TETRAHYDROCANNABINOL AND CANNABIDIOL ARE NEUROPROTECTIVE

INVESTIGATING THE NEUROPROTECTIVE MECHANISMS OF CANNABINOIDS THROUGH ENDOPLASMIC RETICULUM STRESS MODULATION

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LAY ABSTRACT

With the worldwide ageing population increasing, finding new treatments for illnesses that affect the elderly is crucial. Disorders such as Parkinson's and Alzheimer's disease mainly affect older individuals and are caused when brain cells stop working or when brain cells die. These disorders share some common causes. One is the inability to fold proteins properly. The cellular process that is responsible for protein folding and the changes that occur within that process are studied in this project. Also, the impact of the cannabinoids THC and CBD, a major component of cannabis, on the protein folding process is studied. This project found that using cannabinoids before the protein folding system is disrupted helps brain cells survive. This study is a step in understanding how THC and CBD are helpful in brain cell survival in patients suffering from diseases that damage brain cells.

ABSTRACT

The aggregation of misfolded proteins in the endoplasmic reticulum (ER) is a pathological trait shared by many neurodegenerative disorders. This aggregation leads to the persistent activation of the unfolded protein response (UPR) and ultimately apoptosis due to ER stress. Cannabinoids, such as tetrahydrocannabinol (THC) and cannabidiol (CBD), have been reported to be neuroprotective in *in vitro* and *in vivo* models of neurodegeneration through their antioxidant and anti-inflammatory properties. However, little is known about the role of these cannabinoids in the context of ER stress. ST*Hdh*$^{Q7/Q7}$ cells were treated with the ER stress inducer thapsigargin (TG) and cannabinoids in three different experimental paradigms to investigate the effect of 2.5 μM THC and 1 μM CBD monotreatment and cotreatment on ER stress-induced cell death. The mouse striatal neurons survived significantly more when THC or CBD was given before TG exposure. To further investigate this experimental paradigm, the gene and protein expression of UPR proteins was measured to determine the effect of cannabinoid pre-treatment on cell survival through ER stress modulation. A significant increase in the gene expression of the ER chaperone GRP78 and the ER-resident neurotrophic factor MANF in pre-treated samples suggest that with THC or CBD pre-treatment, the protein folding capacity of the cell is improved. Additionally, a decrease in the ER-mediated apoptotic markers such as BIM and caspase 12 with THC or CBD pre-treatment provides further evidence that cannabinoid pre-treatments are neuroprotective through ER stress modulation.

These data suggest that prior cannabinoid monotherapy prepares the cell for future insults to the ER. Understanding the role of ER stress in the neuroprotective properties of THC and CBD provides insight into the therapeutic potential of cannabinoids and the role of ER dysfunction in various neurodegenerative disorders.

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LIST OF ABBREVIATIONS AND SYMBOLS

µM	Micromolar
6-OHDA	6-hydroxydopamine
ATF6	Activating transcription factor 6
BAX	BCL-2-associated X protein
BCL-2	B-cell lymphoma 2
BIM	BCL-2 interacting mediator of cell death
C/EBP	CCAAT/enhancer binding protein
Ca^{2+}	Calcium ion
CB_1	Cannabinoid receptor 1
CBD	Cannabidiol
CDNF	Cerebral dopamine neurotrophic factor
CGN	Cerebellar granule neurons
CHOP	C/EBP homologous protein
CT	Cycle threshold
DMSO	Dimethyl sulfoxide
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
GOI	Gene of interest
GRP78	Glucose-regulated protein-78
H_2O_2	Hydrogen peroxide
IRE1	Inositol-requiring enzyme 1
MANF	Mesencephalic astrocyte-derived neurotrophic factor
MPP^+	1-methyl-4-phenylpyridinium
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
nM	Nanomolar
NRT	No reverse transcriptase
NTC	No template control
NTF	Neurotrophic factor
PBS	Phosphate-buffered saline
PERK	Protein kinase R-like ER kinase
ROS	Reactive oxygen species
RT-qPCR	Reverse transcription quantitative real-time polymerase chain reaction
SERCA	Sarco/endoplasmic reticulum Ca^{2+} ATPase
TG	Thapsigargin
THC	Delta-9-tetrahydrocannabinol
TrkA	Tropomyosin receptor kinase A
UPR	Unfolded protein response

DECLARATION OF ACADEMIC ACHIEVEMENT

The entirety of this work was conceived, performed, analyzed, and written by the author of this thesis, in consultation with Dr. Ram Mishra.

1. INTRODUCTION

Unfolded Protein Response and Endoplasmic Reticulum Stress

The endoplasmic reticulum (ER) is the site for protein synthesis, folding, and transport. It also plays an essential role in maintaining homeostasis by storing calcium and regulating calcium signalling. Disturbances in calcium regulation, protein modification, and the overexpression or mutation of proteins are some factors that may cause ER stress. This occurs when the demand for protein folding exceeds the ER's capacity to fold proteins. To detect and respond to the accumulation of unfolded proteins in the ER, cells have signalling cascades called the unfolded protein response (UPR) (Rao et al., 2004). Ultimately, the UPR will signal cell death or protection in response to the stress.

To monitor the activation of the UPR, ER-chaperone glucose-regulated protein-78 (GRP78) will be measured. GRP78 is the master regulator of ER stress because of its role in protein folding, anti-apoptotic properties, and its interactions with the ER stress sensors, IRE1, PERK, and ATF6 (M. Wang et al., 2009). GPR78 is bound to the ER stress sensors in the absence of ER stress, inhibiting UPR signalling. In the presence of ER stress, GRP78 dissociates from these sensors and the UPR is initiated (Hetz & Saxena, 2017).

Upon activation of the UPR, genes in both the adaptive and terminal UPR pathways are activated. If the cell favours the adaptive UPR pathway, the expression of pro-apoptotic genes and caspases will be lowered to save the cell. Contrarily, if the terminal UPR pathway is more active, the expression of anti-

apoptotic genes will decrease, and pro-apoptotic genes will increase to signal cell death. The pro-apoptotic marker C/EBP homologous protein (CHOP) will be measured because it activates downstream ER stress-induced apoptosis genes. Overexpression of CHOP has been shown to lead to apoptosis (Maytin et al., 2001). GRP78 overexpression had been shown to reduce CHOP induction, therefore reducing ER stress-induced apoptosis (X. Z. Wang et al., 1996).

CHOP interacts directly with B-cell lymphoma 2 (BCL-2) and Bcl-2-associated X protein (BAX). BCL-2 is an anti-apoptotic gene; overexpression of CHOP reduces BCL-2 expression and promotes apoptosis (McCullough et al., 2001). BCL-2 inactivates the pro-apoptotic activity of BAX (Fribley et al., 2009). While BCL-2 and BAX are often used in conjunction with CHOP to measure apoptosis induction, they are mainly associated with the mitochondria-dependent apoptosis pathway.

ER-specific apoptosis markers include BCL2 interacting mediator of cell death (BIM) and caspase 12. Caspase 12 is an ER-resident caspase and is the first caspase activated during ER stress-induced apoptosis (Sakurai et al., 2005). Downstream signalling by caspase 12 leads to the activation of other caspases and triggers apoptosis (Hitomi et al., 2004). Translocation of BIM to the ER is required to activate caspase 12 in response to ER stress (Morishima et al., 2004).

Neurodegeneration and Endoplasmic Reticulum Stress

The continuous overactivation of the UPR has been implicated in neurodegenerative disorders such as Parkinson's disease, Alzheimer's disease,

Huntington's disease, prion disease, and amyotrophic lateral sclerosis (Lastres-Becker et al., 2005a; Moreno et al., 2013). When the cell first encounters ER stress, the adaptive UPR sensors will activate downstream responses to improve protein folding to reduce ER stress. Persistent ER stress will shift the adaptive UPR to a terminal UPR, which will induce apoptosis (Hetz & Saxena, 2017). While there are synthetic therapeutics for the symptoms of neurodegenerative diseases, natural compounds such as cannabinoids have also been suggested to have therapeutic potential.

Cannabinoids

Cannabinoids are chemical components of the cannabis plant. *Cannabis Indica* and *Cannabis sativa* are subspecies of the cannabis plant and both physically and chemically distinct. *Cannabis indica* is shorter with a greater amount of cannabidiol (CBD), while *Cannabis sativa* is taller and has a higher tetrahydrocannabinol (THC) content (Dewey, 1986). Cannabis has been cultivated for over 6000 years and has been used as a recreational drug for more than 4000 years (Li, 1974). The plant has been studied since the nineteenth century when the plant's chemical compounds were isolated (Wood et al., 1899). *Cannabis sativa* is composed of over 60 cannabinoids, the main component is Δ^9-THC, while CBD, Δ^8-THC, and cannabinol are also major components (**Figure 1**) (Gaoni & Mechoulam, 1964; Pertwee, 2008). For millennia, cannabis has had medical, recreational, and spiritual value. Still, its effects on human health and the

pharmacology of its major components have been researched for a little over a century.

Delta-9-tetrahydrocannabinol **Cannabidiol**

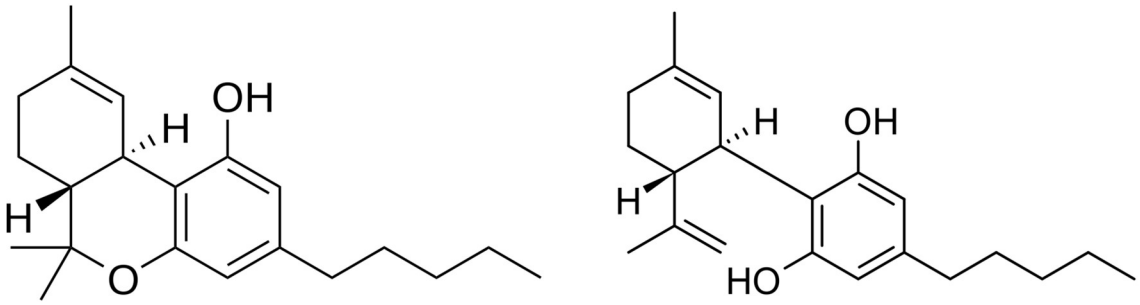

Figure 1: Structure of delta-9-tetrahydrocannabinol (THC) and cannabidiol (CBD). The chemical structure of both THC and CBD is $C_{21}H_{30}O_2$, but they have structural differences.

