM measured at 490nm and 680nm. Percent (%) cytotoxicity was calculated using the following equation: $\frac{((\text{Abs490}-\text{Abs680})-\text{Spontaneous LDH Release}))}{(LDH\ Max\ Release-Spontaneous\ LDH\ Release)} * 100\%$. Significant differences were determined using one way ANOVA followed by a Bonferroni post hoc test. Data points with different letters signify *P < 0.05; ****P < 0.0001 relative to vehicle control.

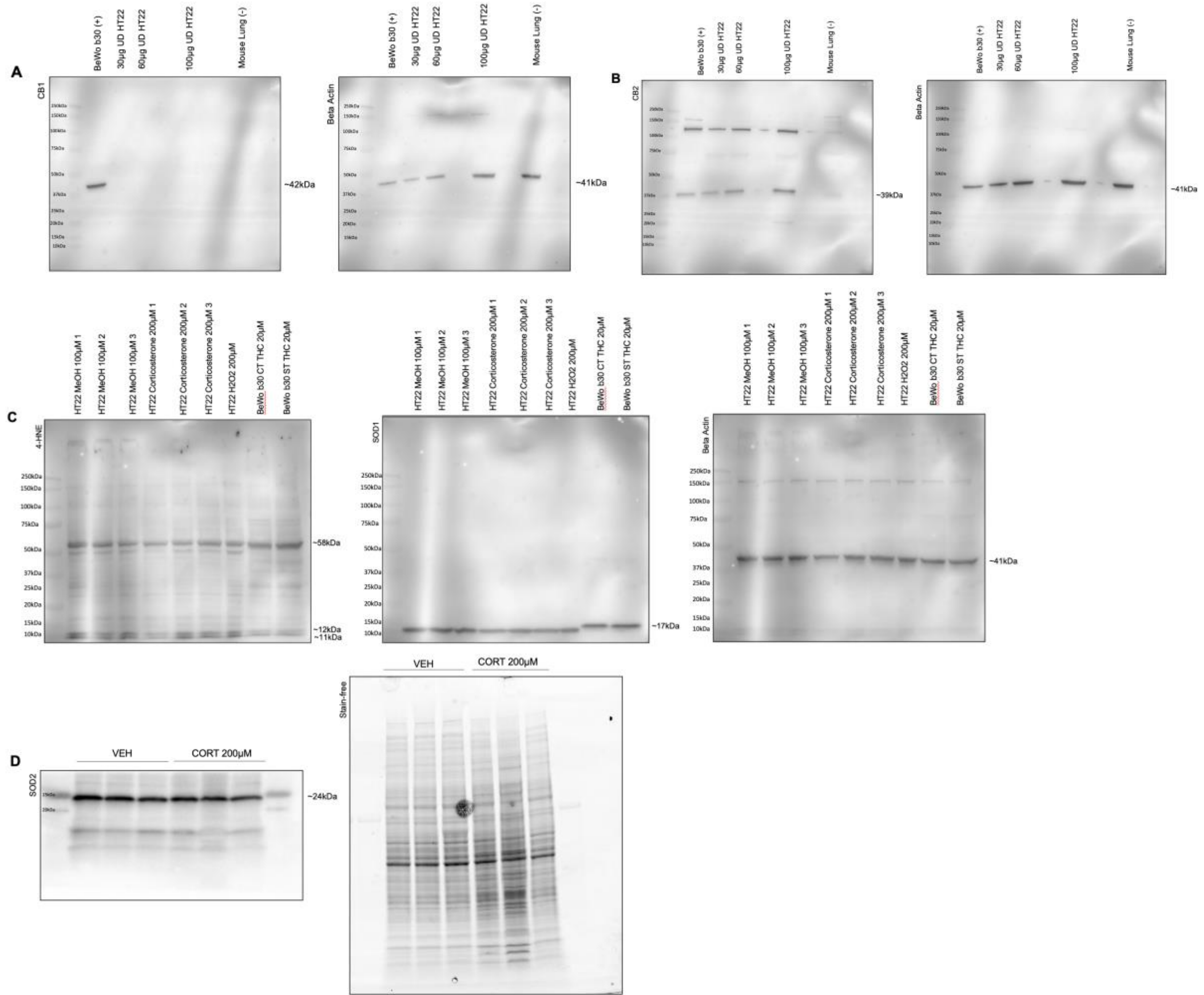

Figure 4. Labelled western blot images.

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