THESIS

SKN-1, NRF HOMOLOG, MEDIATES CANNABIDIOL CELLULAR STRESS RESPONSIVE EFFECTS IN CAENORHABDITIS ELEGANS

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ABSTRACT

SKN-1, NRF HOMOLOG, MEDIATES CANNABIDIOL CELLULAR STRESS RESPONSIVE EFFECTS IN CAENORHABDITIS ELEGANS

Alzheimer's disease (AD) is a neurodegenerative disease that is affecting an increasing number of the aged population worldwide. AD is characterized by the accumulation of amyloid beta (Aβ) and tau hyperphosphorylation along with a failure in redox homeostasis. The hallmarks of neurodegenerative diseases include the increased generation of reactive oxygen species (ROS) which is tightly controlled by an antioxidant defense mechanism under physiological conditions. This research aimed to utilize various strains of the model organism *C. elegans* to understand the mechanism of cannabidiol at the cellular level in stressed models. The SKN-1 gene, the Nrf homolog in *C. elegans*, encodes for three different isoforms, skn-1a, skn-1b, and c. Skn-1b/c, which plays a role in oxidative stress, is negatively regulated by the repressor WDR-23. In *C. elegans*, skn-1a plays a role in proteotoxic stress through upregulation proteosome subunits and is negatively regulated by the abundance of proteosome complex protein. Results show that 10μM of CBD was able to activate isoforms of skn-1, skn-1a and skn-1b/c. The ROS scavenging activity of CBD was dependent on the presence of skn-1b/c. Furthermore, CBD's protective effects under proteotoxic stress were diminished in the absence of skn-1a. Further investigation will be conducted to identify the role of skn-1 in CBD's reduction of Aβ plaques.

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Introduction

1.1 Alzheimer's Disease

Alzheimer's disease (AD) is the most common form of dementia, and it affects more than 50 million people worldwide. It is caused by the apparent conformational change of normally soluble proteins. The aggregation of amyloid beta (Aβ) outside the neuron cells or the formation of neurofibrillary tangles inside the cell contributes to the progression of AD (Scheltens et al., 2021). Aβ plaques and neurofibrillary tangles are major histopathological characteristics associated with AD progression. Amyloid precursor protein (APP), an integral protein on the plasma membrane, regulates survival, growth, and motility of cells. However, its physiological function remains less understood (Tiwari et al., 2019)

Amyloid pathogenesis starts through cleavage of APP by α-, ß- and γ-secretases forming insoluble fibrils of Aβ. Insoluble Aβ fibrils then goes through oligomerization and deposit in synaptic clefts which interferes with synaptic signaling. Furthermore, it polymerizes into insoluble amyloid fibrils that aggregate into plaques (Imbimbo et al., 2005). This polymerization leads to activation of kinases which in turn leads to hyperphosphorylation of the microtubule-associated tau protein and its polymerization into insoluble NFTs. Neurofibrillary tangles (NFTs) are fragments of paired and helically wound protein filaments in the cell cytoplasm of neurons and in their processes (Majd et al., 2015). The aggregation of both plaques and tangles leads to recruitment and activation of microglia, which provokes an inflammatory response and contributes to neurotoxicity.

Pathological signaling pathways in AD have been studied extensively to understand the molecular mechanism of the disease. However, integration of the different pathways and their contribution to the progression of AD became difficult to interpret.

Glycogen synthase kinase-3 (GSK-3) is well-known for its function in tau phosphorylation, transcription factor activation, and glycogen metabolism. Over-activity of GSK-3 is associated with increased tau hyper-phosphorylation and Aβ production (Hooper et al., 2008). Mitogen-activated protein kinases (MAPKs) are serine-threonine kinases that mediate intracellular signaling associated with a variety of cellular activities including cell proliferation, differentiation, survival, and death (Puig et al., 2004). In mammalian cells, three MAPK families have been characterized that include extracellular signal-regulated kinase (ERK), p38, and c-Jun NH2-terminal kinase. Activated MAPKs phosphorylate various substrate proteins, including transcription factors such as Elk-1, c-Jun, ATF2 and p53. MAPK pathways are activated as a result of a series of binary interactions between kinase components or through the formation of signaling complexes containing multiple kinases and controlled by scaffold proteins (McCubrey et al., 2006). In AD, hyper-phosphorylation of tau has been shown to be mediated through several kinases such as JNK, p38, and ERK. Also, oxidative stress triggered by reactive oxygen species (ROS) which are activator of JNK and p38 pathways contribute to the pathogenesis of AD (Marques et al., 2003). The mitochondrial membrane is a possible location for intracellular Aβ localization, which disrupts mitochondrial activity (Wang et al., 2014). Accumulation of Aβ causes the electron transport chain to become blocked, which raises the levels of reactive oxygen species (ROS) and oxidative stress (Good et al., 1996). One of the initial stages of AD is oxidative damage, which manifests in moderate cognitive impairment.

Through the suppression of tau phosphatase, chronic oxidative stress precludes tau dephosphorylation. Additionally, oxidative stress activates the MAPK p38, resulting in an increase in tau phosphorylation.

Several pathways and factors play a role in increasing the deposition of misfolded proteins and progression of neurodegenerative diseases (Shpilka & Haynes, 2018). How protein aggregates contribute to selective neuronal loss in AD remains under investigation.

1.2 Protein Misfolding

The endoplasmic reticulum, a network of branching tubules and flattened sacs, plays a significant role in the synthesis and folding of more than a third of all proteins made in the cell. Proteins that are destined to reside in the ER, plasma membrane, lysosomes, and Golgi apparatus are translated on ER-membrane-bound ribosomes and transferred into the ER lumen (Cabral-Miranda & Hetz, 2018). Proteins that are targeted to the ER lumen have an N-terminal signal sequence which guides them to the ER membrane. In the ER lumen, proteins must be folded into their unique three-dimensional shapes and go through multiple post-translational modifications (such as glycosylation and disulfide bond formation). The ER resident enzymes, such as chaperones, serve as protein-folding machines and are responsible for correctly folding misfolded proteins (Z. Zhang et al., 2019). Despite the presence of ER enzymes to control folding capacity, the success rate of folding proteins is low for proteins translocated to the ER. In a damaged cell, disaggregation of misfolded proteins is not often under physiological conditions as It favors the aggregation state. Improperly folded proteins are further processed by a more stringent quality control system. Unfolded proteins in the ER lumen are translocated to the cytosol, where they are tagged with ubiquitin and degraded by the 26S proteasome

through a process called the ER-associated degradation (ERAD) pathway (Read & Schröder, 2021).

Protein homeostasis, proteostasis, is the network of proteome regulation through the synthesis, folding, and degradation of proteins. Impaired proteostasis and accumulation of misfolded proteins is a hallmarks of aging. Cells can activate the unfolded protein response (UPR) upon detection of misfolded proteins to restore proteostasis.

Adjustment of protein metabolism, inhibition of translation, and upregulation of chaperones are all changes made to restore proteostasis (Ron, 2002).

1.3 The proteosome

The proteasome is a protein complex that plays an essential role in the degradation of misfolded and damaged proteins that harm the neurons. It is made up of two subcomplexes, a catalytic core particle (20S proteasome) and one or two terminal 19S regulatory particles (RP). The 19S RP binds to the 20S proteosome forming an enzymatically active proteasome. The apparent sedimentation coefficient of the active proteasome is 26S; thus, the complex is referred to as the 26S proteasome (Collins & Goldberg, 2017).

Ubiquitin is a pivotal molecule that works with the proteasome. It is a degradation marker that marks damaged or misfolded proteins. The degradation of a protein is initiated by the covalent attachment of a chain containing several copies of ubiquitin. Through the actions of E1 (ubiquitin activator), E2 (ubiquitin conjugation), and E3 (ubiquitin ligase) enzymes, the polymerized ubiquitin chain delivers the target protein to the proteasome where the targeted protein is proteolytically degraded by the proteasome (Lee et al., 2001).

The ubiquitin-proteasome system (UPS) regulates basic cellular processes, cell cycle, progression, signal transduction, cell death, immune response, and protein quality through the degradation of short-lived regulatory or structurally aberrant proteins.

1.4 Oxidative Stress

Despite the fact that the amyloid cascade hypothesis has dominated the field of research for the past decade, another explanation of the disease mechanism has emerged linking mitochondrial dysfunction and increased production of reactive oxygen species to the progression of AD (Singh et al., 2019). Mitochondrial damage caused by ROS production plays a pivotal role in the pathology of neurodegenerative diseases (Murphy, 2009). The term ROS encompasses oxygen free radicals, such as superoxide anion radical ($O_2^{\cdot -}$) and hydroxyl radical ($^{\cdot}OH$), and nonradical oxidants, such as hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$). ROS are generated by stressors such as UV, xenobiotics, and heat. Oxidative stress is the redox state resulting from an imbalance between the generation and detoxification of reactive oxygen species (ROS). ROS are physiological byproducts that are unavoidable but have a dual purpose in the biological system (Zorov et al., 2014). The concept of hormesis has been utilized by many researchers to understand the protective effects of harmful molecules on the cellular level. Even though endogenously generated reactive oxygen species (ROS) have multiple beneficial effects, it is hard to separate the signaling and negative responses that would lead to oxidative stress. Cellular redox imbalance leads to activation of protective or damaging responses such as gene expression, signaling pathways and transcription factor activation. The manifestation of these responses could render the cells adaptive to cellular stress or contribute to their demise (Spitz et al., 2004).

Oxidative stress has been linked to neuronal death and the progression of neurodegenerative diseases. As a consequence of mitochondrial dysfunction, a prominent increase in the genesis and amplification of ROS in these diseases is observed (Yan et al., 2013).

ROS are highly unstable, reactive and possess a short half-life which make them difficult to measure directly. Under physiological conditions, the balance between ROS generation and ROS scavenging is highly controlled. Regulated oxidative stress could initiate diverse cellular responses such as autophagy to shield neighboring mitochondria and cells from the damage (Galluzzi et al., 2014). The generation of ROS is involved in the deterioration of neuronal cells through modulation of the function of biomolecules. These species target different biomolecules (DNA, RNA, lipids, and proteins) and processes (nucleic acid oxidation, lipid peroxidation) in the cell. Lipid peroxidation is an oxidative degradation process that occurs to lipids when ROS attack lipids, polyunsaturated fatty acids, and cause a chain reaction of free radicals that results in the production of lipid peroxidation products (Palmer & Burns, 1994). The most in-depth research on lipid peroxidation products in AD has focused on reactive aldehydes such 4-hydroxynonal, malondialdehyde (MDA), and 2-propenal (acrolein), as well as chemically and metabolically stable isoprostanoids like F2-isoprostanes and F4-neuroprostanes. Oxidative stress and lipid peroxidation have been known to be involved in the development of AD (McGrath et al., 2001). Additionally, ROS can harm proteins directly or reaction with glycation, glycoxidation, and lipid peroxidation products binding. In AD, substantial research has been done on oxidative damage to DNA and RNA. DNA double strand breakage, DNA/protein crosslinking, and base modification are all possible effects (Valko et al., 2007). brain is highly susceptible to oxidative imbalance due to its high energy demand, high oxygen consumption,

rich abundance of easily peroxidizable polyunsaturated fatty acids, high level of potent ROS catalyst iron, and relative paucity of antioxidants and related enzymes.

The mitochondria are important organelles to all eukaryotic cells. They have a high dynamic double membrane-bound structure and their own double stranded mitochondrial DNA (mtDNA). Mitochondria generate ATP through oxidative phosphorylation to meet the high energy demand of cells (Suomalainen & Battersby, 2018). The mitochondrial function has been shown to be inversely related to aging, and it plays a pivotal role in neurodegenerative diseases (Payne & Chinnery, 2015). Neurons are among the cells to have the highest demand for ATP. It is important to maintain ionic gradients for neurotransmission and synaptic plasticity. Damaged mitochondria contribute to the production of free radicals and can cause apoptosis by releasing cytochrome c into the cytoplasm (Fuchs & Steller, 2015). In the AD brain, a prolonged buildup of free radicals is also linked to decreased activity of antioxidant enzymes such glutathione peroxidase (GPx), catalase, superoxide dismutase (SOD), and glutathione reductase (Ionescu-Tucker & Cotman, 2021). Furthermore, the formation and aggregation of misfolded proteins, including aggregates that promote the activities of the APP catalytic enzymes and secretase, will be influenced by increased free radical generation, decreased ATP synthesis, loss of tissue regeneration capacity, and impaired DNA repair mechanisms (Lovell & Markesbery, 2007). This improvement speeds up APP amyloidogenic processing, which promotes the production of Aβ plaques and causes AD-related deficits that include localized neurodegeneration and cognitive decline. This illustrated how delicately the neurons are made vulnerable by the possibility of injury from even a minor decline in mitochondrial function.

ATP is essential for cells to function, and it is recognized as the energy currency for cells. Mitochondria is responsible for the production of ATP through electron transport chain and oxidative phosphorylation (Brand et al., 2013). Since the brain consumes a large amount of energy and oxygen, oxidative stress is prominent in neurons.

Despite the danger imposed by stress on the cells, there are several physiological defense mechanisms that protect the cells. The nuclear factor (erythroid-2)-like (Nrf) transcription factors are members of the Cap'n'Collar basic leucine zipper transcriptional regulators family. Both Nrf-1 and Nrf-2 regulate antioxidant gene expression through binding to the anti-oxidant response element (ARE). These transcription factors are ubiquitously expressed and regulate the transcription of anti-oxidant and Phase II metabolizing enzymes such as UDP-glucuronosyltransferase (UGTs) and sulfotransferase (SULTs), and drug transporters in response to oxidative stress and xenobiotic exposures (Ma, 2013). Even though both transcription factors regulate ARE-containing genes, each one has a specific cellular stress response.

