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Flavonoid and Cannabidiol Neural Glyoxalase Pathway Enhancement Against Aging and Alzheimer's Disease

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FLAVONOID AND CANNABIDIOL NEURAL GLYOXALASE PATHWAY ENHANCEMENT AGAINST AGING AND ALZHEIMER'S DISEASE

by

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A DISSERTATION

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DEDICATION

This work is dedicated to my grandparents who suffered from Alzheimer's Disease:

Dale Frandsen

Leona "Ruth" Shaver and Donald "Ted" Shaver

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University of Nebraska Medical Center, 2020
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ABSTRACT

Alzheimer's Disease is a neurodegenerative condition featuring neural cell death and a decline in cognitive capacity caused by elevated inflammation and production of reactive oxygen species. The glyoxalase pathway is an endogenous antioxidant system that neutralizes reactive methylglyoxal through sequential reactions. Dysfunction of the glyoxalase pathway contributes to oxidative stress and the accumulation of inflammatory metabolic byproducts. Plant-produced compounds with antioxidant activity can enhance endogenous antioxidant pathways and protect cells from elevated ROS production. We hypothesize that flavonoids and limited *Cannabis Sativa*-produced cannabidiol can enhance glyoxalase pathway function through regulation of antioxidant and pro-apoptotic signaling pathways to prevent methylglyoxal-mediated cellular damage. This research investigates the effects of flavonoids and cannabidiol on glyoxalase pathway function. We also investigated the influence of structural modifications in flavonoid morin to improve its inherent antioxidant activity. We evaluated the effect of flavonoids and CBD on expression of glyoxalase constituents and cell signaling pathways in vitro utilizing primary mouse cerebellar neurons, and in vivo with C. elegans. Our research provides evidence of antioxidant compounds enhancing endogenous glyoxalase pathway activity, and the specific mechanism of cellular signaling pathway modulation.

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3xTg-AD	triple-transgenic Alzheimer's disease mice
αS	alpha-synuclein
Aβ	beta amyloid
AD	Alzheimer's disease
AGE	advanced glycation endproducts
APP	amyloid precursor protein
ARE	antioxidant response element
ASD	autism spectrum disorder
ATP	adenosine triphosphate
BBB	blood brain barrier
CREB	C-AMP response element binding protein
CBC	cannabichromene
CBD	cannabidiol
CBN	cannabinol
CBG	cannabigerol
CNS	central nervous system
Cul-3	cullin-3
DBM	di-bromomorin
DLS	dynamic light scattering
DNPH	2,4-dinitrophenylhydrazine
FUdR	5-Fluoro-2'-deoxyuridine
GABA	γ-amino butyric acid
GAD65	glutamic acid decarboxylase
GCL	γ-glutamylcysteine ligase

GFP	green fluorescent protein
Glo-1	glyoxalase 1
Glo-2	glyoxalase 2
Glo-3	glyoxalase 3
GP	glyoxalase pathway
GSH	reduced glutathione
GSSG	oxidized glutathione
GST	glutathione-S-transferase
HO-1	heme oxygenase-1
IκBα	inhibitor of kappa B
IKK	inhibitor of kappa B kinase
Keap1	kelch-like ECH-associated protein 1
MAO-A	monoamine oxidase A
MAP2	microtubule-associated protein-2
MAPK	mitogen activated protein kinase
MG	methylglyoxal
MNP	morin nanoparticle
NADPH	nicotinamide adenine dinucleotide phosphate oxidase
NeuN	neuronal specific nuclear protein
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NFT	neurofibrillary tangles
NGM	nematode growth medium
NLS	nuclear localization signal
NQO1	NAD(P)H dehydrogenase quinone-1
Nrf2	nuclear factor erythroid 2-related factor 2
OS	oxidative stress

PD	Parkinson's disease
PDI	polydispersity index
PNO2	paraoxygenase-2
PPAR-γ	peroxisome proliferator-activated receptors
RAGE	Receptor for advanced glycation endproducts
RNS	reactive nitrogen species
ROS	reactive oxygen species
SAR	structure activity relationship
SAPK/JNK	stress-activated protein kinases/Jun amino-terminal kinases
SOD	superoxide dismutase
THC	Δ9-tetrahydrocannabinol
TNF-α	tumor necrosis factor alpha
VGLUT1	vesicular glutamate transporter-1