THC and CBD

The two major components of cannabis, THC and CBD, have been extensively researched in various contexts. THC and CBD have the same molecular formula, but different functional groups give each compound distinct pharmacological properties (Compton, 1993). THC has an ether group, where CBD has a hydroxyl group. This structural difference changes the interactions of these cannabinoids with the cannabinoid receptor 1 (CB_1). THC binds to the orthosteric site of the receptor and acts as a partial agonist of CB_1, whereas CBD binds to the allosteric site of the receptor and acts as a negative allosteric modulator (Laprairie et al., 2015). THC and CBD exert cellular, behavioural, and pharmacological effects on the central nervous system as well as the immune, cardiovascular,

metabolic, and reproductive systems (Atakan, 2012). There is contradicting evidence on the effects of each cannabinoid on the brain. The effects of each cannabinoid are mainly dependent on the dose, toxin, and cell model used.

Independent Neuroprotective Effects of THC and CBD

Neurodegenerative diseases have various genetic and environmental causes, but they share several common mechanisms that mediate neurodegeneration. These mechanisms include neuroinflammation, excitotoxicity, oxidative stress and reduced trophic support (Fagan & Campbell, 2014). Cannabinoids such as THC and CBD support cell survival by exhibiting anti-inflammatory, antioxidant, and neurotrophic activity. CBD was protective against glutamate neurotoxicity in cortical neurons (Hampson et al., 1998). Independently, THC and CBD were neuroprotective against oxidative stress in hippocampal and cerebellar cell cultures (Marsicano et al., 2002). In glial cells, THC and CBD were protective against 6-hydroxydopamine (6-OHDA), which is used to model Parkinson's disease (Lastres-Becker et al., 2005). This study also found THC and CBD to be protective against 6-OHDA *in vivo*.

Due to the reduced psychoactive effects of CBD compared to THC, CBD's role in neuroprotection has been studied in many cellular models. PC12 cells treated concurrently with 1-methyl-4-phenylpyridinium ($MPP^{+)}$ and CBD for 24 hours had increased cell viability compared to MPP^+ cells (Santos et al., 2015). The study also found that CBD protected against the inhibition of cellular differentiation induced by MPP^+ in PC12 cells but not in SH-SY5Y neuroblastoma

cells. It was suggested that neuroprotection involved the activation of the nerve growth factor receptor, tropomyosin receptor kinase A (trkA). The receptor is not expressed in SH-SY5Y cells, so CBD could not induce neuritogenesis in these neuroblastoma cells. In PC12 cells, CBD was also shown to decrease pro-inflammatory cytokine release and inhibit apoptosis *in vitro* (Santos et al., 2015). CBD (2.5 μM) pre-treatment for 1 hour was also reported to increase cell viability against H_2O_2 in rats' primary cerebellar granule neurons (CGN). The same cells also had increased viability when exposed to rotenone after either a 1-hour or 24-hour treatment of 2.5 μM CBD. These neuroprotective effects of CBD were still observed when neurons were co-incubated with CB_1 or CB_2 antagonists suggesting that the neuroprotection is receptor-independent (Echeverry et al., 2020). Suggested receptor-independent mechanisms were the antioxidative properties and effects of anti-inflammatory activity of THC and CBD (Hampson et al., 1998). Additionally, CBD induced neuroprotection against H_2O_2 in primary hippocampal neurons (Kim et al., 2021). A 24-hour incubation with CBD and H_2O_2 caused a 2.4-fold increase in cell viability compared to H_2O_2. CBD did not completely recover neurite degeneration, but the use of a nonlethal dose is neuroprotective. CBD also protected dopaminergic neurons from cadmium-induced toxicity. SHSY-5Y cells differentiated with retinoic acid were treated with 1 μM CBD for 24 hours following exposure to cadmium. CBD counteracted the cadmium-dependent decrease in cell viability and reactive oxygen species (ROS) increase (Branca et al., 2019).

Studies have shown the neuroprotective effects of THC in cellular and animal models. THC protected rats against ouabain-induced excitotoxicity. Cell swelling decreased in the hippocampus, striatum, and cortex (Stelt et al., 2001). THC was also shown to be protective in SH-SY5Y cells against MPP^+ by restoring proteins involved in mitochondrial biogenesis (Zeissler et al., 2016). While THC and CBD are protective against the aforementioned mechanisms, their role in the ER stress response remains unclear.

Some studies measured the effects of CBD or THC on the expression of UPR genes; however, they usually use them as an additional measure, and ER stress is rarely the primary focus of the study. CBD was shown to increase cell viability in differentiated SHSY5Y cells with a 24-hour pre-treatment of 10 μM CBD before exposure to MPP^+ (Gugliandolo et al., 2020). Gugliandolo et al. also found that the MPP^+-induced decrease in cell viability was inversely correlated with the expression of BAX. Pre-treatment with CBD restored BAX expression to control levels (Gugliandolo et al., 2020). The study by Branca et al. also saw this restoration of BAX to control levels in CBD-mediated neuroprotection from cadmium. While this evidence is promising, the effects of CBD and THC can be toxic or protective depending on the dose, duration of exposure, and cell type.

Combined Neuroprotective Effects of THC and CBD

In vivo studies have demonstrated that the combination of THC and CBD can also lead to neuroprotection. A 1:1 combination of THC and CBD protected striatal neurons against malonate, a toxin that damages neurons through

apoptosis and microglial activation. In rats, the combination of THC and CBD attenuated the effects of malonate, such as edema, increased degeneration, and glial activation. The study also found that these effects were dependent on the cannabinoid receptors (Valdeolivas et al., 2012). Chronic administration of a 1:1 combination of THC and CBD in a transgenic mouse model of Alzheimer's disease reduced learning impairment and amyloid-beta plaques (Aso et al., 2015).

Cytotoxic Effects of THC and CBD Independently

Previous studies which saw neuroprotection at lower doses of CBD saw that increasing the dose was neurotoxic in the same model. In primary hippocampal neurons, 9.85 μM CBD was lethal (Kim et al., 2021). In primary cerebellar granule neurons, 10 μM CBD was cytotoxic (Echeverry et al., 2020). CBD has also been shown to have a toxic effect on optic nerve oligodendrocytes. Increased ROS production was suggested to cause this toxicity, but it is specific to oligodendrocytes but not in cortical neurons (Mato et al., 2010).

Modelling ER Stress in vitro

A sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) inhibitor such as thapsigargin (TG) can be used to introduce ER stress into a cellular model. The SERCA pump transports calcium ions into the ER. The inhibition of this pump with TG will lead to the depletion of calcium ions from the ER lumen, leading to calcium dysregulation and ER stress (Thastrup et al., 1990). In PC12 cells, TG has been shown to increase the expression GRP78 compared to control samples. In the

presence of a neuroprotective compound and TG, the expression of GRP78 was significantly greater than TG alone. Additionally, the expression of BCL-2 decreased with TG alone. This decrease was restore back to control levels in the presence of a neuroprotective compound (Hiroi et al., 2005). TG alone increases MANF expression in U2OS, HEK293, and SH-SY5Y cells (Apostolou et al., 2008). C6 glioma cells also had an increase in GRP78, MANF, and CHOP expression with TG treatment (Yagi et al., 2020).

TG is the ideal toxin to study ER stress because it directly targets the ER. Other toxins such as tunicamycin, Brefeldin A, dithiothreitol, and MG132 also induce ER stress, but these compounds target downstream events such as post-translational modifications to induce ER stress (Hitomi et al., 2004; Oslowski & Urano, 2011). However, TG was used for this study because it causes ER stress through calcium dysfunction, which has been implicated in neurodegenerative disorders (Alexianu et al., 1994; Mattson, 2004; Ramaswamy et al., 2007; Surmeier, 2007).

Study rationale, objective, specific aims, and hypotheses

Neurodegenerative diseases may directly or indirectly cause ER stress. The lack of consensus on research about the direct effects of CBD and THC on ER stress mediation warrants an investigation of the mechanistic effects of these cannabinoids in a cellular model of ER stress. This project aims to investigate the effects of THC and CBD on cell survival and the unfolded protein response by using a cellular model of ER stress. The specific aims are:

1. To use cell viability assays to determine the appropriate THC, CBD, and TG doses in the ST*Hdh*$^{Q7/Q7}$ mouse striatal cells.

   Hypotheses: Doses of CBD (0.1 μM – 1.0 μM) and THC (0.25 μM – 2.5 μM) used in other cellular models will not affect cell viability in ST*Hdh*$^{Q7/Q7}$ cells. Similar to published studies, concentrations greater than 100 nM TG will significantly reduce cell viability after 24 hours in ST*Hdh*$^{Q7/Q7}$ cells.

2. To use cell viability assays to investigate the changes in cell survival in three cell treatment paradigms of cannabinoids and ER stress to study concurrent (a), neuroprotective (b), and neurorestorative (c) effects of THC, CBD, and a combination of THC and CBD against TG.
   a. Measure cell survival when cannabinoids and TG are administered together.
   b. Measure cell survival when cannabinoids are given prior to TG administration.
   c. Measure cell survival when cannabinoids are given after TG administration.

   Hypotheses: THC and CBD independently will increase cell viability against TG-induced toxicity in the concurrent and neuroprotective models because other cellular models have shown neuroprotection with pre-treatment of a cannabinoid or concurrent treatment of the cannabinoid with a toxin. Extrapolating from *in vivo* studies using a combination of the cannabinoids, a pre-treatment will increase cell viability against TG-induced toxicity.

3. To use RT-qPCR to measure the mRNA expression of UPR genes and neurotrophic factors in any cell treatment paradigm which shows significant changes to cell survival caused by cannabinoids. The genes measured will be GRP78, CHOP, BAX, BCL-2, BIM, and caspase 12. Additionally, the expression of the ER-resident neurotrophic factors (NTFs), mesencephalic astrocyte-derived neurotrophic factor (MANF) and cerebral dopamine neurotrophic factor (CDNF) will be measured (Stepanova et al., 2020). MANF has been shown to interact with GRP78 in a calcium-dependent manner to inhibit the UPR (Voutilainen et al., 2015). MANF knockout studies in pancreatic β cells have displayed chronic UPR activation (Lindahl et al., 2014). The structures of MANF and CDNF also suggest they play a role in protein folding in the ER. First, they contain C-terminal signals that resemble the ER retention signal needed to bind to the ER retention receptor (Henderson et al., 2013). Second, they each have a cysteine bridge motif which might assist in protein folding (Parkash et al., 2008).