The Nrf2 transcription factor binds to ARE and regulates the transcription of anti-oxidant and metabolizing enzymes in response to stress and xenobiotic exposure. A superfamily of drug metabolizing enzymes (DMEs) known as cytochrome P450s (P450s) catalyzes the oxidation of a wide range of exogenous and endogenous substances. Nrf-2 mediates the induction of several drug-metabolizing enzymes, such as glutathione S-transferase (GST) and NAD(P)H: quinone oxidoreductase 1 (NQO 1). Overproduction and accumulation of Aβ induce oxidative stress and contribute to neurotoxicity, but the relationship of oxidative stress and the progression of AD is not fully understood (Butterfield et al., 2013). It is crucial to investigate the role of anti-oxidant

pathways in the progression of AD and identify critical pathways pivotal in the resolution of neurotoxicity.

1.5 Proteotoxic Stress

On the other hand, the Nrf1 transcription factor regulates the transcription of proteasome subunit genes that generates proteosome synthesis. This compensatory mechanism is essential as a survival mechanism when the proteasome is inhibited. Nrf1 associates with the ER and extends to the ER lumen through the N-terminal transmembrane domain (Northrop et al., 2020). It translocates from the ER lumen to the cytoplasm for rapid degradation by the proteasome through ER-associated degradation (ERAD) machinery. In impaired proteasome conditions, the half-life of the transcription factor (Nrf1) is increased, and it enters the nucleus to induce up-regulation of proteasome subunit genes. The proteasome subunit genes are direct transcriptional targets of Nrf1. The presence of unfolded or aggregated proteins triggers a response mediated by the Nrf1 transcription factor to increase the expression of proteasome subunit genes. The expression of the proteasome gene is responsive to the need for protein degradation (Albornoz et al., 2019).

Our understanding of the cellular mechanisms by which cells communicate to adjust to disturbances in cellular homeostasis has improved (Fulda et al., 2010; Lockshin & Zakeri, 2001). Cells degenerate when they are unable to adjust to cellular stress (Szegezdi et al., 2006). Several routes are induced, each of which is particular to a different stressor. The unfolded protein response increases chaperone activity and promotes survival of the cells Crosstalk between stress responsive pathways has been investigated. Oxidative stress increases the expression of heat shock protein 27 (HSP 27) which can protect against heavy metal stress

(Gorman et al., 1999). Also, activation of the UPR promotes anti-oxidant pathways. Through PERK-dependent phosphorylation of Nrf-2, it mediates upregulation of antioxidant genes (Swindell et al., 2007). Despite the specific signaling components of cellular stress pathways, they eventually disturb the cellular homeostasis and lead to death, thus; investigating the molecular mechanism would allow restoration of cellular homeostasis.

1.6 Caenorhabditis elegans

Nematodes are free living roundworms. They have a high degree of differentiation, muscles, intestines, gonads, glands, neurons, and excretory systems. Because of their small size, short life cycle, and high fertility, nematodes can be used for screening for potential new drugs. C. elegans genome possess homolog of approximately 66% of all human disease genes; thus, using this model organism would provide a theoretical basis for the translation of human disease treatment (Iliff & Xu, 2020). Transgenic C. elegans expressing amyloid beta has been constructed, which gave an opportunity for researchers to understand the pathology and etiology of AD in this model organism (Link, 2006). Several transgenic strains of C. elegans have been used in this research.

In mammalian cells, Nrf1 and Nrf2 have similar DNA binding domains and may regulate an overlapping set of downstream targets. However, only Nrf1 is required for the upregulation of proteasome subunit genes following proteasome disruption (Blackwell et al., 2015). Upon toxic stress, C. elegans induce transcription of proteasome subunit, detoxification, and immune response genes. The transcriptional response to stress involves skn-1, which encodes multiple isoforms of transcriptional functions similar to the mammalian Nrf1 and Nrf2. C. elegans skn-1 encodes three protein isoforms (skn-1A, B, and C) that share an identical C-terminal CnC DNA

binding domain but possess different N-termini and expression patterns. Skn-1A is expressed in all tissues. Skn-1B is expressed in two sensory neurons. Skn-1C is expressed in the intestines. Oxidative stress induces nuclear localization of skn-1c; thus, it may function as an analog to Nrf2. Under normal conditions, skn-1a localizes to the ER, where it undergoes N-linked glycosylation and rapid degradation by the proteasome.

However, when the proteasome function is impaired, skn-1a enters the nucleus, where it can induce the transcription of genes encoding proteasome subunits. The proteasome is a critical quality control guard to maintain proteostasis in neurodegenerative diseases.

1.7 Cannabinoids

Cannabis has been used in human medicine for ages, both for medicinal and recreational purposes. Over 100 of the 500 compounds found in the cannabis plant are known as cannabinoids. The most prevalent are Cannabidiol (CBD), which is non-psycoactive, and 9-tetrahydrocannabinol (THC), which possess psychoactive properties (Alves et al., 2020). The endocannabinoid system (ECS) is a complex biological system found throughout the body. It is essential for maintaining internal balance in the brain, skin, digestive tract, and liver, as well as the respiratory, cardiovascular, and reproductive systems (Alves et al., 2020). It plays a role in brain development, neurotransmission, and the release of cytokines from microglia and directly affect emotions, cognition, and fertility (Lowe et al., 2021). Additionally, it has been found that changes in the ECS components are related to several pathological illnesses including cancer, neurodegenerative diseases, and cardiovascular diseases, thus; the pharmacological modulation of the system has captured the attention of medical research (Campolongo & Trezza, 2012). Most of the effects of Cannabinoids are mediated through two G-protein coupled

receptors, CB1 and CB2. Cannabinoid receptors 1 (CB1) are present mostly in the brain while CB2 are in the periphery and immune cells (Basavarajappa et al., 2017). Endogenous cannabinoids, anandamide and 2-arachidonoyl glycerol (2-AG), are lipids that activate cannabinoid receptors. 2-AG is an agonist for both CB1 and CB2 receptors while anandamide has low affinity for both receptors. Fatty acid amino hydrolase (FAAH) is responsible for the degradation of multiple fatty acid amides including the endogenous cannabinoids (Mackie, 2008). THC works through a G-protein-mediated method to suppress the intracellular synthesis of cyclic adenosine monophosphate (cAMP), thereby activating the CB1 receptor.

The psychotropic effects of cannabis such as hypo-locomotion, hypothermia, catalepsy, and analgesia which are correlated with the activation of the CB1 receptor by THC (Lu & Mackie, 2016). Furthermore, THC possesses anti-inflammatory, antispasmodic, and neuroprotective properties that are mediated by activating several receptors, including peroxisome proliferator-activated receptor (PPAR) and CB2. THC is metabolized in the liver through cytochrome P450 (CYP 450) isozymes CYP2C9, CYP2C19 and CYP3A4, where it is changed into 11-hydroxy-THC or 11-nor-9-carboxy-THC. Both metabolites of THC are thought to have psychoactive properties (Amin & Ali, 2019).

In contrast, the non-psychoactive cannabis compound CBD has no effect on motor or cognitive function. It has weak affinity for both CB1 and CB2 receptors, and it acts as an inverse agonist on CB2 receptor which helps it have anti-inflammatory properties (Lucas et al., 2018). CBD is also hepatically metabolized to 7-hydroxy-cannabidiol (7-OH-CBD) via isozymes CYP2C19 and CYP3A4 (Li et al., 2020). The pharmacological properties of CBD's metabolites are not well established (Gaston & Friedman, 2017). In human post-mortem samples and animal models of

AD, the expression of ECS components is changed, particularly in the cerebral cortex and hippocampus (Ujváry & Hanuš, 2016). The amount of CB1 receptors in neurons has been well documented as being low, whereas evidence from animal models suggests that CB2 receptors may act as a protective mechanism against neuroinflammation due to their overexpression in microglial cells (Fride, 2004). Prior studies on dogs showed that CBD was tolerable and examined its pharmacokinetic and pharmacodynamic characteristics (Bartner et al., 2018). Additionally, patients with AD had higher levels of FAAH expression in their brains (Benito et al., 2003). These results imply that alterations in the ECS may be involved in the pathological development of AD. In familial AD model, a two-week regimen of high dose CBD reduced accumulation of Aβ and improved cognition (Khodadadi et al., 2021). Aβ neurotoxicity is a prominent feature in AD, which includes neuronal inflammation and loss of neuronal function. CBD has been shown to ameliorate Aβ neurotoxicity in AD models along with a ROS scavenging property (Atalay et al., 2019). Furthermore, it's critical to emphasize that a variety of receptors, including serotonin, vanilloid, adenosine, peroxisome proliferator activated (PPAR), opioid, and dopamine receptors, are activated by CBD. Through modulation of the activities of these receptors, CBD exhibits multiple therapeutic benefits including neuroprotective, antiepileptic, and anti-inflammatory effects. Despite the promise that CBD gives as a potential drug candidate for AD, the cellular mechanism by which CBD protects against neurotoxicity has never been established. In this research, we hypothesize that CBD acts through Nrf1 and Nrf2 transcription factors to reduce the proteotoxic load generated by the misfolded proteins in AD. The amelioration of stress with CBD is through its' ROS scavenging activity and induction of anti-

oxidant enzymes through Nrf2. Furthermore, CBD acts on Nrf1 to upregulate the synthesis of the proteasome under proteotoxic stress.

Materials and Methods

2.1 C. elegans strains

The C. elegans strains utilized in this study were obtained from the Caenorhabditis Genetics Center (CGC) at the University of Minnesota and consisted of QV225, GR2198, GR2245, GR3149, DLM23, LD1, and N2. The N2 (wild type) is the ancestral strain that does not have any disease or mutation. QV225 strain has a hypomorphic allele of skn-1. GR2198 expresses skn-1a with a C-terminal GFP tag. GR2245 strain has a specific skn-1a mutation. GR3149 strain has a muscle-specific proteasome dysfunction. DLM24 is resistant to hydrogen sulfide due to the deletion of skn-1 repressor (wdr-23). LD1 expresses. Skn-1b/c with C-terminal GFP tag.

2.2 Maintenance

The nematodes were grown on Nematode Growth Media (NGM) agar plates and stored in a 20 °C incubator. Nematodes were fed OP50, a nonpathogenic *Escherichia coli* (*E. coli*) strain, once a week. The maintenance for all the various strains was performed in the same manner. Chunking, which involves using a sterilized metal spatula to cut a small square in the NGM agar plate that is then placed onto a new NGM plate, was employed for the maintenance and propagation of the strains.

2.3 Synchronization

For longevity studies, we required age-specific model organisms. For this, the nematodes were first washed from the NGM plates using M9 buffer and glass serological pipettes and then collected. The nematodes were then synchronized using 1M NaOH and 5.25% hypochlorite bleaching treatments and centrifuged for 3 min at 2000 rpm. The reaction is interrupted by the

addition of an M9 buffer. Bleached pellets were washed two times with M9, followed by a final wash with S-medium. Nematode pellets were then transferred from the tubes onto Petri dishes containing agar using 5-in glass aspirator pipettes. Nematodes were then fed with 200 µL of OP50 after 24 hr to ensure all eggs hatched simultaneously to allow the nematodes to age at the same rate.

2.4 Compounds and other materials

Cannabidiol: Cayman Chemical Company, Batch – 0592969-62. N-Acetylcysteine: Sigma-Aldrich Company, Batch- A9165-25G. 5-Fluoro-2′-deoxyuridine: Research Products International, CAS No. 50-91-9 Lot 119676-154090. Bortezomib: Cayman Chemical Company, Batch-0513569. Dihydroethidium: Thermo-Fisher Scientific, Lot 2335596. UV-Crosslinker: CL-1000 Ultraviolet Crosslinker. S-medium: 5 ml 1 M Potassium citrate - pH 6.0, 5 ml Trace Metals solution 1.5 ml 1M MgCl2 1.5 ml 1M CaCl2 Trace Metals Solution Disodium EDTA (5 mM) FeSO4 7H2O (2.5 mM) MnCl2 4H2O (1 mM) ZnSO4 7H2O (1 mM) CuSO4 5H2O (0.1 mM) Dissolve in 1L H_2O.

M9 buffer: (3 g KH_2PO_4, 6 g Na2HPO4, 5 g NaCl, and 1mL 1 M $MgSO_4$ in 1 L of H_2O)

2% agarose pad: To immobilize the nematodes for imaging, we prepared a 2% agarose pad on which nematodes were mounted for imaging. Immobilizing agents were not an option since they paralyze the animals, which would affect the stress assays conducted. One gram of agarose was added to 50 ml of distilled water and autoclaved. Using a warmed Pasteur pipet, two to three drops of 2% agarose were added to glass slides, and the slide was covered immediately with another slide to allow the drops to spread before they hardened.

2.5 Assays

2.5.1 Longevity Assay

A synchronous population of nematodes was transferred from the NGM plates to a 96-well microliter plate containing an S-medium buffer. At day zero, FudR was added to the wells to inhibit the offspring and allow for proper/synchronous growth of the population. On day one, drugs of interest were added to the wells. On day 2, nematodes were exposed to stressors of interest. Plates were placed on a microliter plate shaker for 2-3 minutes and scored under a dissecting microscope. Animals that move were scored alive and dead if not moving. Plates were kept in the 20 °C incubator. Counting was repeated every 2-4 days until all the animals ceased to move (dead).

2.5.2 Bortezomib Sensitivity Assay

Bortezomib sensitivity was assessed by the ability of L1 Larvae to develop in the presence of various concentrations of Bortezomib. A synchronous population of the strain of interest was used to assay the nematodes' ability to escape the developmental delay under proteotoxic stress. Approximately 10 L1 Larvae mixed with E. coli OP50 suspended in S-medium were transferred to a 96-well microliter plate. All treatment conditions contained 0.01% DMSO. The drugs/stressors of interest were added at L1 Larvae. The plates were kept for three days in a 20 °C incubator. Animals' growth was manually scored under a dissecting microscope. Growth of C. elegans was scored into three categories: (1) Normal: reached the young adulthood stage/ indistinguishable from control; (2) Delayed: Most animals are L2 or L3 larvae; (3) Larval arrest/ death: animals did not develop beyond L1 larvae or dead. A heat map was generated to display the results.

2.5.3 ROS Scavenging Assay

Dihydroethidium (DHE) is a superoxide indicator that exhibits blue fluorescence in the absence of reactive oxygen species (ROS) and red fluorescence in the presence of ROS. Animals were placed on NGM plates that contained compounds of interest and incubated in a 20 °C incubator for 24 hr. After 24 hr, worms were washed off the plates using M9 buffer and centrifuged for 3 minutes at 2,000 RPM. The pellets were collected from each tube and placed in a new tube that contained 30 uM DHE and incubated for 1 hr. Nematodes were subsequently washed with PBS three times, followed by a final wash with M9 buffer. Under sterile and non-stressful techniques, animals were aspirated from the tubes and mounted on 2% agarose slides.

2.5.4 GFP Imaging Assay

Strains that express GFP-tagged protein were imaged using Zeiss microscopy. Animals were placed on NGM plates that contained compounds of interest and incubated in 20 °C incubator for 24 hr. For skn-1b/c activation assay, worms that were incubated with CBD for 24 hr were stressed on the following day using the UV crosslinker. Imaging was conducted on the second day. For skn-1a activation assay, worms that were incubated with CBD for 24 hr were washed with M9 buffer three times on the second day and placed on NGM plates that either contained Bortezomib or vehicle. Imaging was conducted on the third day. Statistical analysis was performed using GraphPad Prism, comparing mean fluorescence intensity.

2.5.5 Paralysis Assay

For each assay, 15 synchronized young adult animals were placed on NGM plates that contained FUDR to maintain a synchronous population throughout the assay and incubated for

24 hr in a 20 °C incubator. On day 1, animals were placed on NGM plates that either contained CBD or vehicle and incubated for 24 hr in a 20 °C incubator. On day 2, animals from the two groups were placed on NGM plates that had Bortezomib or vehicle and incubated for 24 hr in a 20 °C incubator. On day 3, animals were scored for paralysis every 24 hr. Animals were scored as paralyzed if they showed no sign of movement after tapping the plate or gently prodding the animal.

Results

3.1 Oxidative Stress

3.1.1 Effect of Cannabidiol on Lifespan Under Oxidative Stress

The ability to sense light is a molecular feature found in many organisms, and it serves a wide range of functions, including energy synthesis, DNA repair, and modulation of circadian rhythm (Bhatla et al., 2015). Modulation of C. elegans daily light exposure affects life span. It induces oxidative stress, which activates several stress response pathways (Ward et al., 2008). Visible light decreases the life span of C. elegans. It generates photooxidative stress and activates the UPR pathway (De Magalhaes Filho et al., 2018). We used various exposure doses of UV light to induce oxidative stress and study its effect on the lifespan of the nematodes. The goal was to choose a dose that would mimic the oxidative stress generated by misfolded proteins. A dose that would not kill the animals or change their morphology but induce stress that would show a reduction in their lifespan. Lifespan experiments showed a reduction in lifespan in animals exposed to all the chosen exposure doses. We chose 3 J/cm^2 for further experiments (Figure 3.1.1)

Misfolded proteins have been known to cause further stress to the neurons. The aggregation of misfolded proteins such as Aβ is an essential step of the pathology underlying several neurodegenerative diseases such as AD (Abramov et al., 2020). The toxicity resulting from misfolded proteins depends on the stage and form of aggregates. Increased production of free radicals is proven to be involved in the pathology of neurodegenerative diseases (Kim & Jin, 2015). The presence of oxidative stress could further aid the misfolding of proteins and increase the demand for anti-oxidants. CBD has been shown to decrease the expression of amyloid beta

in neurodegenerative models, but the mechanism by which CBD does that is yet to be investigated. N-acetyl-L-cysteine (NAC) is a sulfur-containing amino acid derivative with an acetyl group attached to the L-cysteine nitrogen. NAC is a potent anti-oxidant and has liver-protecting activity (Yedjou & Tchounwou, 2007). Supplementation with NAC increases resistance to oxidative stress caused by ROS by elevating intracellular levels of glutathione (Daraie et al., 2012). NAC is also involved in the cellular detoxification of heavy metal ions, such as lead, mercury, and arsenic (Takagi et al., 2002). Dietary supplementation of NAC increases resistance to heat and oxidative stress. It extended the lifespan of animals irradiated with 20/ J/cm^2/minute (Oh et al., 2015). We used wild-type animals and used NAC doses from 1mM to 20mM under UV stress. Animals treated with NAC had higher resistance to UV stress compared to vehicle (Figure 3.1.1). At lower doses, NAC did not extend the lifespan of worms that were exposed to UV stress. However, doses higher than 3mM had a higher impact on the lifespan of treated animals (Figure 3.1.1). 10mM NAC was able to induce an appropriate resistance and decrease the death rate of stressed animals.

Utilizing the wild-type strain, we stress the animals using a UV crosslinker to mimic the stress generated by misfolded proteins. NAC is used as a positive control to compare its ROS scavenging activity to CBD. Animals treated with 10 µM CBD were able to resist the oxidative stress caused by UV (Figure 3.1.1). The oxidative stress resistance in animals treated with CBD was superior to NAC's anti-oxidant effects. CBD was able to extend the lifespan of nematodes under stressful conditions similar to those generated by misfolded proteins (Figure 3.1.1).

We investigated if the stress resistance properties of CBD are independent of skn-1b/c. Since skn-1 is crucial for the development of the animals, a complete knockout of skn-1 would be

lethal to the worms. QV225 is a strain that has a hypomorphic allele of skn-1. CBD's ROS scavenging properties were not sufficient to extend the lifespan of skn-1 hypomorphic animals (Figure 3.1.1). This proves that the UV stress resistance properties of CBD seen with the wild type are dependent on the activation of skn-1.

Under normal conditions, skn-1c is kept in the cytoplasm by its' repressor, wdr-23. However, when stress is present, wdr-23 dissociates from skn-1c, allowing it to translocate to the nucleus and induce the transcription of anti-oxidant enzymes (Spatola et al., 2019). We used a strain that has a mutation in the repressor (to investigate if CBD's activation of skn-1c is dependent on inhibiting its repressor. 10 µM CBD did not induce significant lifespan extension compared to the vehicle (Figure 3.1.1). The current data suggest that CBD activity could be partially dependent on inhibiting the repressor from inducing translocation of skn-1 to the nucleus and further activation of anti-oxidant pathways. Also, since skn-1 is constantly activated in the absence of the repressor, it would be difficult to say that CBD's activity under stressful conditions is dependent on wdr-23.

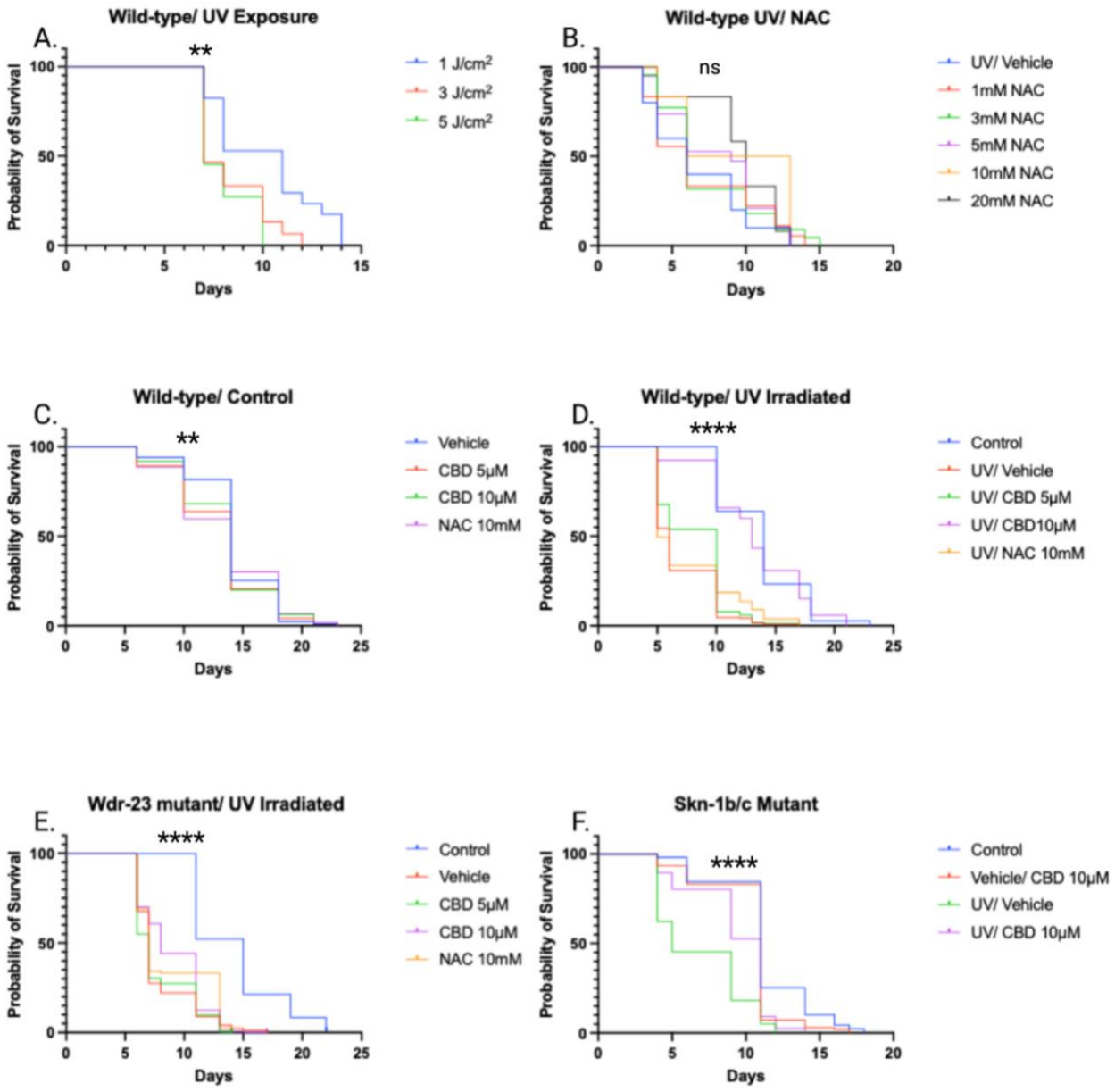

Figure 3.1.1. Effect of cannabidiol on lifespan of several strains in the presence or absence of oxidative stress. (A) Wild-type animals exposed to three doses in the UV-crosslinker chamber. 3J/cm^2 is the exposure dose used for subsequent assays.
(B) UV-irradiated wild-animals that were pre-treated with several doses of NAC. 10mM is the dose used for subsequent assays. (C) Un-irradiated (control) wild-type animals treated with the selected doses of the drugs of interest. (D) UV-irradiated wild-type animals treated with the selected doses of the drugs of interest. CBD significantly extended the lifespan of irradiated

wild-type animals. (E) Skn-1 repressor (wdr-23) mutant animals treated with the selected doses of the drugs of interest. (F) Control/ UV-irradiated skn-1b/c animals pre-treated with 10 µM CBD. CBD did not extend the lifespan of irradiated skn-1b/c mutant animals. Results are presented as a Mantel–Cox test. P< .0001****: panels D, E, and F. P**<.05: panels A and C. P>.05 non-significant (ns): panel B. N= 100-300. Three biological replicas.

3.1.2 Skn-1b/c Activation Assay

Many studies have shown that enhancing resistance to stress can extend the lifespan of C. elegans. Cannabidiol has been shown to protect against AD through ROS scavenging of its phenol hydroxyl group in C. elegans (Y. Zhang et al., 2022). Since CBD has a low affinity to CB receptors, it is unlikely that its neuroprotective effects are mediated through these receptors (Elsaid et al., 2019). Also, CBD reduces Aβ deposits in nematodes expressing ab minigene and reduces Aβ microglial activation. The ROS scavenging activity of CBD was independent of skn-1, but the isoforms of interest were not investigated. We utilized a skn-1b/c, which corresponds to mammalian Nrf-2, green fluorescent protein (GFP) protein tagged strain to investigate if CBD could activate skn-1b/c under normal conditions. It is shown that 10µM CBD activates skn-1b/c in normal conditions (Figure 3.1.2). However, CBD did not induce further activation of skn-1b/c under UV stress. Since this strain does not model neurodegenerative disease, it is safe to assume that the anti-oxidant system is functioning properly. Also, the presence of CBD would not induce further activation of skn-1b/c because it is already reaching the maximum capacity. Anti-oxidant systems, Nrf-2, have been known to decline with aging and drive the cells to a more aged phenotype.