   Hypothesis: An increase in cell viability will correlate with an increase in pro-survival genes GRP78, MANF, CDNF, and BCL-2, and a decrease in the pro-apoptotic genes CHOP, BAX, BIM, and caspase 12.

4. To use a western blot to measure the changes in the expression of the GRP78 protein to verify mRNA expression results.

   Hypothesis: An increase in GRP78 protein expression will correlate with an increase in cell viability.

2. METHODS

Cell Culture

ST*Hdh*$^{Q7/Q7}$ cells were obtained from the Coriell Institute for Medical Research. These are a striatal neuronal cell line derived from an Hdh7/7 wild-type knock-in mouse. Cells were grown as an adherent monolayer with 80-95% confluency in DMEM media, with 10% fetal bovine serum (FBS), 1% G418, and 1% penicillin/streptomycin solution. Cells were grown at 33°C in a temperature and moisture-controlled incubation chamber supplemented with 5% CO_2 and 95% O_2. 50% of the media was renewed every three days. The passage number of the cells was kept below P14.

Reagent Preparation

Thapsigargin (Tocris, catalogue #1138) was dissolved in 0.1% dimethyl sulfoxide (DMSO) at a concentration of 100 nM. THC (Sigma, catalogue #T4764) was dissolved in 0.1% methanol at a concentration of 2.5 μM. CBD (Sigma, catalogue #C-045) was dissolved in 0.1% methanol at a concentration of 1 μM. Vehicle controls were either 0.1% DMSO or 0.1% methanol. All reagents were prepared fresh.

Treatment

Cells were seeded in six-well plates at a density of $2.5x10^5$ cells/well. Cells were counted using the Trypan blue method on a glass hemocytometer. Treatment times for cell viability experiments are described in figure captions. All gene and

protein expression experiments were measured after 24 hours of treatment with THC and/or CBD, followed by 24 hours of TG alone.

Cell Viability

Cells were plated in 96-well plates at a density of 7.5 x10^3 cells/well. Cells were counted using the Trypan blue method on a glass hemocytometer. Cell viability was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma, catalogue #M5655). 5 mg of MTT powder was dissolved in 5 ml phosphate-buffered saline (PBS), 10 µl was added to each well. Following 2 hours of incubation at 33^{o}C/5% CO_2, the solution was removed from each well, and the resulting precipitate was dissolved in 100% DMSO. After 15 minutes of incubation with agitation, optical density values were obtained at 570nm on the Bio-Tek EPOCH microplate spectrophotometer. Each value conducted in MTT assays was analyzed in triplicate wells. Absorbance values were expressed as a percentage of control samples.

RNA Isolation

Cells were harvested using TRIzol reagent. Total RNA was extracted as per the manufacturer's protocol using TRIzol (Thermo Scientific, catalogue #15596018). RNA was diluted to the same concentration across all samples and was further purified using DNAse I enzyme (Thermo Fisher Scientific, catalogue #EN0521). A NanoDrop spectrophotometer was used to evaluate RNA integrity and purity. Samples were deemed pure if the A260/A280 value was greater than 2.0 and the A260/A230 value was close to 2.0. qScript cDNA SuperMix (Quantabio,

catalogue # 95048) was used for reverse transcription following the manufacturer's protocol. All samples were stored at -80°C until use.

Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)

Primers were synthesized at the Mobix Laboratory at McMaster University (**Table 1**). Gene expression was measured using RT-qPCR using SYBR Green Supermix (Bio-Rad, catalogue # 1725271) on the Stratagene MX3000P cycler following the manufacturer's protocol. Each reaction had a volume of 20 µl, and primers were used at a working concentration of 500 nM. The polymerase activation and DNA denaturation step was performed at 95°C for 30 seconds, followed by 40 cycles of amplification. Each cycle started with denaturation at 95°C for 10 seconds, and annealing and extension were combined for 30 seconds at 60°C. The negative controls used for every plate were the no reverse transcriptase (NRT) and no template control (NTC), which were used to check for contamination during RNA preparation and PCR, respectively. The relative gene expression levels were measured using the comparative CT method using GAPDH as the internal control. The following equation was used to calculate relative gene expression for each gene of interest (GOI) (Schmittgen & Livak, 2008):

$$2^{-\Delta\Delta C_T} = 2^{-[\,(C_{T_{GOI}} - C_{T_{GAPDH}})Sample - (Avg\ C_{T_{GOI}} - Avg\ C_{T_{GAPDH}})Control]}$$

Table 1: RT-qPCR mouse primer sequences

Gene	Sequence
GAPDH	F: CAACTCACTCAAGATTGTCAGCAA

	R: GGCATGGACTGTGGTCATGA
GRP78	F: TGCAGCAGGACATCAAGTTC
	R: TACGCCTCAGCAGTCTCCTT
MANF	F: CGGTTGTGCTACTACATTGGA
	R: CTGGCTGTCTTTCTTCTTCAGC
CDNF	F: AGCTGCTCAACTTTTGCTCA
	R: TAGGATCTTGGTGGCTGCAT
CHOP	F: CTGCCTTTCACCTTGGAGAC
	R: CGTTTCCTGGGGATGAGATA
BAX	F: TGCTACAGGGTTTCATCCAG
	R: GTCAGCAATCATCCTCTG
BCL-2	F: TCGCCCTGTGGATGACTGA
	R: CAGAGACAGCCAGGAGAAATCA
BIM	F: CGGATCGGAGACGAGTTCA
	R: GTCTTCAGCCTCGCGGTAAT
CASPASE 12	F: GAAGGAATCTGTGGGGTGAA
	R: TCAGCAGTGGCTATCCCTTT

Western Blot

Cells were harvested using CellLytic™ M Reagent (Sigma, catalogue #C2978). Protein concentration was determined using the Pierce 660nm Protein

assay (Thermo Scientific, catalogue #22662). 20 µl of the sample was prepared with 40 µg of protein. The samples were incubated at 95°C for 10 minutes and separated by electrophoresis on a 4-15% Mini-PROTEAN TGX stain-free precast gel as per the manufacturer's protocol (Bio-Rad, catalogue #456-8084). Separated protein gels were activated and transferred to a polyvinylidene membrane and blocked for 10 minutes in EveryBlot blocking buffer (Bio-Rad, catalogue #12010020) at room temperature. The membrane was then incubated at room temperature for 1.5 hours with anti-GRP78 (Proteintech, catalogue #11587-1-AP) at a concentration of 1:3000 in EveryBlot blocking buffer followed by five washes in 1X TBT-T for five minutes per wash. The membrane was incubated at room temperature for 1.5 hours in anti-rabbit IgG horseradish peroxidase-linked whole secondary antibody (Proteintech, catalogue #SA00001-7L) at a concentration of 1:16000 in EveryBlot blocking buffer. The membrane was washed six times in 1X TBS-T for five minutes per wash. The membrane was imaged using enhanced chemiluminescence substrate (Bio-Rad, catalogue #1705061) on the ChemiDoc MP Imaging System. The Image Lab program was used to determine band intensity, and all samples were normalized to total protein. Expression values were calculated as a percentage of control samples.

Statistical Analysis

All results were analyzed using GraphPad Prism 9.2.0. Statistical significance for cell viability, PCR, and western blot results were determined using a one-way ANOVA with Dunnett's or Tukey's multiple comparisons tests, and $p <$

0.05 was considered statistically significant. All outliers were excluded using the ROUT method.

3. RESULTS

Measuring the cytotoxicity of reagents

First, the published concentrations of THC and CBD were used to determine the effects of the compounds alone on ST*Hdh*$^{Q7/Q7}$ cells. THC was used at concentrations of 0.25, 1.25, and 2.50 μM and incubated for 24 hours (A) (**Figure 2**). CBD was used at concentrations of 0.10, 0.50, and 1.00 μM and incubated for 24 hours (B). The doses were not cytotoxic to ST*Hdh*$^{Q7/Q7}$ cells after 24 hours, so 2.5 μM THC and 1 μM CBD were used for all subsequent experiments.

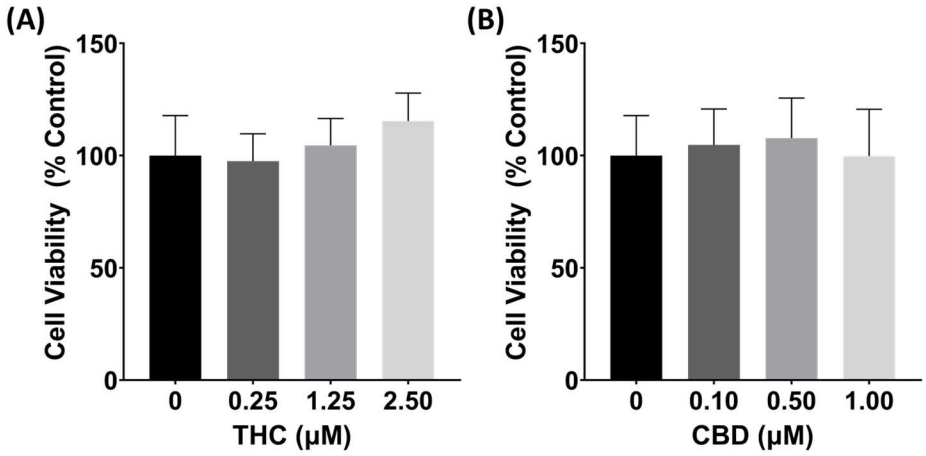

Figure 2: Cell viability of published doses of THC and CBD. (A) Viability of ST*Hdh*$^{Q7/Q7}$ cells did not change significantly after THC treatment (0.25μM, 1.25μM, 2.5μM) for 24 hours ($F(3,41) = 2.335$, $p = 0.0879$). (B) CBD (0.1μM, 0.5μM, 1μM) did not change cell viability significantly after 24 hours ($F(3,50) = 0.6019$, $p = 0.6168$). One-way ANOVA and Dunnett's multiple comparisons tests were used and data represent mean ± SEM. $n_{control} = 18$, $n_{treatment} = 12$, ROUT method of identifying outliers was used to remove outliers.