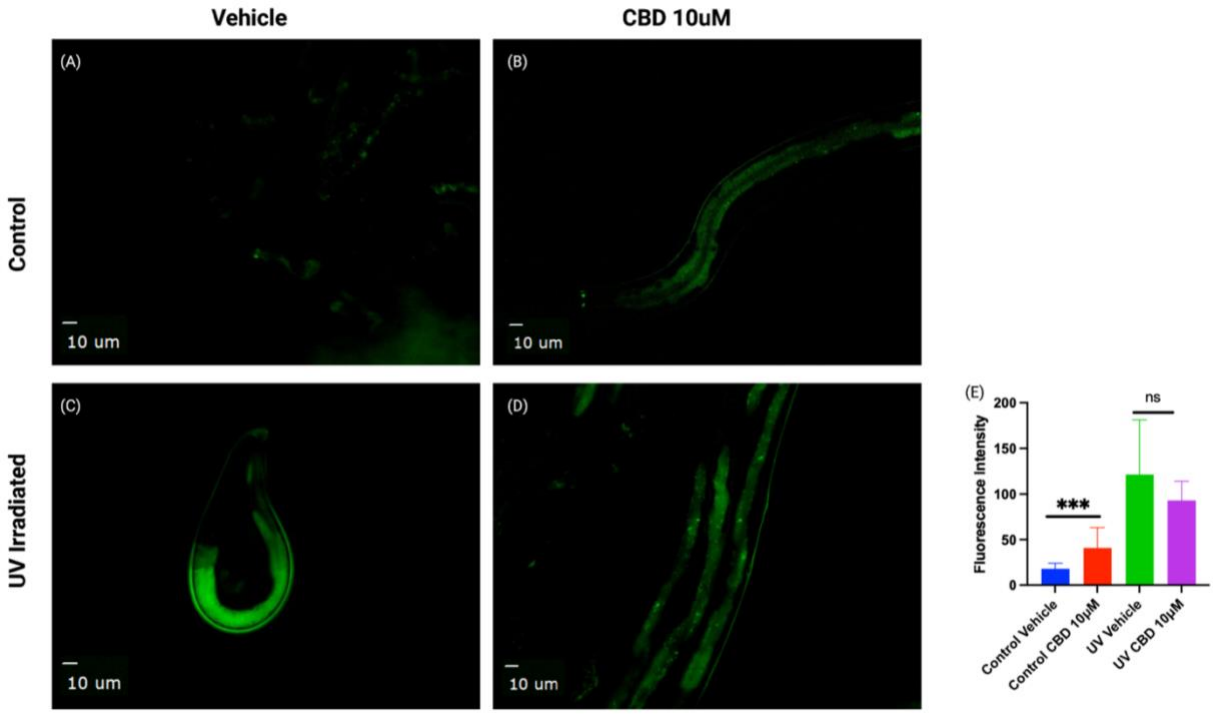

Figure 3.1.2. Skn-1b/c activation assay under control and stressful settings. (A) Control group. (B) Animals pretreated with 10µM CBD and kept in the incubator to prevent light exposure. CBD significantly activated skn-1b/c. (C) Animals pretreated with vehicle and exposed to oxidative stress through the UV-crosslinker. UV exposure significantly activated skn-1b/c. (D) Animals pretreated with 10µM CBD and exposed to oxidative stress through the UV-crosslinker. CBD did not significantly activate skn-1b/c in UV irradiated compared to UV exposed worms. (E) One-Way ANOVA statistical analysis (***= < 0.005/ ns= non-significant). N=30-50. Three biological replicas.

3.1.3 Cannabidiol's ROS Scavenging Activity

The presence of reactive oxygen species following UV stress can be overwhelming to the point that anti-oxidant pathways would not be able to clear it (Miranda-Vizuete & Veal, 2017). In addition to CBD's ability to activate skn-1c, it possesses a ROS scavenging activity (Pereira et al., 2021). To visualize the presence of ROS in the animals, we used a fluorescing dye that allows for ease of staining of the animals. Dihydroethidium (DHE) is a dye that fluoresces blue in the absence of reactive oxygen species. However, DHE dye fluoresces red in the presence of

reactive oxygen species (Senchuk et al., 2018). We hypothesize that CBD's activation of skn-1c and its scavenging ability would clear the reactive oxygen species present in the animals. We used wild-type animals (N2) and incubated them in NGM plates containing vehicle, CBD, or NAC for 24 hours. After incubation, nematodes were either irradiated with 3 joules/cm^2 or kept in the incubator until imaging staining with the dye. All the animals intended to be imaged were incubated in an M9 buffer containing 30 µM DHE for 1 hour. Worms were washed three times with PBS after incubation with DHE to clear any remaining bacteria before imaging. It is shown that CBD and NAC were able to scavenge most of the reactive oxygen species in the UV-stressed animals (Figure 3.1.3).

To confirm if CBD's anti-oxidant activity is dependent on skn-1c. We used a strain that has a hypomorphic allele of skn-1. The ROS scavenging activity of CBD that was seen in the wild-type of irradiated worms was diminished in the absence of skn-1. 10uM CBD was not able to clear the ROS generated by UV stress in skn-1 hypo-morphic animals (Figure 3.1.3). It suggests skn-1 is of paramount importance to alleviate oxidative stress.

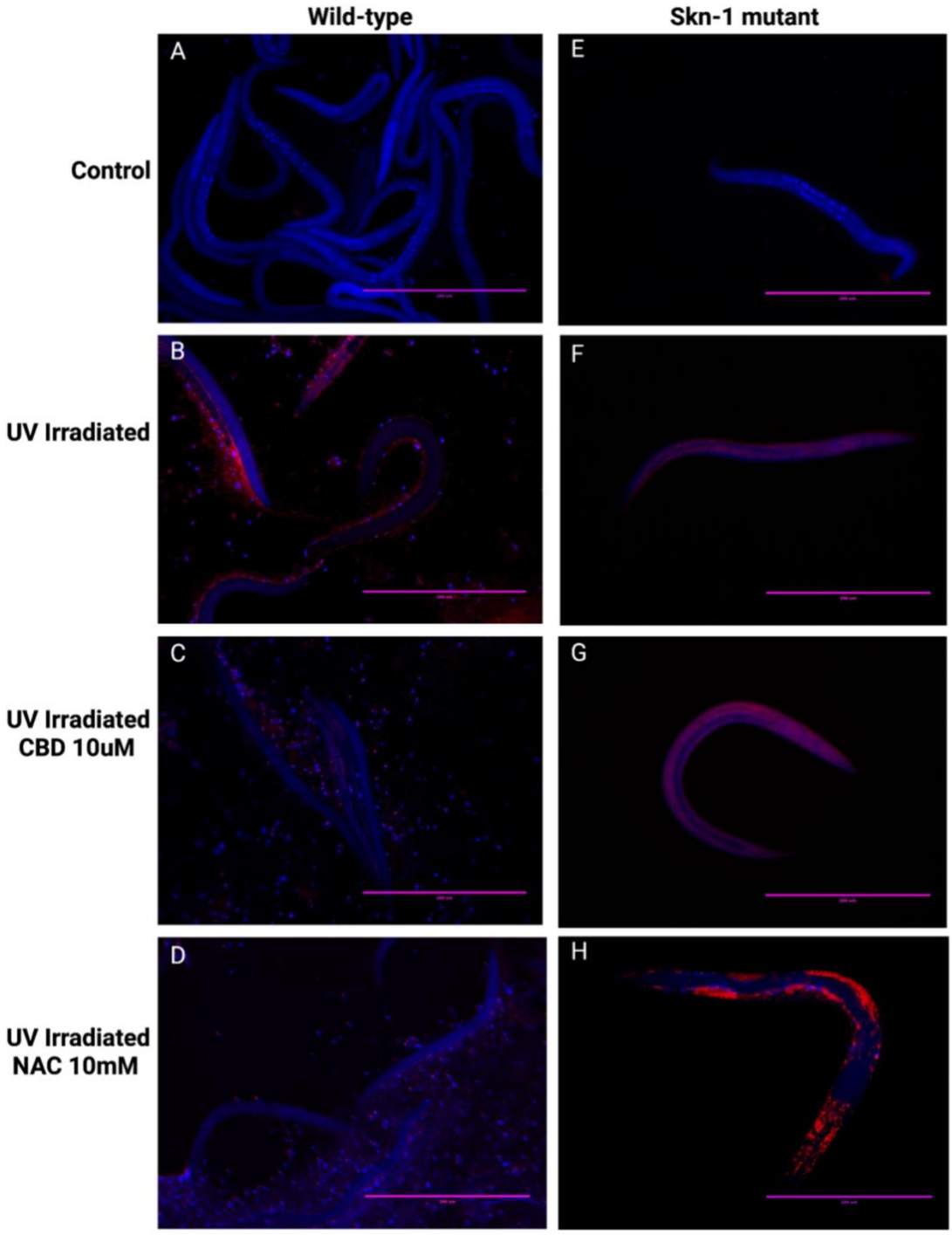

Figure 3.1.3. Effect of cannabidiol on reactive oxygen species (ROS) generation. (A) Wild-type control group. (B) Wild-type animals pretreated with vehicle and exposed to oxidative stress through the UV-crosslinker.
ROS are visible in UV exposed wild-type (C) Wild-type animals pretreated with 10µM CBD and exposed to oxidative stress through the UV-crosslinker. (D) Wild-type animals pretreated with 10mM NAC and exposed to oxidative stress through the UV-crosslinker. (E) Skn-1 mutant animals control group. (F) Skn-1 mutant animals pretreated with vehicle and exposed to

oxidative stress through the UV-crosslinker. (G) Skn-1 mutant animals pretreated with 10µM CBD and exposed to oxidative stress through the UV-crosslinker. (H) Skn-1 mutant animals pretreated with 10mM NAC and exposed to oxidative stress through the UV-crosslinker. N=20-50. Two biological replicas.

3.2 Proteotoxic Stress

3.2.1 Bortezomib Sensitivity Assay

The proteasome is a known line of defense for misfolded proteins that were not correctly folded by the chaperones (Anderson et al., 2022). It degrades misfolded proteins that were tagged by ubiquitin. However, the accumulation of misfolded proteins can stress the proteasome and impair its function (Rao & Bredesen, 2004). Impaired proteasome function is associated with aging and age-dependent neurodegenerative diseases. Bortezomib, an anti-neoplastic drug, inhibits the 26S proteasome complex (Fricker, 2020)

It prevents the degradation of various pro-apoptotic factors, which leads to apoptosis in cancer cells. We used Bortezomib to mimic the proteasome stress caused by the accumulation of misfolded proteins. C. elegans go through four developmental stages before reaching the adult stage. At L1 stage, animals were incubated with either Bortezomib or CBD/Bortezomib and kept in the incubator for 48 hours. Nematodes were scored for escaping the developmental delay caused by stress (L4 larvae), delayed development (L1 or L2), or arrested development (death). Using the wild type (N2), animals were incubated in a 96-well plate in S-medium containing OP50. Animals were either kept as control or incubated with various concentrations of Bortezomib to investigate the developmental delay caused by the proteasome stressor. L1 animals were incubated with Bortezomib 1, 3, 5, 7, 10, and 20µM to determine the concentration of the stressor that would stress the animals and cause developmental delay without causing death. At 1 and 3uM Brotezomib, most of the animals in the two groups

reached the L4 stage with partial developmental delay (Figure 3.2.1). Almost all the animals that received 5 and 7µM concentrations had delayed development. Death caused by Bortezomib was seen first with 10µM concentration. With 20µM concentration, animals had an increased death rate compared to the other groups. 10µM CBD was used to investigate at which Bortezomib dosage CBD would be able to rescue the animals. At 5µM Bortezomib, CBD was able to rescue most of the delayed animals.

Skn-1a induces the expression of proteasome subunit genes under proteotoxic stress. It is essential to keep the nematodes alive in proteasome dysfunction. We used an skn-1a mutant strain to investigate if the developmental delay escape induced by CBD is dependent on skn-1a. Due to the nature of the mutated skn-1a animals, some of the control cohorts had developmental delays. 10µM CBD was not able to rescue developmental delay/ death in animals stressed with 5µM Bortezomib (Figure 3.2.1). This suggests that skn-1a is crucial for CBD to help worms reach L4 stage under proteotoxic stress.

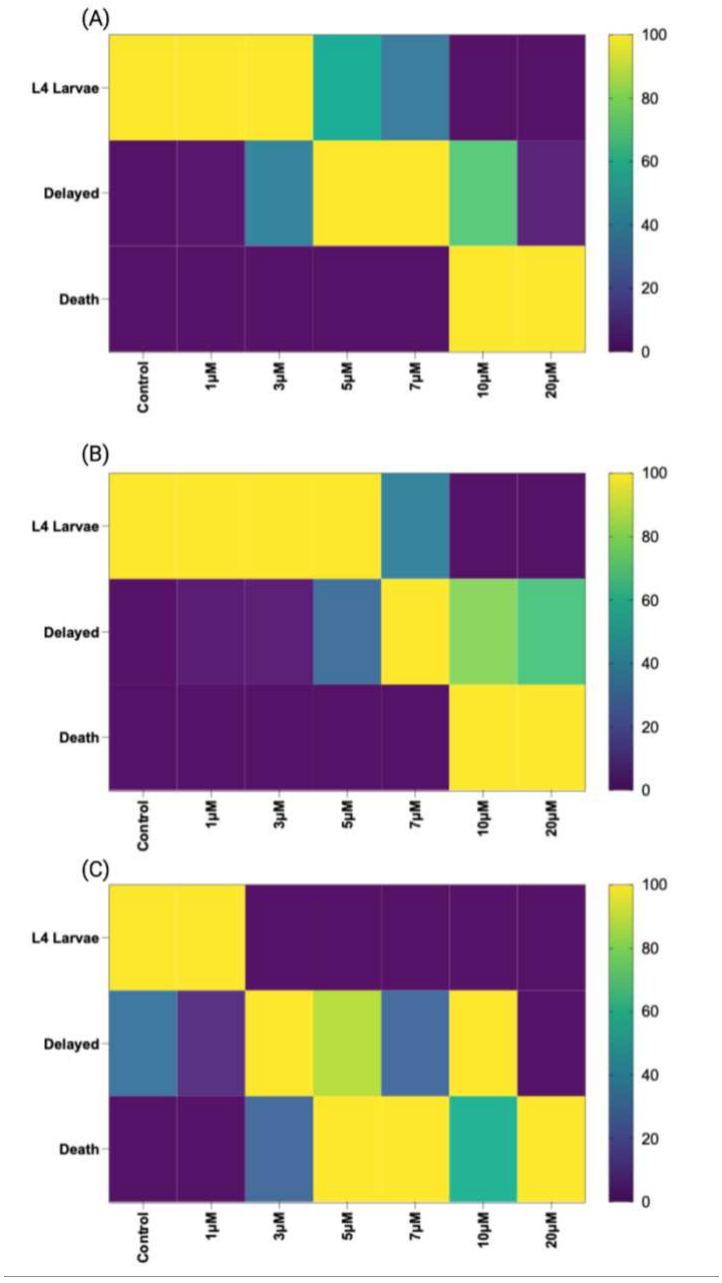

Figure 3.2.1. Bortezomib sensitivity assay of wild-type and skn-1a mutant nematodes. Animals were exposed to different concentration of Bortezomib or kept as a control. (A) Wild-type animals exposed to different concentrations of Bortezomib. (B) Wild-type animals exposed different concentrations of Bortezomib with 10µM CBD in each group except for the control. (C) skn-1a mutant animals exposed different concentrations of Bortezomib with 10µM CBD in each group except for the control. N=100-300. Three biological replicas.

3.2.2 Skn-1a Activation Assay

The proteasome is tightly controlled through the ERAD pathway and skn-1a (Lehrbach & Ruvkun, 2016). Under normal conditions, skn-1a is sequestered and degraded. When the proteasome is impaired, skn-1a translocates to the nucleus and upregulates the proteasome subunit genes. We used a strain that has a GFP-tagged protein to skn-1a. Animals were placed on NGM plates that contained either 10µM CBD or vehicle for 24 hours. Animals in the CBD/vehicle plates were placed on either 5µM Bortezomib or the vehicle for another 24 hours. We used a paralysis agent to try to immobilize the movement of the worms during imaging, but it caused more stress and resulted in uninterruptable results. Agarose pads were sufficient to slow the movement of the worms on the slides. It allowed us to get clearer images rather than using a paralysis agent.

On the day of imaging, nematodes were washed with M9 buffer three times and placed on agarose slides to immobilize their movement during imaging without affecting them. Under normal conditions, 10µM CBD was able to activate skn-1a (Figure 3.2.2). Under proteotoxic stress, CBD increased the activation of skn-1a. This suggests that under proteotoxic conditions that mimic stress caused by misfolded proteins, CBD was able to increase the expression of proteasome subunit genes through skn-1a activation.

3.2.3 Proteotoxic Stress Induce ROS Generation

The accumulation of misfolded proteins can stress the cells and cause further damage (Abramov et al., 2020). We used DHE dye on wild-type animals that were exposed to proteotoxic stress. The presence of stress on the proteasome can induce the generation of ROS (Figure 3.2.3).

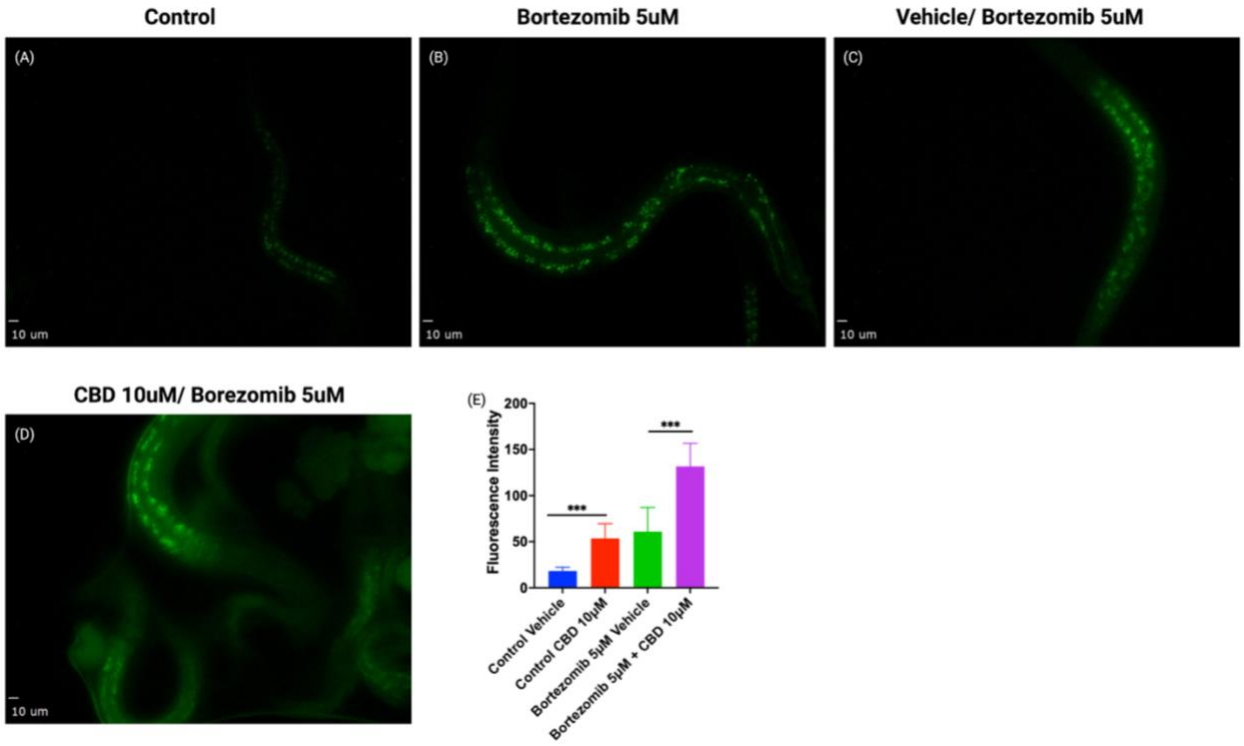

Figure 3.2.2. Skn-1a activation assay under control and stressful settings. (A) Skn-1a GFP tagged Control group. (B) Skn-1a GFP tagged animals treated with 10µM CBD in the absence of proteotoxic stress. (C) Skn-1a GFP tagged animals treated with 5µM Bortezomib. (D) Skn-1a GFP tagged animals pretreated with 10µM CBD followed by 5µM Bortezomib treatment. (E) One-Way ANOVA statistical analysis (***= < 0.005/ ns= non-significant). N=30-50. Three biological replicas.

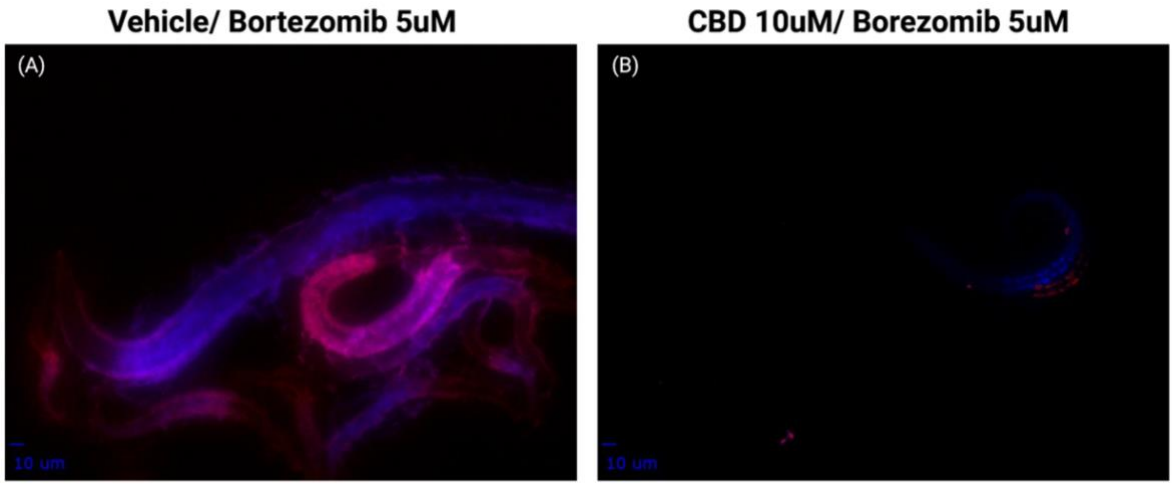

Figure 3.2.3. Proteosome impairment generates oxidative stress. (A) 5µM Bortezomib stressed wild-type animals incubated with DHE. (B) 10µM CBD/5µM Bortezomib treated wild-type animals incubated with DHE. N=20-30. Two biological replicas.

3.2.4 Effect of Cannabidiol on Lifespan Under Proteotoxic Stress

Accumulation of misfolded and aggregated proteins is thought to cause a decline in cellular function and health during aging (David et al., 2010) (López-Otín et al., 2013) (Walther et al., 2015). We have shown that CBD increases the expression of proteosome subunit genes, but the translational effect of skn-1a activation caused by CBD treatment under proteotoxic stress were not investigated. Wild type animals were used to investigate the effects of CBD under proteotoxic stress. 10µM CBD was able to recue animals and significantly extend their life span compared to vehicle (Figure 3.2.4). Mutations that affect both SKN-1A and SKN-1C reduce lifespan. However, the individual contribution of skn-1a in CBD-treated animals was not investigated. We used skn-1a mutant animals and stressed them with Bortezomib. CBD was not able to rescue the stressed animals, and the results were non-significant (Figure 3.2.4). The current data suggests that CBD resolution of proteotoxic stress caused by misfolded proteins is

dependent on skn-1a. Moreover, skn-1a has a crucial role in alleviating the proteotoxic effects that mimic misfolding proteins.

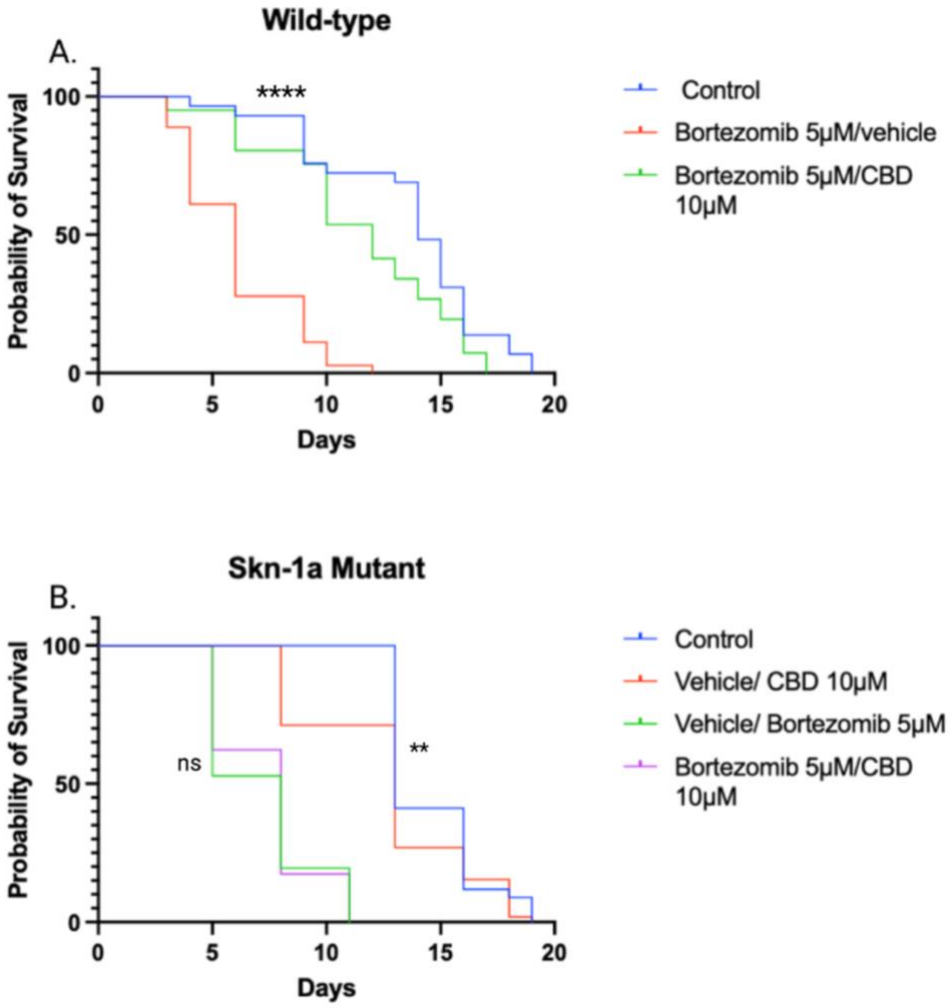

Figure 3.2.4. Effect of cannabidiol on lifespan of several strains in the presence or absence of proteotoxic stress. (A) wild-type animals kept as a control, exposed to 5μM Bortezomib, or pre-treated with 10μM CBD in the presence of proteotoxic stress. (B) Skn-1a mutant animals kept as a control or pre-treated with 10μM CBD in the presence or absence of stress. Results are presented as a Mantel–Cox test. P< .00001****: panel A and B. P< .05**: panel B. P > .05 non-significant (ns): Panel B. N=100-200. Two biological replicas.