Previously published doses of TG in other cell lines were used in ST*Hdh*$^{Q7/Q7}$ cells to determine which concentration leads to significantly decreased cell viability (Eesmaa et al., 2021; Hiroi et al., 2005; Yagi et al., 2020). Cell viability of TG at various concentrations from 5 nM to 1 µM and incubated for 24 hours (**Figure 3**). Cell viability was significantly decreased to 77% at 50 nM and was further reduced as the dose increased. 1 µM TG resulted in 58% cell viability. The dose used for subsequent experiments was 100 nM which decreased cell viability to 70%. This dose was chosen to remain consistent with existing research.

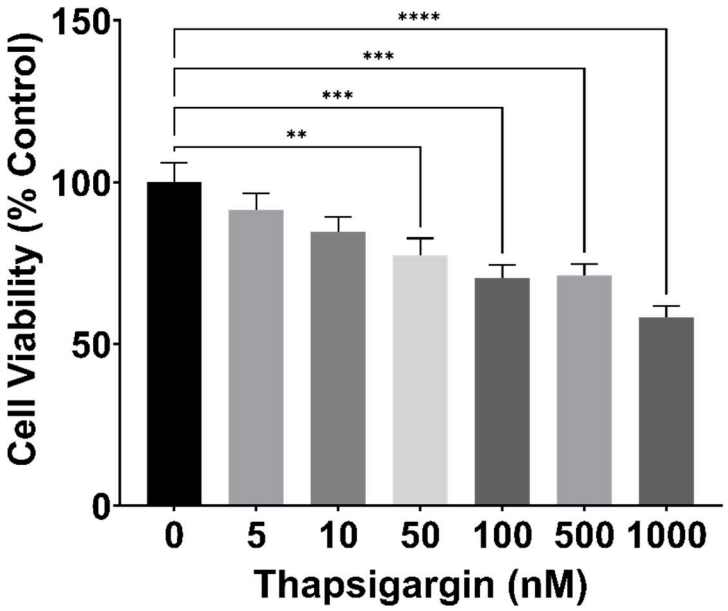

Figure 3: Cell viability of published doses of thapsigargin (TG). The viability of ST*Hdh*$^{Q7/Q7}$ cells decreased significantly after 24 hours at doses greater than 50 nM. Previously published dose of 100 nM TG reduced cell viability to 70% ($p = 0.0003$). One-way ANOVA ($F(6, 67) = 9.235$, $p < 0.0001$) and Dunnett's multiple

comparisons tests were used, and data represent mean ± SEM. n = 11 for all groups, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$, ROUT method of identifying outliers was used to remove outliers.

Testing the effects of THC and CBD on cell viability in three treatment paradigms of ER stress

Based on previous studies that investigated cannabinoid-mediated neuroprotection, three treatment paradigms were used to measure the effects of cannabinoids (independently and combined) on consequent, concurrent, and prior ER stress. A cell viability assay was used to test the effects of THC and CBD on ST*Hdh*$^{Q7/Q7}$ cell survival in the presence of ER stress. THC, CBD, or a combination of THC and CBD was administered simultaneously was TG for 24 hours to determine if inducing ER stress at the same time as cannabinoid treatment results in changes in neuronal survival (**Figure 4**). (A) THC administered concurrently with TG did not result in any changes in the cell death induced by TG. THC alone has no impact on cell viability. (B) CBD administered concurrently with TG did not result in any changes in the cell death induced by TG. CBD alone has no impact on cell viability. (C) THC and CBD administered concurrently with TG did not change the cell death caused by TG. THC and CBD cotreatment in the absence of TG reduced cell viability to 73%. This treatment paradigm was not investigated further because there were no changes in cell viability resulting from a cotreatment of cannabinoids and TG.

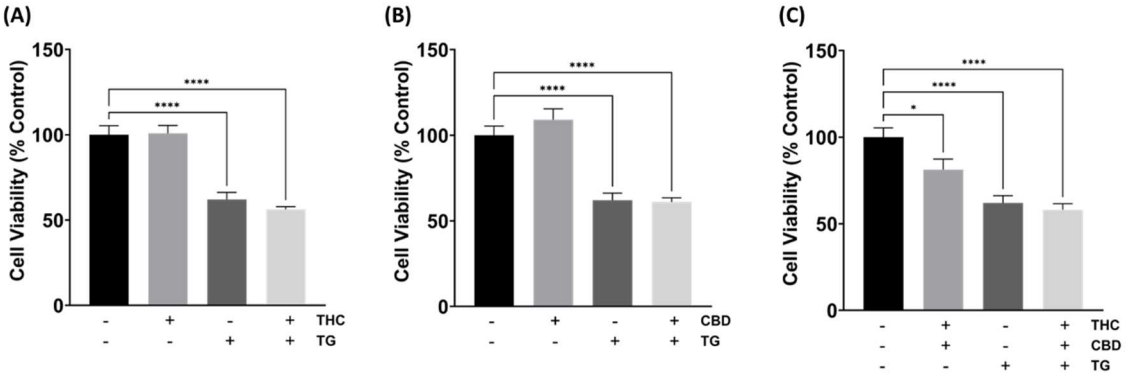

Figure 4: Changes in cell viability with concurrent treatment of THC, CBD, or a combination of THC and CBD with TG. The viability of ST*Hdh*$^{Q7/Q7}$ neurons decreases significantly after concurrent treatment of THC (A), CBD (B), and a combination of THC and CBD (C), with TG for 24 hours. Cell viability decreases to 62% with TG alone ($p < 0.0001$) and is not changed with cotreatment with THC, CBD, THC, or a mixture of THC and CBD. The combination of THC and CBD in the absence of TG reduced cell viability to 73% ($p = 0.0163$). One-way ANOVA and Tukey's multiple comparisons tests were used, and data represent mean ± SEM. n = 12 for all groups, *$p < 0.05$, ****$p < 0.0001$, ROUT method of identifying outliers was used to remove outliers.

Some studies pre-treat cells with cannabinoids to test if they can protect the cell from future insults instead of concurrent treatment. Therefore, a cell viability assay was used to test the effects of prior exposure (pre-treatment) of THC and

CBD on ST*Hdh*$^{Q7/Q7}$ cell survival before ER stress induction. (**Figure 5**). (A) THC administered prior to TG exposure resulted in less cell death compared to TG alone. (B) CBD administered prior to TG exposure resulted in less cell death compared to TG alone. (C) THC and CBD administered prior to TG did not result in any changes in cell death compared to TG. The changes resulting from independent pre-treatment of cannabinoids against TG led to further investigation of the transcriptional changes that occur in this treatment paradigm.

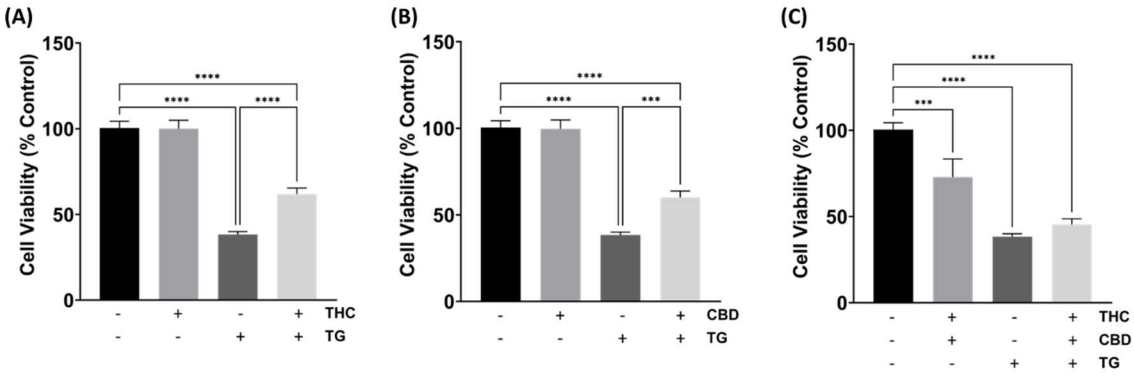

Figure 5: Changes in cell viability with a pre-treatment of THC, CBD, or a combination of THC and CBD, followed by TG exposure. The viability of ST*Hdh*$^{Q7/Q7}$ neurons exposed to TG for 24 hours increased significantly with a 24-hour pre-treatment of THC (A) or CBD (B) and did not change significantly with a 24-hour pre-treatment of a THC and CBD combination (C). Cell viability decreases to 38% with TG alone ($p < 0.0001$) and increase to 61% ($p = 0.0004$) and 60% ($p < 0.0001$) with THC and CBD pre-treatment, respectively. One-way ANOVA and Tukey's multiple comparisons tests were used, and data represent mean ± SEM.

n = 25 for all groups, ***p < 0.001, ****p < 0.0001, ROUT method of identifying outliers was used to remove outliers.

Lastly, the cannabinoid-induced cell survival against TG led to the investigation of whether cannabinoids could rescue striatal neurons from previous exposure to ER stress. To address this aim, a cell viability assay was used to test the effects of subsequent exposure (post-treatment) of THC and CBD on ST*Hdh*$^{Q7/Q7}$ cell survival after ER stress induction (**Figure 6**). (A) THC administered after incubation with TG resulted in no changes to cell survival compared to TG alone. (B) CBD administered after incubation with TG resulted in no changes to cell survival compared to TG alone. (C) THC and CBD co-administered after incubation with TG resulted in no changes to cell survival compared to TG alone.

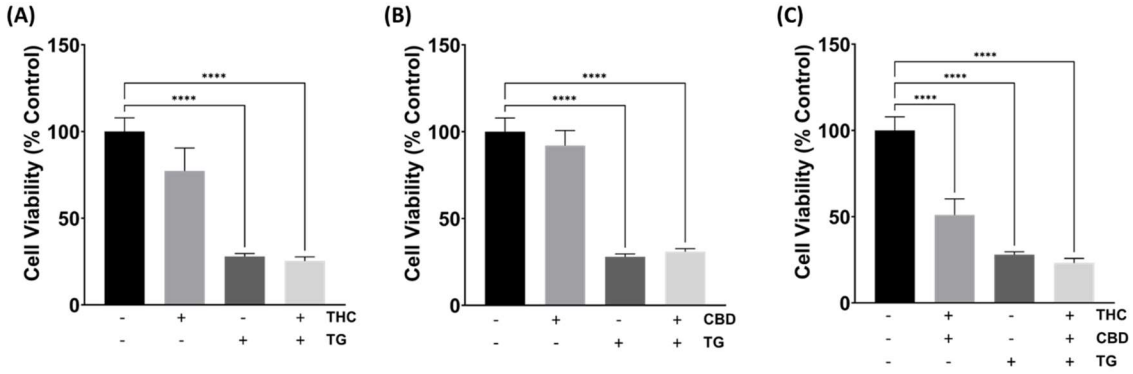

Figure 6: Changes in cell viability with a post-treatment of THC, CBD, or a combination of THC and CBD, following TG exposure. The viability of ST*Hdh*$^{Q7/Q7}$ neurons decreased significantly with a 24-hour exposure to TG. It was not rescued with subsequent treatment of THC (A), CBD (B), or a combination of THC and CBD for 24 hours following a concurrent treatment of THC (A), CBD (B),

and a combination of THC and CBD (C) for 24 hours. Cell viability decreases to 28% with TG alone ($p < 0.0001$). One-way ANOVA and Tukey's multiple comparisons tests were used, and data represent mean ± SEM. n = 12 for all groups, ****$p < 0.0001$, ROUT method of identifying outliers was used to remove outliers.

Transcriptional changes resulting from cannabinoid pre-treatment followed by ER stress induction

Changes in ER-resident chaperone and neurotrophic factors

The chaperone GRP78 is the master regulator of ER stress, so it was measured first to determine if TG neurotoxicity observed in the cell viability assay impacted ER function. Additionally, it was measured to determine if cannabinoid pre-treatment leads to any changes in the ER's ability to respond to stress. GRP78 mRNA expression increased by 5.15-fold when cells were exposed to TG alone (**Figure 7**). Cells pre-treated with THC prior to TG exposure had a 7.66-fold increase in GRP78 expression, while cells pre-treated with CBD prior to TG exposure had a 12.24-fold increase in GRP78 expression. A combined pre-treatment of THC and CBD prior to TG exposure resulted in a 16.74-fold increase in GRP78 expression. GRP78 expression was not altered in any cells which were not exposed to TG. These results confirmed that TG indeed impacted the ER'S protein folding ability. Furthermore, combined and individual cannabinoid pre-treatments lead to even greater induction of GRP78 expression in response to TG

toxicity. However, the increase is seen by all three pre-treatments. Therefore the changes in cell viability with THC or CBD pre-treatment alone are not solely caused by an increase in GRP78 expression. To further investigate the role of ER-resident genes in neuroprotection, the expression of MANF and CDNF will be measured because of their structural composition, which suggests that they may assist in protein folding.

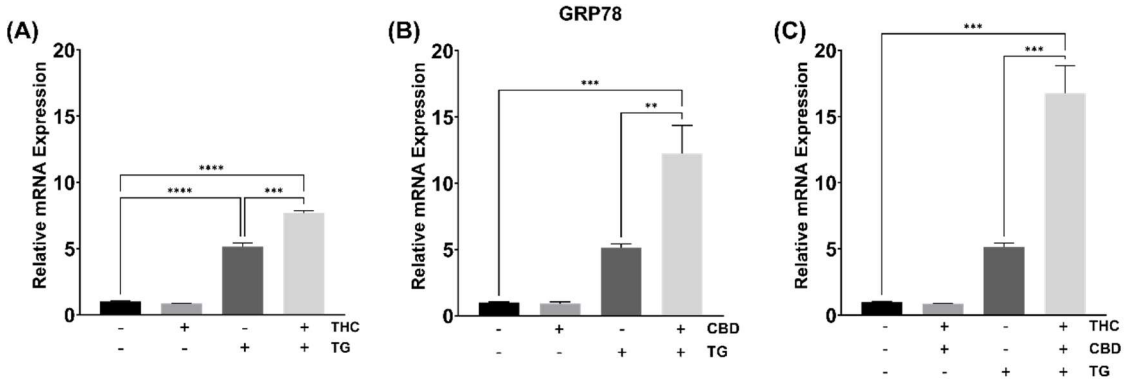

Figure 7: Changes in GRP78 gene expression with cannabinoid pre-treatment followed by TG exposure. GRP78 mRNA expression was measured after ST*Hdh*$^{Q7/Q7}$ neurons were treated with THC (A), CBD (B), or a combination of THC and CBD (C) for 24 hours, followed by a 24-hour incubation with TG. TG alone increases GRP78 expression to 5.15 ($p < 0.0001$). Cells pre-treated with THC before TG exposure had a GRP78 expression of 7.66 ($p = 0.0001$). Cells pre-treated with CBD before TG exposure had a GRP78 expression of 12.24 ($p = 0.0070$). Before TG exposure, cells pre-treated with a combination of THC and CBD had a GRP78 expression of 16.74 ($p = 0.0008$). One-way ANOVA and Tukey's multiple comparisons tests were used, and data represent mean ± SEM.

n = 3 for all groups, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$, ROUT method of identifying outliers was used to remove outliers.

MANF expression was measured to determine the changes in gene expression in ST*Hdh*$^{Q7/Q7}$ neurons caused by the pre-treatment with cannabinoids compared to TG alone (**Figure 8**). MANF mRNA expression increased by 2.48-fold when cells were exposed to TG alone. Cells pre-treated with THC prior to TG exposure had a 3.58-fold increase in MANF expression, while cells pre-treated with CBD prior to TG exposure had a 5.14-fold increase in MANF expression. A combined pre-treatment of THC and CBD prior to TG exposure resulted in a 6.26-fold increase in MANF expression. MANF expression was not altered in any cells which were not exposed to TG. MANF expression follows a similar trend as GRP78 expression, suggesting that changes in downstream UPR genes may cause changes in neuronal survival.

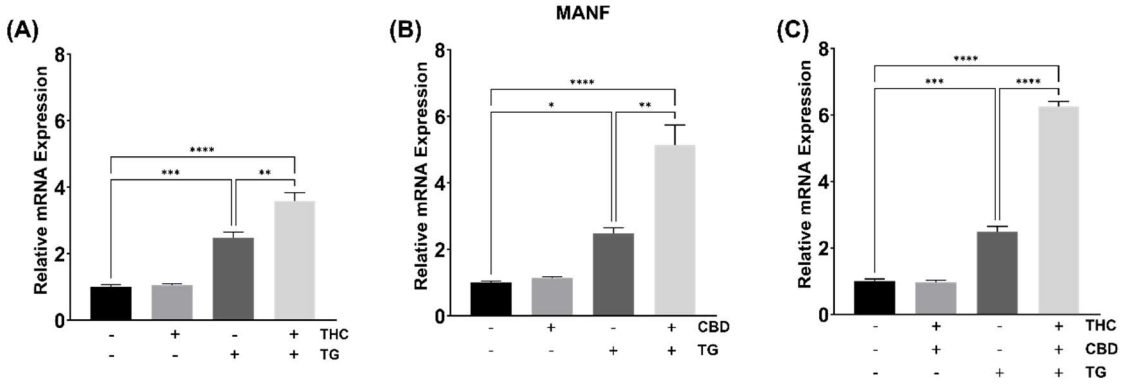

Figure 8: Changes in MANF gene expression with cannabinoid pre-treatment followed by TG exposure. MANF mRNA expression was measured after ST*Hdh*$^{Q7/Q7}$ neurons were treated with THC (A), CBD (B), or a combination of THC

and CBD (C) for 24 hours, followed by a 24-hour incubation with TG. TG alone increases MANF expression to 2.48 ($p = 0.0003$). Cells pre-treated with THC before TG exposure had a MANF expression of 3.58 ($p = 0.0040$). Cells pre-treated with CBD before TG exposure had a MANF expression of 5.14 ($p = 0.0015$). Cells pre-treated with a combination of THC and CBD before TG exposure had a MANF expression of 6.26 ($p < 0.0001$). One-way ANOVA and Tukey's multiple comparisons tests were used, and data represent mean ± SEM. n = 3 for all groups, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$, ROUT method of identifying outliers was used to remove outliers.

Similar to MANF, CDNF expression was measured to determine if transcriptional changes in CDNF result from cannabinoid pre-treatment compared to TG alone (**Figure 9**). Compared to controls, CDNF expression did not differ in any treatment group. These results do not provide any details on the role of CDNF in cannabinoid-mediated cell survival against TG and strengthen the notion that changes to UPR genes downstream of GRP78 contribute to neuronal survival.

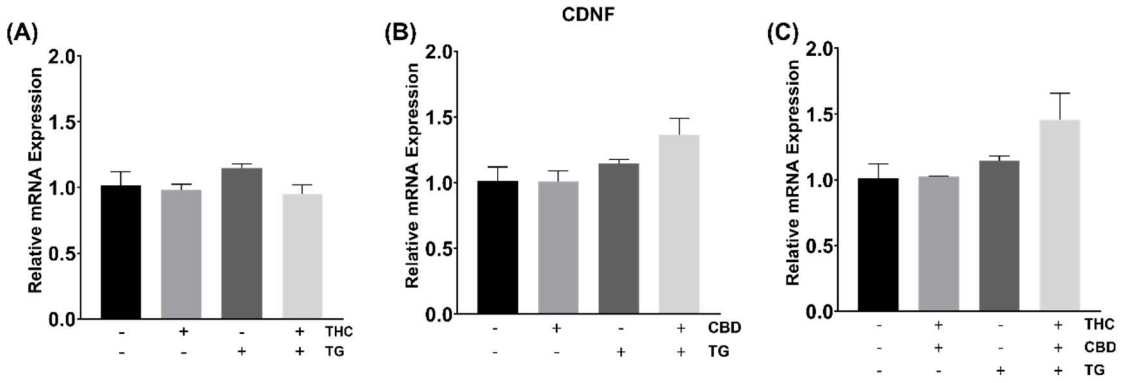

Figure 9: Changes in CDNF gene expression with cannabinoid pre-treatment followed by TG exposure. CDNF mRNA expression was measured after ST*Hdh*$^{Q7/Q7}$ neurons were treated with THC (A), CBD (B), or a combination of THC and CBD (C) for 24 hours, followed by a 24-hour incubation with TG. Compared to control, CDNF expression did not change when treated with TG alone ($p = 0.8654$). CDNF expression did not differ from TG alone when cells were pre-treated with THC, CBD, or a combination of THC and CBD for 24 hours. One-way ANOVA and Tukey's multiple comparisons tests were used, and data represent mean ± SEM. n = 3 for all groups, ROUT method of identifying outliers was used to remove outliers.