3.2.5 Paralysis Assay

In c. elegans, expression of human Aβ in muscle cells leads to progressive adult-onset paralysis (Wu et al., 2006). Accumulation of Aβ in C. elegans muscle cells lead to aggregation and formation of amyloid fibrils, features also associated with adult-onset neurodegeneration in Alzheimer's disease (Link, 1995). Adult-onset paralysis caused by human Aβ in C. elegans muscle is enhanced in skn-1a(mg570) mutants (Lehrbach & Ruvkun, 2019). Overexpression of SKN-1A reduces the paralysis caused by muscle-specific Aβ expression in the wild type. The current literature suggests that the enhanced adult-onset paralysis in animals that lack SKN-1A is caused by higher levels of Aβ accumulation and aggregation. Utilizing wild-type animals, we asked if CBD would be able to alleviate the paralysis caused by proteotoxic stress. Synchronized animals were grown on NGM plates until they reached the young adult stage and treated with FUDR to inhibit the offspring. On day 1, animals were plated on NGM plates that were seeded with either 10µM CBD or vehicle. After 24 hours, animals from each group were washed with M9 buffer and placed on new NGM plates that were seeded with 5µM Bortezomib or vehicle for another 24 hours. Bortezomib did cause significant paralysis in wild-type animals compared to the control (Figure 3.2.5).

Worms that were treated with 10µM CBD/ 5µM Bortezomib showed a significant amelioration of the proteotoxic stress caused by Bortezomib. The current data suggest that CBD is beneficial in proteasome impairment conditions. We then asked if CBD's alleviation of muscle paralysis would be dependent on skn-1a that increases the expression of proteosome subunit genes. A significant increase in muscle paralysis is shown in Bortezomib treated group compared to the

control group. However, CBD did not significantly reduce the paralysis in stressed skn-1a mutant animals (Figure 3.2.5).

Furthermore, we asked if CBD's alleviation of muscle paralysis would be dependent on the muscle-specific proteasome. To answer this question, we used a strain that has a muscle-specific proteasome dysfunction. CBD did not show any superior benefit in the absence of the muscle proteasome (Figure 3.2.5). The findings prove that CBD causes resistance to the paralysis induced by proteotoxic stress, and it is dependent on the presence of skn-1a and muscle proteasome.

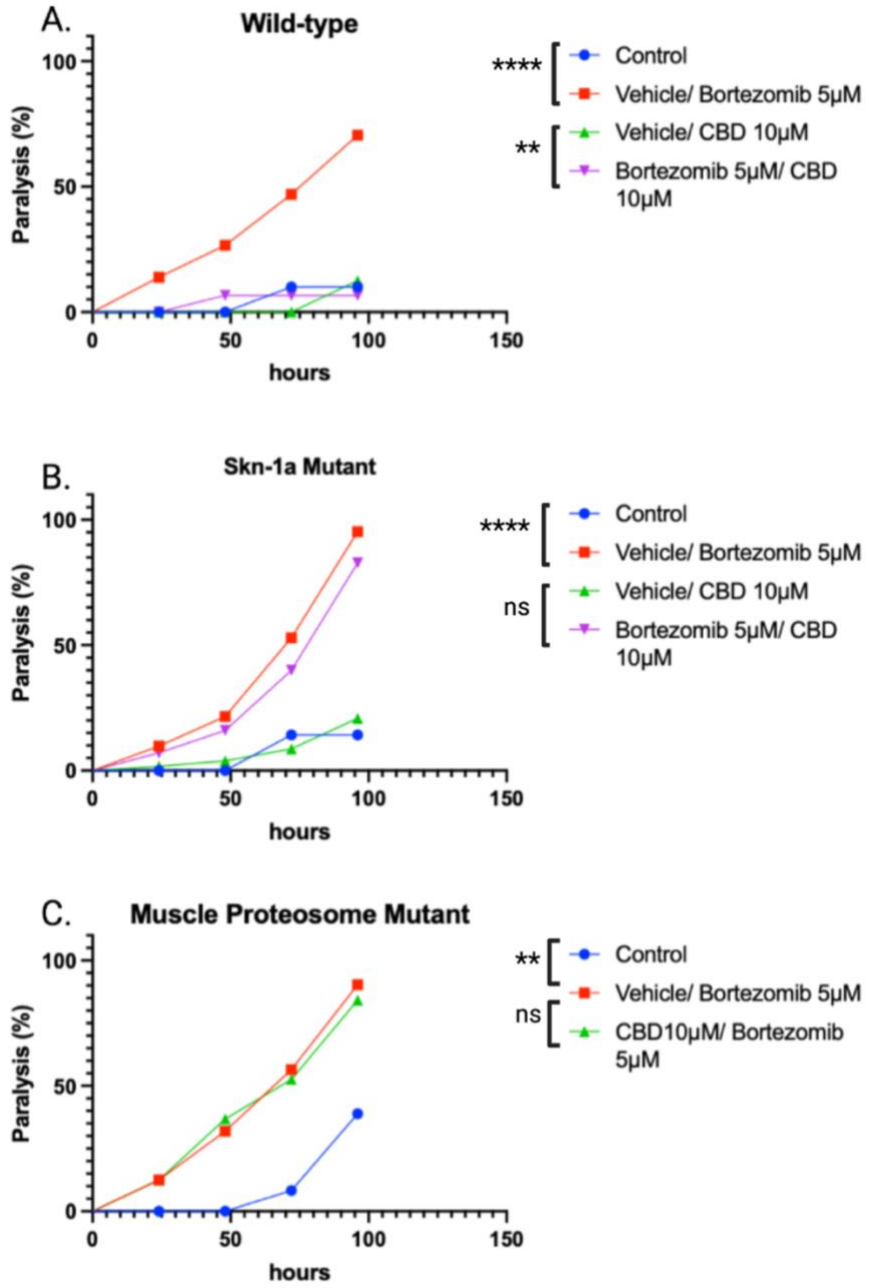

Figure 3.2.5. Effect of Cannabidiol on muscle induced paralysis. Paralysis assays wild-type, skn-1a mutant, and proteosome muscle mutant animals. (A) Wild-type animals kept as a control, treated with 10µM CBD, exposed to 5µM Bortezomib, or pre-treated with 10µM CBD followed by exposure to 5µM Bortezomib.
(B) Skn-1a mutant animals kept as a control, treated with 10µM CBD, exposed to 5µM Bortezomib, or pre-treated with 10µM CBD followed by exposure to 5µM Bortezomib. (C) Proteosome muscle mutant kept as a control, exposed to 5µM Bortezomib, or with 10µM CBD followed by exposure to 5µM Bortezomib. Results are presented as a Mantel–Cox test. P<

.00001****: panel A, B. P< .05**: panel C. P > .05 non-significant (ns): Panel B and C. N=50-100. Two biological replicas.

Conclusion

There is growing evidence that put cannabinoids as a potential treatment for neurodegenerative diseases (Lim et al., 2017). CBD, a Phyto cannabinoid compound extracted from cannabis sativa, has been investigated for its potential effects on neurodegenerative diseases (Viana et al., 2022). It has been reported that CBD acts partially through the endocannabinoid system and through the glycogen synthase kinase 3b (GSK-3b) hyper-phosphorylation caused by Aβ (Li et al., 2020).

LPS-induced microglial inflammation was shown to be inhibited by CBD through ROS/ NF-κB signaling (Dos-Santos-Pereira et al., 2020). Neurodegenerative diseases are characterized by the accumulation and aggregation of misfolded proteins (Vaquer-Alicea & Diamond, 2019). Our previous work showed that the combinational treatment of Cannabidiol and Trazadone significantly improved the neuronal integrity of transgenic animals that expressed aggregation of either $A\beta_{1-42}$ or P-tau. In this study, we investigated the molecular targets by which CBD exerts neuroprotective properties.

In keratinocytes, CBD was found to activate Nrf-2 target genes. It was validated that CBD induces the expression of several Nrf-2 target genes in primary human keratinocytes (Casares et al., 2020). In c. elegans, glutathione transferase-4 (GST-4) has been shown to correspond to skn-1 activation under oxidative stress (Kahn et al., 2008). We showed that CBD directly activates skn-1b/c under normal conditions. Also, we showed that CBD's extension of lifespan under oxidative stress is dependent on the presence of skn-1. The ROS scavenging activity of CBD is shown to be dependent on skn-1b/c. Several genetic and environmental insults render the cells unable to properly fold and modify secretory and transmembrane proteins, which

leads to a buildup of misfolded proteins in the ER (Fagone & Jackowski, 2009). To match the increased demand for protein folding, an intracellular signaling pathway is activated. The unfolded protein response (UPR) induces several transcriptional and translational events to restore ER folding capacity. However, under constant activation of the UPR, cells undergo self-destruction(Anelli & Sitia, 2008).

Pretreatment with CBD in ER-stressed mouse striatal STHdh cells significantly increased the expression of pro-survival ER chaperone *GRP78* and ER-resident neurotrophic factor, MANF. It also decreased ER-mediated pro-apoptotic markers such as BIM and caspase-12 (Patel et al., 2022). Despite the fact that CBD increased chaperone properties, its role in ameliorating proteotoxicity in neurodegenerative diseases is not well investigated. Proteosome impairment can occur as a result of oxidative stress, ER stress, and mitochondrial dysfunction (Bulteau et al., 2001). The functional condition of the proteasome declines in the presence of stressors. Under normal conditions, the proteasome efficiently copes with the cellular demand to degrade misfolded proteins. However, oxidative stress or ER stress slowly compromises the proteolytic activity of the proteasome (Menéndez-Benito et al., 2005). This may explain the slowly progressive nature of neurodegenerative diseases. We hypothesized that skn-1a is one of the crucial targets for CBD to protect against oxidative stress generated by misfolded proteins and increase the expression of proteasome subunit genes. The aggregation of misfolded proteins leads to the opening of mitochondrial permeability transition pores and cell death (Abramov et al., 2020). Our data suggest that CBD activity under proteotoxic stress is dependent on skn-1a. Using wild-type animals, we have shown that proteotoxicity can lead to increased production of ROS. We showed that the presence of CBD allows wild-type animals to

escape the developmental delay induced by Bortezomib. This effect was not present with the use of transgenic worms that have a mutation of skn-1a. Also, skn-1a is shown to be a direct target for CBD under normal and proteotoxic conditions; thus, it allows for the constant expression of proteasome subunit under proteotoxic conditions.

Lifespan assays confirm the crucial role of skn-1a in CBD's mitigation of proteotoxicity. CBD extension of lifespan in wild-type animals was diminished in skn-1a mutant animals. Pretreatment with CBD protects wild-type animals from paralysis induced by Bortezomib. However, this effect was negligible in the absence of both skn-1a and proteosomes in the muscles. Our data suggest that CBD activity is constant among different stressors and put skn-1 is a direct molecular target of CBD under condition that mimic the stress generated by misfolded proteins. Further investigation is needed to further explore the role skn-1 in neurodegeneration. The association between skn-1 isoforms and Aβ accumulation is not well established. Utilizing higher organisms, we would be able to further investigate the molecular mechanism of CBD activation of skn-1 and how each isoform contributes to mitigation of neurotoxicity.

References

Abramov, A. Y., Potapova, E. V., Dremin, V. V., & Dunaev, A. V. (2020). Interaction of Oxidative Stress and Misfolded Proteins in the Mechanism of Neurodegeneration. *Life (Basel, Switzerland)*, *10*(7), 101. https://doi.org/10.3390/life10070101

Albornoz, N., Bustamante, H., Soza, A., & Burgos, P. (2019). Cellular Responses to Proteasome Inhibition: Molecular Mechanisms and Beyond. *International Journal of Molecular Sciences*, *20*(14), 3379. https://doi.org/10.3390/ijms20143379

Alves, P., Amaral, C., Teixeira, N., & Correia-da-Silva, G. (2020). Cannabis sativa: Much more beyond Δ9-tetrahydrocannabinol. *Pharmacological Research*, *157*, 104822. https://doi.org/10.1016/j.phrs.2020.104822

Amin, M. R., & Ali, D. W. (2019). Pharmacology of Medical Cannabis. *Advances in Experimental Medicine and Biology*, *1162*, 151–165. https://doi.org/10.1007/978-3-030-21737-2_8

Anderson, R. T., Bradley, T. A., & Smith, D. M. (2022). Hyperactivation of the proteasome in Caenorhabditis elegans protects against proteotoxic stress and extends lifespan. *The Journal of Biological Chemistry*, *298*(10), 102415. https://doi.org/10.1016/j.jbc.2022.102415

Anelli, T., & Sitia, R. (2008). Protein quality control in the early secretory pathway. *The EMBO Journal*, *27*(2), 315–327. https://doi.org/10.1038/sj.emboj.7601974

Atalay, S., Jarocka-Karpowicz, I., & Skrzydlewska, E. (2019). Antioxidative and Anti-Inflammatory Properties of Cannabidiol. *Antioxidants (Basel, Switzerland)*, *9*(1), 21. https://doi.org/10.3390/antiox9010021

Bartner, L. R., McGrath, S., Rao, S., Hyatt, L. K., & Wittenburg, L. A. (2018). Pharmacokinetics of cannabidiol administered by 3 delivery methods at 2 different dosages to healthy dogs. *Canadian Journal of Veterinary Research = Revue Canadienne De Recherche Veterinaire*, *82*(3), 178–183.