Changes in ER-specific apoptosis markers

The expression of ER-specific apoptosis markers was measured to determine if the cannabinoid-induced increase in cell viability against TG is led to downstream changes in the terminal UPR pathway. The first caspase activated during chronic ER stress is caspase 12, an ER-associated caspase (**Figure 10**). Caspase 12 mRNA expression increased by 1.34-fold when cells were exposed to TG alone. Cells pre-treated with THC prior to TG exposure had a 0.95-fold change from control which significantly differed from the increase seen in cells exposed to TG alone. Similarly, cells pre-treated with CBD prior to TG exposure also had a 0.95-fold change from control which significantly differed from the increase seen in cells exposed to TG alone. A combined pre-treatment of THC and CBD prior to TG

exposure resulted in a 1.83-fold increase in caspase 12 expression. Caspase 12 expression was not altered in any cells which were not exposed to TG. These results show that the terminal UPR pathway is attenuated in the neuroprotection resulting from THC or CBD pre-treatment against TG-induced ER stress. Caspase 12 activity correlates with BIM activity, so the expression of BIM was also measured.

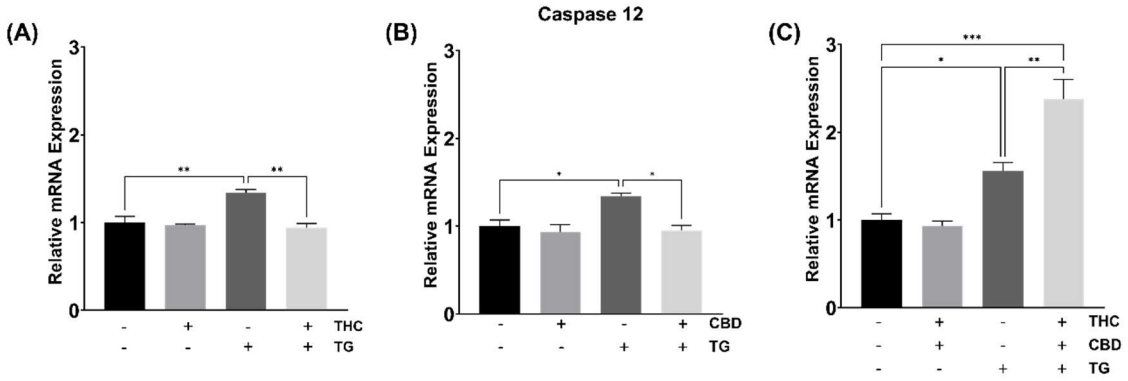

Figure 10: Changes in caspase 12 gene expression with cannabinoid pre-treatment followed by TG exposure. Caspase 12 mRNA expression was measured after ST*Hdh*$^{Q7/Q7}$ neurons were treated with THC (A), CBD (B), or a combination of THC and CBD (C) for 24 hours, followed by a 24-hour incubation with TG. TG alone increases caspase 12 expression to 1.34 ($p = 0.0271$). Cells pre-treated with THC before TG exposure had a caspase 12 expression of 0.95 ($p = 0.0028$). Cells pre-treated with CBD before TG exposure had a caspase 12 expression of 0.95 ($p = 0.0230$). Cells pre-treated with a combination of THC and CBD before TG exposure had a caspase 12 expression of 1.83 ($p = 0.0519$). One-way ANOVA and Tukey's multiple comparisons tests were used, and data

represent mean ± SEM. n = 3 for all groups, *p < 0.05, **p < 0.01, ***p < 0.001, ROUT method of identifying outliers was used to remove outliers.

In the presence of chronic ER stress, BIM expression increases alone with caspase 12 to signal apoptosis. BIM expression was measured to determine the changes in gene expression in ST*Hdh*$^{Q7/Q7}$ neurons caused by the pre-treatment with cannabinoids compared to TG (**Figure 11**). BIM mRNA expression increased by 1.68-fold when cells were exposed to TG alone. Cells pre-treated with THC prior to TG exposure had a 1.05-fold change from control which significantly differed from the increase seen in cells exposed to TG alone. Similarly, cells pre-treated with CBD prior to TG exposure had a 1.39-fold change from control which significantly differed from the increase seen in cells exposed to TG alone. A combined pre-treatment of THC and CBD prior to TG exposure resulted in a 2.73-fold increase in BIM expression. BIM expression was not altered in any cells which were not exposed to TG. THC or CBD pre-treatment before TG exposure leads to a decrease in ER-specific apoptosis markers compared to TG alone.

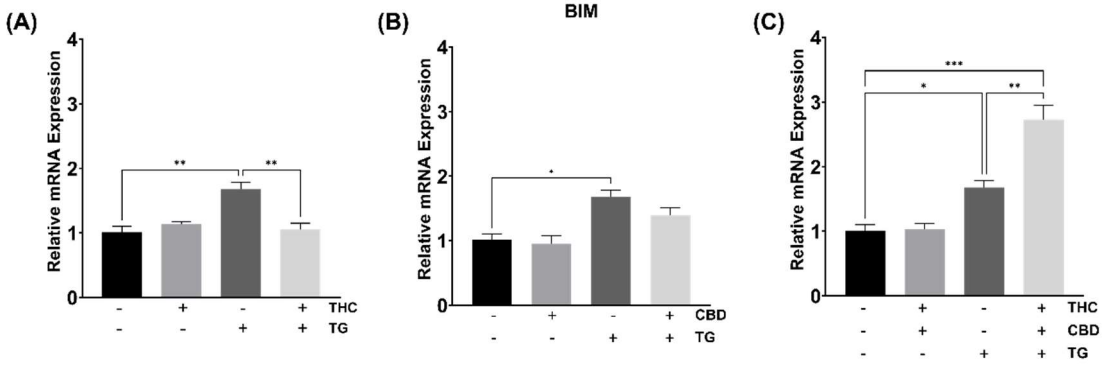

Figure 11: Changes in BIM gene expression with cannabinoid pre-treatment followed by TG exposure. BIM mRNA expression was measured after ST*Hdh*$^{Q7/Q7}$ neurons were treated with THC (A), CBD (B), or a combination of THC and CBD (C) for 24 hours, followed by a 24-hour incubation with TG. TG alone increases BIM expression to 1.68 (p = 0.0236). Cells pre-treated with THC before TG exposure had a BIM expression of 1.05 (p = 0.0090). Cells pre-treated with CBD before TG exposure had a BIM expression of 1.39 (p = 0.3281). Cells pre-treated with a combination of THC and CBD before TG exposure had a BIM expression of 2.73 (p = 0.0046). One-way ANOVA and Tukey's multiple comparisons tests were used, and data represent mean ± SEM. n = 3 for all groups, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ROUT method of identifying outliers was used to remove outliers.

Changes in additional UPR genes and apoptosis markers

The role of the ER-specific apoptosis markers in cell survival led to an investigation of other apoptosis markers in the UPR, which play a role in both ER and oxidative stress modulation. The expression of CHOP, BAX, and BCL-2 was measured to determine the effects of cannabinoid pre-treatment on both pro-apoptotic and anti-apoptotic genes. CHOP is a key regulator of the terminal UPR pathway and was measured to determine the changes in gene expression in ST*Hdh*$^{Q7/Q7}$ neurons caused by the pre-treatment with cannabinoids compared to TG (**Figure 12**). CHOP mRNA expression increased by 6.50-fold when cells were

exposed to TG alone. Cells pre-treated with THC prior to TG exposure had a decrease in CHOP expression but was not statistically significant. In contrast, cells pre-treated with CBD prior to TG exposure had no change in CHOP expression. A combined pre-treatment of THC and CBD prior to TG exposure resulted in a 9.15-fold increase in CHOP expression. CHOP expression was not altered in any cells which were not exposed to TG. The lack of changes in CHOP in cannabinoid pre-treatment groups which showed neuroprotection led to investigating genes downstream of CHOP to determine whether CHOP-activated apoptosis factors play a role in ER stress-induced neuronal death.

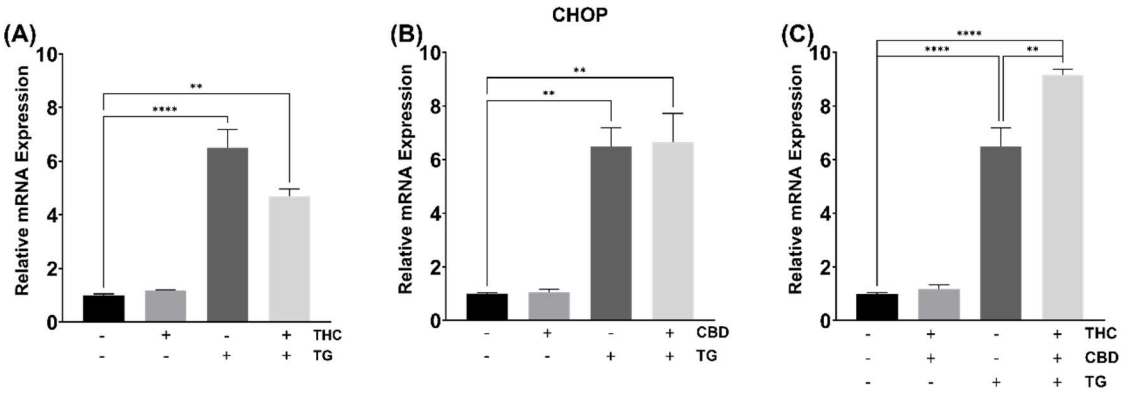

Figure 12: Changes in CHOP gene expression with cannabinoid pre-treatment followed by TG exposure. CHOP mRNA expression was measured after ST*Hdh*$^{Q7/Q7}$ neurons were treated with THC (A), CBD (B), or a combination of THC and CBD (C) for 24 hours, followed by a 24-hour incubation with TG. TG alone increases CHOP expression to 6.50 ($p < 0.0001$). Cells pre-treated with THC before TG exposure had a CHOP expression of 4.70 ($p = 0.0735$). Cells pre-treated with CBD before TG exposure had a CHOP expression of 6.65 ($p = 0.9981$). Cells

pre-treated with a combination of THC and CBD before TG exposure had a CHOP expression of 9.15 ($p = 0.0079$). One-way ANOVA and Tukey's multiple comparisons tests were used, and data represent mean ± SEM. n = 3 for all groups, $^{**}p < 0.01$, $^{****}p < 0.0001$, ROUT method of identifying outliers was used to remove outliers.

CHOP activates pro-apoptotic markers such as BIM and BAX. The role of BIM in cannabinoid-mediated neuroprotection and the increase in CHOP with TG exposure alone prompted the measurement of BAX gene expression (**Figure 13**). Compared to controls, BAX expression did not differ in any treatment group. The inconclusive findings of this apoptotic factor led to the investigation of the expression of the anti-apoptotic marker BCL-2.

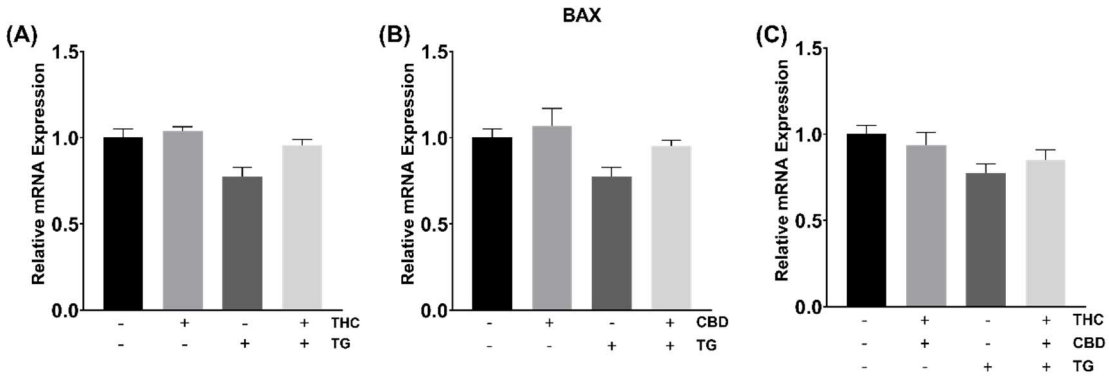

Figure 13: Changes in BAX gene expression with cannabinoid pre-treatment followed by TG exposure. BAX mRNA expression was measured after ST*Hdh*$^{Q7/Q7}$ neurons were treated with THC (A), CBD (B), or a combination of THC and CBD (C) for 24 hours, followed by a 24-hour incubation with TG. Compared to control, BAX expression did not change when treated with TG alone ($p = 0.1328$).

BAX expression did not differ from TG alone when cells were pre-treated with THC, CBD, or a combination of THC and CBD for 24 hours. One-way ANOVA and Tukey's multiple comparisons tests were used, and data represent mean ± SEM. n = 3 for all groups, ROUT method of identifying outliers was used to remove outliers.

CHOP directly inhibits the activity of the anti-apoptotic marker BCL-2. BCL-2 also inhibits pro-apoptotic markers such as BIM and BAX. Therefore, the expression was measured to determine the changes in gene expression in ST*Hdh*$^{Q7/Q7}$ neurons caused by the pre-treatment with cannabinoids compared to TG (**Figure 14**). Compared to controls, BCL-2 expression did not change in cells treated with TG alone. Cells pre-treated with THC or CBD prior to TG exposure had a significant increase in BCL-2 expression, while cells pre-treated a combination of THC and CBD prior to TG exposure had no change in BCL-2 expression. Similar to BIM and caspase 12, the changes in BCL-2 were only observed in the samples pre-treated with THC or CBD but not the samples pre-treated with a combination of the two.

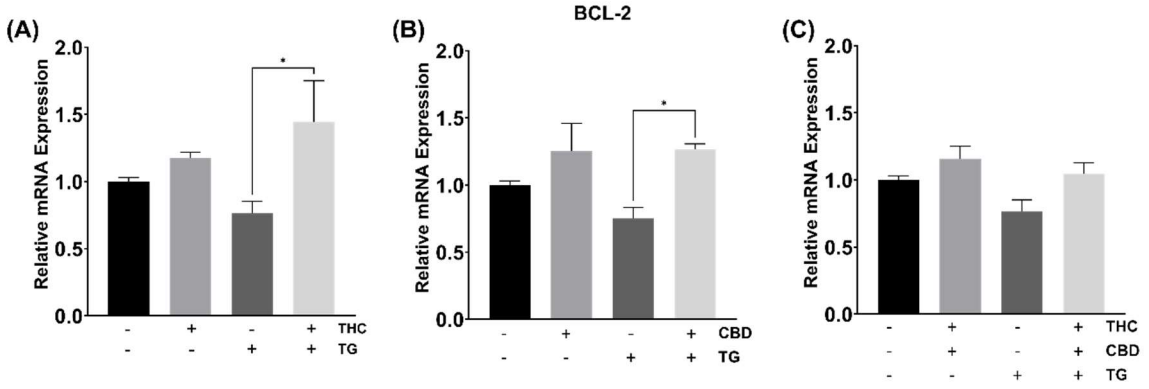

Figure 14: Changes in BCL-2 gene expression with cannabinoid pre-treatment followed by TG exposure. BCL-2 mRNA expression was measured after ST*Hdh*$^{Q7/Q7}$ neurons were treated with THC (A), CBD (B), or a combination of THC and CBD (C) for 24 hours, followed by a 24-hour incubation with TG. TG alone decreases BCL-2 expression to 0.75 ($p = 0.1821$). Cells pre-treated with THC before TG exposure had a BCL-2 expression of 1.45 ($p = 0.0219$). Cells pre-treated with CBD before TG exposure had a BCL-2 expression of 1.27 ($p = 0.0492$). Cells pre-treated with a combination of THC and CBD before TG exposure had a BCL-2 expression of 1.05 ($p = 0.1001$). One-way ANOVA and Tukey's multiple comparisons tests were used, and data represent mean ± SEM. n = 3 for all groups, *$p < 0.05$, ROUT method of identifying outliers was used to remove outliers.

Changes in GRP78 protein expression in cells pre-treated with cannabinoids before ER stress induction

To determine if the changes seen in gene expression are translated to changes in protein expression, a western blot was used to measure the protein

expression of GRP78 (**Figure 15**). Cells pre-treated with THC prior to TG exposure had a 13.11 -fold increase in GRP78 protein expression, while cells pre-treated with CBD prior to TG exposure had a 23.68-fold increase in GRP78 protein expression. These results demonstrate that the changes in GRP78 expression occur at both the transcription and translation stages.

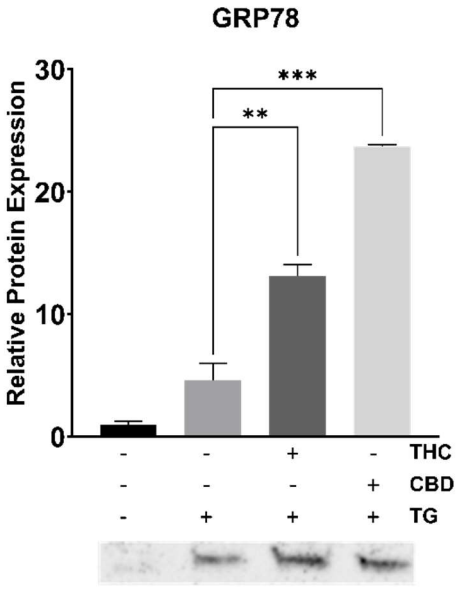

Figure 15: Changes in GRP78 protein expression with cannabinoid pre-treatment followed by TG exposure. GRP78 protein expression was measured after ST*Hdh*$^{Q7/Q7}$ neurons were treated with THC (A), CBD (B), or a combination of THC and CBD (C) for 24 hours, followed by a 24-hour incubation with TG. TG alone increases GRP78 protein expression to 4.58 ($p = 0.1497$). Cells pre-treated with THC before TG exposure had a GRP78 protein expression of 13.11 ($p = 0.0038$). Cells pre-treated with CBD before TG exposure had a GRP78 protein expression of 23.68 ($p = 0.0001$). One-way ANOVA and Tukey's multiple comparisons tests

were used, and data represent mean ± SEM. n = 3 for all groups, $^{**}p < 0.01$, $^{***}p < 0.001$, ROUT method of identifying outliers was used to remove outliers.

4. DISCUSSION

This study shows THC and CBD monotherapy are protective against ER stress in mouse striatal neurons. The decrease in cell viability caused by TG (**Figure 3**) is partially restored with pre-treating with THC or CBD alone for 24 hours prior to incubation with TG (**Figure 5A-B**). These findings are consistent with results from other cell lines, which have shown increased cell viability when treated with CBD before inducing stress. CBD pre-treatments of 2.5 μM ranging from 1 hour to 24 hours in primary cerebellar granule neurons were protective against H_2O_2 and rotenone (Echeverry et al., 2020). A 24-hour pre-treatment of 1 μM CBD was also protective against cadmium in differentiated SH-SY5Y neuroblastoma cells (Branca et al., 2019). In the same neuroblastoma cells, a 24-hour pre-treatment of 10 μM CBD was protective against MPP^+ (Gugliandolo et al., 2020). Previously, CBD pre-treatment was protective against toxins that cause oxidative stress in cerebellar neurons and neuroblastoma cells, this study found that CBD pre-treatment is also protective against ER stress in striatal neurons.