Basavarajappa, B. S., Shivakumar, M., Joshi, V., & Subbanna, S. (2017). Endocannabinoid system in neurodegenerative disorders. *Journal of Neurochemistry*, *142*(5), 624–648. https://doi.org/10.1111/jnc.14098

Benito, C., Núñez, E., Tolón, R. M., Carrier, E. J., Rábano, A., Hillard, C. J., & Romero, J. (2003). Cannabinoid CB2 receptors and fatty acid amide hydrolase are selectively overexpressed in neuritic plaque-associated glia in Alzheimer's disease brains. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *23*(35), 11136–11141. https://doi.org/10.1523/JNEUROSCI.23-35-11136.2003

Bhatla, N., Droste, R., Sando, S. R., Huang, A., & Horvitz, H. R. (2015). Distinct Neural Circuits Control Rhythm Inhibition and Spitting by the Myogenic Pharynx of C. elegans. *Current Biology: CB*, *25*(16), 2075–2089. https://doi.org/10.1016/j.cub.2015.06.052

Blackwell, T. K., Steinbaugh, M. J., Hourihan, J. M., Ewald, C. Y., & Isik, M. (2015). SKN-1/Nrf, stress responses, and aging in Caenorhabditis elegans. *Free Radical Biology & Medicine*, *88*(Pt B), 290–301. https://doi.org/10.1016/j.freeradbiomed.2015.06.008

Brand, M. D., Orr, A. L., Perevoshchikova, I. V., & Quinlan, C. L. (2013). The role of mitochondrial function and cellular bioenergetics in ageing and disease. *The British Journal of Dermatology*, *169 Suppl 2*(0 2), 1–8. https://doi.org/10.1111/bjd.12208

Bulteau, A. L., Lundberg, K. C., Humphries, K. M., Sadek, H. A., Szweda, P. A., Friguet, B., & Szweda, L. I. (2001). Oxidative modification and inactivation of the proteasome during coronary occlusion/reperfusion. *The Journal of Biological Chemistry*, *276*(32), 30057–30063. https://doi.org/10.1074/jbc.M100142200

Butterfield, D. A., Swomley, A. M., & Sultana, R. (2013). Amyloid β-peptide (1-42)-induced oxidative stress in Alzheimer disease: Importance in disease pathogenesis and progression. *Antioxidants & Redox Signaling*, *19*(8), 823–835. https://doi.org/10.1089/ars.2012.5027

Cabral-Miranda, F., & Hetz, C. (2018). ER Stress and Neurodegenerative Disease: A Cause or Effect Relationship? *Current Topics in Microbiology and Immunology*, *414*, 131–157. https://doi.org/10.1007/82_2017_52

Campolongo, P., & Trezza, V. (2012). The endocannabinoid system: A key modulator of emotions and cognition. *Frontiers in Behavioral Neuroscience*, *6*, 73. https://doi.org/10.3389/fnbeh.2012.00073

Casares, L., García, V., Garrido-Rodríguez, M., Millán, E., Collado, J. A., García-Martín, A., Peñarando, J., Calzado, M. A., de la Vega, L., & Muñoz, E. (2020). Cannabidiol induces antioxidant pathways in keratinocytes by targeting BACH1. *Redox Biology*, *28*, 101321. https://doi.org/10.1016/j.redox.2019.101321

Collins, G. A., & Goldberg, A. L. (2017). The Logic of the 26S Proteasome. *Cell*, *169*(5), 792–806. https://doi.org/10.1016/j.cell.2017.04.023

Daraie, B., Pourahmad, J., Hamidi-Pour, N., Hosseini, M.-J., Shaki, F., & Soleimani, M. (2012). Uranyl acetate induces oxidative stress and mitochondrial membrane potential collapse

in the human dermal fibroblast primary cells. *Iranian Journal of Pharmaceutical Research: IJPR*, *11*(2), 495–501.

David, D. C., Ollikainen, N., Trinidad, J. C., Cary, M. P., Burlingame, A. L., & Kenyon, C. (2010). Widespread protein aggregation as an inherent part of aging in C. elegans. *PLoS Biology*, *8*(8), e1000450. https://doi.org/10.1371/journal.pbio.1000450

De Magalhaes Filho, C. D., Henriquez, B., Seah, N. E., Evans, R. M., Lapierre, L. R., & Dillin, A. (2018). Visible light reduces C. elegans longevity. *Nature Communications*, *9*(1), 927. https://doi.org/10.1038/s41467-018-02934-5

Dos-Santos-Pereira, M., Guimarães, F. S., Del-Bel, E., Raisman-Vozari, R., & Michel, P. P. (2020). Cannabidiol prevents LPS-induced microglial inflammation by inhibiting ROS/NF-κB-dependent signaling and glucose consumption. *Glia*, *68*(3), 561–573. https://doi.org/10.1002/glia.23738

Elsaid, S., Kloiber, S., & Le Foll, B. (2019). Effects of cannabidiol (CBD) in neuropsychiatric disorders: A review of pre-clinical and clinical findings. *Progress in Molecular Biology and Translational Science*, *167*, 25–75. https://doi.org/10.1016/bs.pmbts.2019.06.005

Fagone, P., & Jackowski, S. (2009). Membrane phospholipid synthesis and endoplasmic reticulum function. *Journal of Lipid Research*, *50 Suppl*(Suppl), S311-316. https://doi.org/10.1194/jlr.R800049-JLR200

Fricker, L. D. (2020). Proteasome Inhibitor Drugs. *Annual Review of Pharmacology and Toxicology*, *60*, 457–476. https://doi.org/10.1146/annurev-pharmtox-010919-023603

Fride, E. (2004). The endocannabinoid-CB(1) receptor system in pre- and postnatal life. *European Journal of Pharmacology*, *500*(1–3), 289–297. https://doi.org/10.1016/j.ejphar.2004.07.033

Fuchs, Y., & Steller, H. (2015). Live to die another way: Modes of programmed cell death and the signals emanating from dying cells. *Nature Reviews. Molecular Cell Biology*, *16*(6), 329–344. https://doi.org/10.1038/nrm3999

Fulda, S., Gorman, A. M., Hori, O., & Samali, A. (2010). Cellular stress responses: Cell survival and cell death. *International Journal of Cell Biology*, *2010*, 214074. https://doi.org/10.1155/2010/214074

Galluzzi, L., Pietrocola, F., Levine, B., & Kroemer, G. (2014). Metabolic control of autophagy. *Cell*, *159*(6), 1263–1276. https://doi.org/10.1016/j.cell.2014.11.006

Gaston, T. E., & Friedman, D. (2017). Pharmacology of cannabinoids in the treatment of epilepsy. *Epilepsy & Behavior: E&B*, *70*(Pt B), 313–318. https://doi.org/10.1016/j.yebeh.2016.11.016

Good, P. F., Werner, P., Hsu, A., Olanow, C. W., & Perl, D. P. (1996). Evidence of neuronal oxidative damage in Alzheimer's disease. *The American Journal of Pathology*, *149*(1), 21–28.

Gorman, A. M., Heavey, B., Creagh, E., Cotter, T. G., & Samali, A. (1999). Antioxidant-mediated inhibition of the heat shock response leads to apoptosis. *FEBS Letters*, *445*(1), 98–102. https://doi.org/10.1016/s0014-5793(99)00094-0

Hooper, C., Killick, R., & Lovestone, S. (2008). The GSK3 hypothesis of Alzheimer's disease. *Journal of Neurochemistry*, *104*(6), 1433–1439. https://doi.org/10.1111/j.1471-4159.2007.05194.x

Iliff, A. J., & Xu, X. Z. S. (2020). C. elegans: A sensible model for sensory biology. *Journal of Neurogenetics*, *34*(3–4), 347–350. https://doi.org/10.1080/01677063.2020.1823386

Imbimbo, B. P., Lombard, J., & Pomara, N. (2005). Pathophysiology of Alzheimer's disease. *Neuroimaging Clinics of North America*, *15*(4), 727–753, ix. https://doi.org/10.1016/j.nic.2005.09.009

Ionescu-Tucker, A., & Cotman, C. W. (2021). Emerging roles of oxidative stress in brain aging and Alzheimer's disease. *Neurobiology of Aging*, *107*, 86–95. https://doi.org/10.1016/j.neurobiolaging.2021.07.014

Kahn, N. W., Rea, S. L., Moyle, S., Kell, A., & Johnson, T. E. (2008). Proteasomal dysfunction activates the transcription factor SKN-1 and produces a selective oxidative-stress response in Caenorhabditis elegans. *The Biochemical Journal*, *409*(1), 205–213. https://doi.org/10.1042/BJ20070521

Khodadadi, H., Salles, É. L., Jarrahi, A., Costigliola, V., Khan, M. B., Yu, J. C., Morgan, J. C., Hess, D. C., Vaibhav, K., Dhandapani, K. M., & Baban, B. (2021). Cannabidiol Ameliorates Cognitive Function via Regulation of IL-33 and TREM2 Upregulation in a Murine Model of Alzheimer's Disease. *Journal of Alzheimer's Disease: JAD*, *80*(3), 973–977. https://doi.org/10.3233/JAD-210026

Kim, K. W., & Jin, Y. (2015). Neuronal responses to stress and injury in C. elegans. *FEBS Letters*, *589*(14), 1644–1652. https://doi.org/10.1016/j.febslet.2015.05.005

Lee, C., Schwartz, M. P., Prakash, S., Iwakura, M., & Matouschek, A. (2001). ATP-dependent proteases degrade their substrates by processively unraveling them from the degradation signal. *Molecular Cell*, *7*(3), 627–637. https://doi.org/10.1016/s1097-2765(01)00209-x

Lehrbach, N. J., & Ruvkun, G. (2016). Proteasome dysfunction triggers activation of SKN-1A/Nrf1 by the aspartic protease DDI-1. *ELife*, *5*, e17721. https://doi.org/10.7554/eLife.17721

Lehrbach, N. J., & Ruvkun, G. (2019). Endoplasmic reticulum-associated SKN-1A/Nrf1 mediates a cytoplasmic unfolded protein response and promotes longevity. *ELife*, *8*, e44425. https://doi.org/10.7554/eLife.44425

Li, H., Liu, Y., Tian, D., Tian, L., Ju, X., Qi, L., Wang, Y., & Liang, C. (2020). Overview of cannabidiol (CBD) and its analogues: Structures, biological activities, and neuroprotective mechanisms in epilepsy and Alzheimer's disease. *European Journal of Medicinal Chemistry*, *192*, 112163. https://doi.org/10.1016/j.ejmech.2020.112163

Lim, K., See, Y. M., & Lee, J. (2017). A Systematic Review of the Effectiveness of Medical Cannabis for Psychiatric, Movement and Neurodegenerative Disorders. *Clinical Psychopharmacology and Neuroscience: The Official Scientific Journal of the Korean College of Neuropsychopharmacology*, *15*(4), 301–312. https://doi.org/10.9758/cpn.2017.15.4.301

Link, C. D. (1995). Expression of human beta-amyloid peptide in transgenic Caenorhabditis elegans. *Proceedings of the National Academy of Sciences of the United States of America*, *92*(20), 9368–9372. https://doi.org/10.1073/pnas.92.20.9368

Link, C. D. (2006). C. elegans models of age-associated neurodegenerative diseases: Lessons from transgenic worm models of Alzheimer's disease. *Experimental Gerontology*, *41*(10), 1007–1013. https://doi.org/10.1016/j.exger.2006.06.059

Lockshin, R. A., & Zakeri, Z. (2001). Programmed cell death and apoptosis: Origins of the theory. *Nature Reviews. Molecular Cell Biology*, *2*(7), 545–550. https://doi.org/10.1038/35080097

López-Otín, C., Blasco, M. A., Partridge, L., Serrano, M., & Kroemer, G. (2013). The hallmarks of aging. *Cell*, *153*(6), 1194–1217. https://doi.org/10.1016/j.cell.2013.05.039

Lovell, M. A., & Markesbery, W. R. (2007). Oxidative DNA damage in mild cognitive impairment and late-stage Alzheimer's disease. *Nucleic Acids Research*, *35*(22), 7497–7504. https://doi.org/10.1093/nar/gkm821

Lowe, H., Toyang, N., Steele, B., Bryant, J., & Ngwa, W. (2021). The Endocannabinoid System: A Potential Target for the Treatment of Various Diseases. *International Journal of Molecular Sciences*, *22*(17), 9472. https://doi.org/10.3390/ijms22179472