However, a pre-treatment with a combination of THC and CBD does not result in an increase in cell viability against TG (**Figure 5C**). While a combination of THC and CBD was protective in an animal model of Alzheimer's disease, this cellular model saw the opposite result. There was no change in TG-induced cell death in striatal neurons. However, the animals were injected daily with cannabinoids for five weeks prior to performing behavioural and biological analyses (Aso et al., 2015). In another animal study, striatal neuron degeneration was

reduced with a combined treatment of THC and CBD. This study injected the cannabinoids 30 minutes prior to and two hours following the excitotoxicity-inducing toxin injection; brain tissue was harvested 48 hours following the final injection (Valdeolivas et al., 2012). The chronic treatment of the cannabinoids in the Alzheimer's disease model or multiple treatments in the excitotoxicity model may have contributed to the attenuation of degeneration in the animal models. The inability to attenuate TG-induced toxicity with a combination of THC and CBD pre-treatment may be due to the single pre-treatment administered in this study.

Concurrent treatment of THC, CBD, or a combination of THC and CBD, with TG, did not result in any changes in cell viability compared to TG alone (**Figure 4**). While previous studies have not studied the concurrent effects of THC or a combination of THC and CBD against neurotoxins, some studies have investigated the effects of concurrent CBD treatment. PC12 cells, derived from a pheochromocytoma of the adrenal medulla, were treated concurrently with 1 μM CBD and MPP^+ increased cell viability compared to MPP^+ alone after 24 hours. A similar effect is seen with concurrent treatment with 5 μM CBD or 10 μM CBD (Santos et al., 2015). Additionally, a 24-hour concurrent treatment of 5 μM CBD and H_2O_2 was neuroprotective against H_2O_2-induced toxicity in primary hippocampal neurons (Kim et al., 2021). However, hippocampal neurons viability did not change significantly with concurrent treatment with 1 μM CBD and H_2O_2. Both MPP^+ and H_2O_2 increase the production of ROS, resulting in oxidative stress. Therefore a 24-hour concurrent treatment with CBD may be neuroprotective

against an oxidative stress inducer but is not protective against an ER stress inducer.

This study was the first to investigate the effect of cannabinoid treatment after ER stress induction in striatal neurons. A treatment of THC, CBD or a combination of THC and CBD after ER stress induction using TG also did not rescue cell viability (**Figure 6**). No previous studies had investigated this treatment paradigm, and the results from this study suggest that THC and CBD, independently or combined, cannot restore neuron function following ER stress.

The protective changes resulting from a pre-treatment THC or CBD against TG led to investigating the transcriptional changes in the UPR genes. Compared to control, TG alone increased the expression on GRP78, MANF, caspase 12, BIM, and CHOP (**Figures 7, 8, 10-12**) and did not alter the expression of CDNF, BAX, and BCL-2 (**Figures 9, 13, 14**). Alone, TG has been shown to significantly increase GRP78, MANF, CHOP, and BIM mRNA levels in various cell lines(Hiroi et al., 2005; Wang et al., 2014; Yagi et al., 2020). TG also decreases BCL-2 mRNA levels in PC12 cells(Hiroi et al., 2005). Additionally, TG has induced GRP78-mediated procaspase 12 cleavage (Nakagawa et al., 2000). The results of this study are consistent with the observed effects of TG in other neuronal and kidney cell lines.

This study was the first to investigate the transcriptional changes caused by cannabinoid treatment in a striatal neuron model of ER stress. The genes measured directly interact with misfolded proteins or play a role in restoring ER function to facilitate protein folding. This study found that THC pre-treatment prior

to TG exposure increased the expression of GRP78, MANF, and BCL-2 compared to TG alone (**Figures 7A, 8A, 14A**). Additionally, caspase 12 and BIM expression significantly decreased with THC pre-treatment compared to TG alone (**Figures 10A and 11A**). Compared to TG alone, CBD pre-treatment prior to TG exposure increased the expression of GRP78, MANF and BCL-2 (**Figures 7B, 8B, 14B**) and decreased the expression of caspase 12 (**Figure 10B**). When THC and CBD were combined for the pre-treatment before TG, there was an increase in GRP78, MANF, CHOP and BIM expression compared to TG alone (**Figures 7C, 8C, 11C, 12C**).

While no previous studies have measured changes in all these genes simultaneously, some have focused on the role of cannabinoids in transcriptional changes in UPR genes. In the liver, hepatic stellate cells treated with 2 μM CBD resulted in apoptosis and an increase in the expression of CHOP (Lim et al., 2011). THC or CBD alone have not resulted in neuronal harm in this study, but the cytotoxic effect of CBD in Lim et al. may be due to the increased concentration of CBD used. The expression of cannabinoid receptors is also different in the liver compared to the brain, so the receptor-mediated effects of CBD may have played a role in the observed cytotoxicity. In the current study, striatal cells pre-treated with CBD before TG exposure did not display any changes in CHOP expression compared to TG alone.

Additionally, MPP^+-induced oxidative stress was attenuated by CBD. This resulted in an increase in cell viability with CBD treatment and a decrease in BAX

expression (Gugliandolo et al., 2020). BAX expression was not altered in any treatment groups of this study. This difference with existing literature may be due to the toxins used in each model. MPP^+ primarily induces oxidative stress, while TG induces ER stress. The role of BAX is not directly related to ER function; it is activated downstream of CHOP. Therefore, the decrease in BAX observed in the CBD-mediated neuroprotection against MPP^+ may be due to the critical role of BAX in initiating oxidative stress-induced apoptosis (Steckley et al., 2007). The apoptotic activity of BIM in ER stress may be replacing the role of BAX in oxidative stress. This is supported by the decrease BIM expression with THC or CBD pre-treatment compared to TG alone.

Lastly, GRP78 protein expression results emulated the gene expression results showing an increase in the expression of GRP78 with THC or CBD pre-treatment compared to TG (**Figure 15**). One study has measured the protein expression changes in GRP78 caused by TG in ST*Hdh*$^{Q7/Q7}$ cells. However, the study did not report the band intensity because the TG-induced change in GRP78 expression was not the primary focus (Sbodio et al., 2018).

This study adds to our understanding of the role of cannabinoids in neuroprotection, specifically ER stress modulation. While ST*Hdh*$^{Q7/Q7}$ cells have been used for cannabinoid-related research, this is the first study to use them as a model for ER stress in combination with the cannabinoids THC and CBD. The protective effect of THC and CBD treatment against ER stress observed in these striatal neurons suggests their therapeutic benefit to decelerate the loss of neurons

caused by ER dysfunction in neurodegenerative disorders. Additionally, this study also investigated the effects of THC and CBD on UPR proteins and the neurotrophic factors MANF and CDNF. The observed increase in adaptive UPR genes and decrease in terminal UPR genes suggests that cannabinoid pre-treatment pre-empts the cell for an insult to the ER. It does so by increasing the expression of GRP78 and MANF to facilitate protein folding capacity. Also, the decrease in expression of apoptotic markers such as BIM and caspase 12 suggests that the cannabinoids may be shifting the UPR to its adaptive pathway to promote cell survival instead of the terminal pathway, which leads to cell death. Lastly, these results support the notion that cannabinoids are not neurotoxic in striatal cells because cannabinoid monotherapy does not affect cell viability and gene expression on its own. Changes are only caused in the presence of TG. These findings provide insights into the role of cannabinoids in ER stress modulation and neuronal survival.

5. LIMITATIONS

While this study provides a detailed look at the effects of cannabinoids on ER stress modulation, there are some limitations of the study design. Firstly, the cell line used was derived from the striatum. The conclusions of this study can only be extrapolated to the role of ER stress-mediated striatal dysfunction in disorders. However, the extensive research done on the role of the striatum in conditions such as Parkinson's disease, progressive supranuclear palsy, Huntington's disease, the extrapyramidal symptoms of amyotrophic lateral sclerosis, and frontotemporal dementia supports the conclusions made in this study (Alexianu et al., 1994; Mattson, 2004; Ramaswamy et al., 2007; Surmeier, 2007).

The effects of thapsigargin on other cellular processes could also limit the conclusions of this study. Thapsigargin was chosen as the toxin in this cellular model because of its direct effects on the ER. It explicitly affects calcium homeostasis, making it an ideal toxin to study neurodegenerative disorders with calcium dysfunction. While the results in the study support the notion that neuronal survival with THC or CBD pre-treatment is mediated through the ER, other mechanisms of cell survival may also play a role in increasing the cell viability observed. For example, the modulation of oxidative stress and inflammation by THC and CBD pre-treatment may also play a role in cell survival. While ER stress modulation plays a role in cannabinoid-mediated neuroprotection against thapsigargin, it may not be the sole factor contributing to protection.

6. FUTURE DIRECTIONS

These findings create various opportunities for research in the role of cannabinoid-mediated cell survival against ER stress. This study focused on the UPR effects downstream of GRP78 dissociation. However, the role of cannabinoid receptor signalling in the ER stress-induced cell death remains uninvestigated. The distinct pharmacological properties of THC and CBD lead to different receptor-mediated effects in vitro. Previous studies had found that cannabinoid-mediated neuroprotection was not observed when receptors were blocked. So, adding CB_1 and CB_2 receptor inhibitors to this treatment paradigm or replicating the experiments in a cell line that does not express CB receptors.

Also, the role of intracellular secondary messengers such as Ca^{2+} should be researched. TG induces ER stress through calcium dysfunction. Therefore, the effect of THC and CBD acute and chronic treatment on calcium should be studied. Using calcium fluorescence assays to determine the changes in ER calcium concentration caused by THC, CBD, and TG would provide insights into the role of calcium homeostasis in cannabinoid-mediated neuroprotection.

Additionally, the limitation of the use of TG and the chronic effects of the compound on other cellular processes should also be addressed in future studies. By studying the changes in oxidative stress markers, pro-inflammatory cytokines, and anti-inflammatory mediators, the effect of prolonged TG exposure on multiple cellular processes can be determined. These markers could also be measured in the pre-treatment paradigm investigated in the study to determine if the increase

from THC or CBD pre-treatment leads to attenuation of oxidative stress or inflammation caused by TG.

Lastly, to increase the generalizability of these findings, similar experiments should be conducted in cell lines derived from other brain regions implicated in neurodegenerative disorders. For example, replicating these experiments in cells derived from different brain regions within the frontal cortex, the hippocampus, and the basal forebrain would provide insights into the role of ER stress-mediated neuronal loss and the potential of cannabinoid-mediated neuroprotection in those regions. This study sets a foundation for the experimental approach and markers that can be used in future experiments investigating the role of cannabinoids and ER stress in neurodegenerative disorders.

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