Lu, H.-C., & Mackie, K. (2016). An Introduction to the Endogenous Cannabinoid System. *Biological Psychiatry*, *79*(7), 516–525. https://doi.org/10.1016/j.biopsych.2015.07.028

Lucas, C. J., Galettis, P., & Schneider, J. (2018). The pharmacokinetics and the pharmacodynamics of cannabinoids. *British Journal of Clinical Pharmacology*, *84*(11), 2477–2482. https://doi.org/10.1111/bcp.13710

Ma, Q. (2013). Role of nrf2 in oxidative stress and toxicity. *Annual Review of Pharmacology and Toxicology*, *53*, 401–426. https://doi.org/10.1146/annurev-pharmtox-011112-140320

Mackie, K. (2008). Cannabinoid receptors: Where they are and what they do. *Journal of Neuroendocrinology*, *20 Suppl 1*, 10–14. https://doi.org/10.1111/j.1365-2826.2008.01671.x

Majd, S., Power, J. H., & Grantham, H. J. M. (2015). Neuronal response in Alzheimer's and Parkinson's disease: The effect of toxic proteins on intracellular pathways. *BMC Neuroscience*, *16*, 69. https://doi.org/10.1186/s12868-015-0211-1

Marques, C. A., Keil, U., Bonert, A., Steiner, B., Haass, C., Muller, W. E., & Eckert, A. (2003). Neurotoxic mechanisms caused by the Alzheimer's disease-linked Swedish amyloid precursor protein mutation: Oxidative stress, caspases, and the JNK pathway. *The Journal of Biological Chemistry*, *278*(30), 28294–28302. https://doi.org/10.1074/jbc.M212265200

McCubrey, J. A., Lahair, M. M., & Franklin, R. A. (2006). Reactive oxygen species-induced activation of the MAP kinase signaling pathways. *Antioxidants & Redox Signaling*, *8*(9–10), 1775–1789. https://doi.org/10.1089/ars.2006.8.1775

McGrath, L. T., McGleenon, B. M., Brennan, S., McColl, D., McILroy, S., & Passmore, A. P. (2001). Increased oxidative stress in Alzheimer's disease as assessed with 4-hydroxynonenal but not malondialdehyde. *QJM: Monthly Journal of the Association of Physicians*, *94*(9), 485–490. https://doi.org/10.1093/qjmed/94.9.485

Menéndez-Benito, V., Verhoef, L. G. G. C., Masucci, M. G., & Dantuma, N. P. (2005). Endoplasmic reticulum stress compromises the ubiquitin-proteasome system. *Human Molecular Genetics*, *14*(19), 2787–2799. https://doi.org/10.1093/hmg/ddi312

Miranda-Vizuete, A., & Veal, E. A. (2017). Caenorhabditis elegans as a model for understanding ROS function in physiology and disease. *Redox Biology*, *11*, 708–714. https://doi.org/10.1016/j.redox.2016.12.020

Murphy, M. P. (2009). How mitochondria produce reactive oxygen species. *The Biochemical Journal*, *417*(1), 1–13. https://doi.org/10.1042/BJ20081386

Northrop, A., Byers, H. A., & Radhakrishnan, S. K. (2020). Regulation of NRF1, a master transcription factor of proteasome genes: Implications for cancer and neurodegeneration. *Molecular Biology of the Cell*, *31*(20), 2158–2163. https://doi.org/10.1091/mbc.E20-04-0238

Oh, S.-I., Park, J.-K., & Park, S.-K. (2015). Lifespan extension and increased resistance to environmental stressors by N-acetyl-L-cysteine in Caenorhabditis elegans. *Clinics (Sao Paulo, Brazil)*, *70*(5), 380–386. https://doi.org/10.6061/clinics/2015(05)13

Palmer, A. M., & Burns, M. A. (1994). Selective increase in lipid peroxidation in the inferior temporal cortex in Alzheimer's disease. *Brain Research*, *645*(1–2), 338–342. https://doi.org/10.1016/0006-8993(94)91670-5

Patel, V., Abu-Hijleh, F., Rigg, N., & Mishra, R. (2022). Cannabidiol Protects Striatal Neurons by Attenuating Endoplasmic Reticulum Stress. *Cannabis and Cannabinoid Research*. https://doi.org/10.1089/can.2022.0090

Payne, B. A. I., & Chinnery, P. F. (2015). Mitochondrial dysfunction in aging: Much progress but many unresolved questions. *Biochimica Et Biophysica Acta*, *1847*(11), 1347–1353. https://doi.org/10.1016/j.bbabio.2015.05.022

Pereira, S. R., Hackett, B., O'Driscoll, D. N., Sun, M. C., & Downer, E. J. (2021). Cannabidiol modulation of oxidative stress and signalling. *Neuronal Signaling*, *5*(3), NS20200080. https://doi.org/10.1042/NS20200080

Puig, B., Gómez-Isla, T., Ribé, E., Cuadrado, M., Torrejón-Escribano, B., Dalfó, E., & Ferrer, I. (2004). Expression of stress-activated kinases c-Jun N-terminal kinase (SAPK/JNK-P) and p38 kinase (p38-P), and tau hyperphosphorylation in neurites surrounding betaA plaques in APP Tg2576 mice. *Neuropathology and Applied Neurobiology*, *30*(5), 491–502. https://doi.org/10.1111/j.1365-2990.2004.00569.x

Rao, R. V., & Bredesen, D. E. (2004). Misfolded proteins, endoplasmic reticulum stress and neurodegeneration. *Current Opinion in Cell Biology*, *16*(6), 653–662. https://doi.org/10.1016/j.ceb.2004.09.012

Read, A., & Schröder, M. (2021). The Unfolded Protein Response: An Overview. *Biology*, *10*(5), 384. https://doi.org/10.3390/biology10050384

Ron, D. (2002). Translational control in the endoplasmic reticulum stress response. *The Journal of Clinical Investigation*, *110*(10), 1383–1388. https://doi.org/10.1172/JCI16784

Scheltens, P., De Strooper, B., Kivipelto, M., Holstege, H., Chételat, G., Teunissen, C. E., Cummings, J., & van der Flier, W. M. (2021). Alzheimer's disease. *Lancet (London, England)*, *397*(10284), 1577–1590. https://doi.org/10.1016/S0140-6736(20)32205-4

Senchuk, M. M., Dues, D. J., Schaar, C. E., Johnson, B. K., Madaj, Z. B., Bowman, M. J., Winn, M. E., & Van Raamsdonk, J. M. (2018). Activation of DAF-16/FOXO by reactive oxygen species contributes to longevity in long-lived mitochondrial mutants in Caenorhabditis elegans. *PLoS Genetics*, *14*(3), e1007268. https://doi.org/10.1371/journal.pgen.1007268

Shpilka, T., & Haynes, C. M. (2018). The mitochondrial UPR: Mechanisms, physiological functions and implications in ageing. *Nature Reviews. Molecular Cell Biology*, *19*(2), 109–120. https://doi.org/10.1038/nrm.2017.110

Singh, A., Kukreti, R., Saso, L., & Kukreti, S. (2019). Oxidative Stress: A Key Modulator in Neurodegenerative Diseases. *Molecules (Basel, Switzerland)*, *24*(8), 1583. https://doi.org/10.3390/molecules24081583

Spatola, B. N., Lo, J. Y., Wang, B., & Curran, S. P. (2019). Nuclear and cytoplasmic WDR-23 isoforms mediate differential effects on GEN-1 and SKN-1 substrates. *Scientific Reports*, *9*(1), 11783. https://doi.org/10.1038/s41598-019-48286-y

Spitz, D. R., Azzam, E. I., Li, J. J., & Gius, D. (2004). Metabolic oxidation/reduction reactions and cellular responses to ionizing radiation: A unifying concept in stress response biology. *Cancer Metastasis Reviews*, *23*(3–4), 311–322. https://doi.org/10.1023/B:CANC.0000031769.14728.bc

Suomalainen, A., & Battersby, B. J. (2018). Mitochondrial diseases: The contribution of organelle stress responses to pathology. *Nature Reviews. Molecular Cell Biology*, *19*(2), 77–92. https://doi.org/10.1038/nrm.2017.66

Swindell, W. R., Huebner, M., & Weber, A. P. (2007). Transcriptional profiling of Arabidopsis heat shock proteins and transcription factors reveals extensive overlap between heat and non-heat stress response pathways. *BMC Genomics*, *8*, 125. https://doi.org/10.1186/1471-2164-8-125

Szegezdi, E., Logue, S. E., Gorman, A. M., & Samali, A. (2006). Mediators of endoplasmic reticulum stress-induced apoptosis. *EMBO Reports*, *7*(9), 880–885. https://doi.org/10.1038/sj.embor.7400779

Takagi, M., Satofuka, H., Amano, S., Mizuno, H., Eguchi, Y., Hirata, K., Miyamoto, K., Fukui, K., & Imanaka, T. (2002). Cellular toxicity of cadmium ions and their detoxification by heavy metal-specific plant peptides, phytochelatins, expressed in Mammalian cells. *Journal of Biochemistry*, *131*(2), 233–239. https://doi.org/10.1093/oxfordjournals.jbchem.a003093

Tiwari, S., Atluri, V., Kaushik, A., Yndart, A., & Nair, M. (2019). Alzheimer's disease: Pathogenesis, diagnostics, and therapeutics. *International Journal of Nanomedicine*, *14*, 5541–5554. https://doi.org/10.2147/IJN.S200490

Ujváry, I., & Hanuš, L. (2016). Human Metabolites of Cannabidiol: A Review on Their Formation, Biological Activity, and Relevance in Therapy. *Cannabis and Cannabinoid Research*, *1*(1), 90–101. https://doi.org/10.1089/can.2015.0012

Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T. D., Mazur, M., & Telser, J. (2007). Free radicals and antioxidants in normal physiological functions and human disease. *The International Journal of Biochemistry & Cell Biology*, *39*(1), 44–84. https://doi.org/10.1016/j.biocel.2006.07.001

Vaquer-Alicea, J., & Diamond, M. I. (2019). Propagation of Protein Aggregation in Neurodegenerative Diseases. *Annual Review of Biochemistry*, *88*, 785–810. https://doi.org/10.1146/annurev-biochem-061516-045049

Viana, M. de B., de Aquino, P. E. A., Estadella, D., Ribeiro, D. A., & Viana, G. S. de B. (2022). Cannabis sativa and Cannabidiol: A Therapeutic Strategy for the Treatment of

Neurodegenerative Diseases? *Medical Cannabis and Cannabinoids*, *5*(1), 207–219. https://doi.org/10.1159/000527335

Walther, D. M., Kasturi, P., Zheng, M., Pinkert, S., Vecchi, G., Ciryam, P., Morimoto, R. I., Dobson, C. M., Vendruscolo, M., Mann, M., & Hartl, F. U. (2015). Widespread Proteome Remodeling and Aggregation in Aging C. elegans. *Cell*, *161*(4), 919–932. https://doi.org/10.1016/j.cell.2015.03.032

Wang, X., Wang, W., Li, L., Perry, G., Lee, H., & Zhu, X. (2014). Oxidative stress and mitochondrial dysfunction in Alzheimer's disease. *Biochimica Et Biophysica Acta*, *1842*(8), 1240–1247. https://doi.org/10.1016/j.bbadis.2013.10.015

Ward, A., Liu, J., Feng, Z., & Xu, X. Z. S. (2008). Light-sensitive neurons and channels mediate phototaxis in C. elegans. *Nature Neuroscience*, *11*(8), 916–922. https://doi.org/10.1038/nn.2155

Wu, Y., Wu, Z., Butko, P., Christen, Y., Lambert, M. P., Klein, W. L., Link, C. D., & Luo, Y. (2006). Amyloid-beta-induced pathological behaviors are suppressed by Ginkgo biloba extract EGb 761 and ginkgolides in transgenic Caenorhabditis elegans. *The Journal of Neuroscience: The Official Journal of the Society for Neuroscience*, *26*(50), 13102–13113. https://doi.org/10.1523/JNEUROSCI.3448-06.2006

Yan, M. H., Wang, X., & Zhu, X. (2013). Mitochondrial defects and oxidative stress in Alzheimer disease and Parkinson disease. *Free Radical Biology & Medicine*, *62*, 90–101. https://doi.org/10.1016/j.freeradbiomed.2012.11.014

Yedjou, C. G., & Tchounwou, P. B. (2007). N-acetyl-l-cysteine affords protection against lead-induced cytotoxicity and oxidative stress in human liver carcinoma (HepG2) cells.

International Journal of Environmental Research and Public Health, *4*(2), 132–137. https://doi.org/10.3390/ijerph2007040007

Zhang, Y., Li, H., Jin, S., Lu, Y., Peng, Y., Zhao, L., & Wang, X. (2022). Cannabidiol protects against Alzheimer's disease in C. elegans via ROS scavenging activity of its phenolic hydroxyl groups. *European Journal of Pharmacology*, *919*, 174829. https://doi.org/10.1016/j.ejphar.2022.174829

Zhang, Z., Zhang, L., Zhou, L., Lei, Y., Zhang, Y., & Huang, C. (2019). Redox signaling and unfolded protein response coordinate cell fate decisions under ER stress. *Redox Biology*, *25*, 101047. https://doi.org/10.1016/j.redox.2018.11.005

Zorov, D. B., Juhaszova, M., & Sollott, S. J. (2014). Mitochondrial reactive oxygen species (ROS) and ROS-induced ROS release. *Physiological Reviews*, *94*(3), 909–950. https://doi.org/10.1152/physrev.00026.2013