INTRODUCTION

The progression of neurodegenerative disorders is heavily influenced by the cellular microenvironment, where excessive production of reactive oxygen species (ROS) results in states of oxidative stress (OS) and inflammation.[1-8] Elevated ROS is a contributing factor to the severity and pathology of neurodegenerative disorders, and is a prominent feature in Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), multiple sclerosis (MS), and ischemia. Prolonged states of OS are a causative and underlying factor in many inflammatory diseases, however it is especially insidious in neural tissues, where the brain's high metabolic requirements render the organ highly vulnerable to damage mediated by excessive concentrations of ROS and other inflammatory metabolic byproducts.[4-7,9-14] OS results from the imbalance between pro-oxidant and antioxidant compounds, causing a sustained inflammatory response in cells and tissues.[1,6,8,10,14-18] Chronic states of inflammation irreversibly damages cells, disrupts reduction-oxidation (redox) signaling, prevents integral cellular processes, and directly impacts and influences the progression and severity of disease.[6,13,19] Retention of endogenous neural antioxidant systems is paramount to proper maintenance and function of the brain and cognitive processes, which are susceptible to the accumulation of inflammatory compounds. Ubiquitously produced metabolic byproduct methylglyoxal (MG) is a dicarbonyl, oxoaldehyde compound and highly reactive glycating agent. [20-26] Due to its highly reactive nature, it is able to spontaneously and irreversibly bind to proteins, lipids, nucleic acids, metal ions, and other cellular macromolecules forming advanced glycation endproducts (AGEs).[26-29] Compared to their unmodified counterparts, AGEs have altered chemical structures which prevents their proper functioning in cellular processes.[21,30-34] Accumulation of MG damages cellular

components through inducement of chronic inflammation and OS.[21,24,25,33,35,36] The body possesses multiple mechanisms to prevent accumulation of carbonyl compounds, but none are more important or integral in detoxification of MG than the glyoxalase pathway (GP).[1,3,20,21,26,34,37,38]

CHAPTER 1: FLAVONOIDS AS GLYOXALASE PATHWAY ENHANCING AGENTS

This chapter will provide information about the function of the GP and its role in aging and disease, and the mechanism of flavonoid mediated neuroprotection and GP enhancement. The GP is a well-conserved antioxidant defense system found in all cells of the body that facilitates the neutralization of highly reactive and oxidizing dicarbonyl molecules, with MG being the most critical target.[3,21,22,37] Through a series of reactions – involving reduced glutathione (GSH), glyoxalase 1 (Glo-1), and glyoxalase 2 (Glo-2) – MG is neutralized and detoxified into D-lactate.[1,21,22,34,37,39] (A less understood mechanism of MG detoxification utilizes glyoxalase 3 (Glo-3) to directly convert MG into D-lactate, which will be examined further in Chapter 4.) Efficient GP function is crucial in preventing ROS-mediated inflammatory cellular environments, and is implicated in a wide range of maladies including the previously mentioned neurodegenerative conditions, psychiatric and neurological disorders, diabetes mellitus, cancer, and cardiovascular diseases.[21,22,27,36,40-44] The brain is susceptible to MG accumulation due to the high rate of metabolism and low content of antioxidant systems, and is a target for MG-mediated cellular damage due to the high concentration of oxidizable substrates, including metal ions, lipids, and polyunsaturated fatty acids.[1,21,32,34,37,45-47] GP dysfunction results in accumulation and decreased neutralization of MG; conversely, accumulation of MG can be prevented through enhancement of the GP making it a valid target for pharmacological intervention.[22,38,48,49] Increasing the capacity and efficiency of the GP appears to be an effective means of reducing the onset and severity of aging and neurodegenerative disease. [22,38,48,49] Pathological hallmarks of these diseases are also shared in GP dysfunction: production of ROS and inflammatory cytokines, decreased expression of antioxidant proteins, elevated macromolecule oxidation, and apoptosis.[2,15,17,23,39]

Flavonoids are a class of polyphenol compounds and naturally occurring secondary plant metabolites, with over 5000 identified compounds possessing a wide range of biological activity. [50-52] These potent antioxidant compounds exhibit the ability to directly bind and neutralize ROS and MG, however their primary mechanism of cellular protection is enhancement of endogenous antioxidant systems - including the GP - and modulation of anti and pro-apoptotic signaling pathways.[53-56] [1,37,50,51] Compounds in this chemical family are currently being investigated as a means to prevent and mitigate OS-mediated cellular damage commonly found in neurodegenerative disorders.[1,37,54,57-63]

Glyoxalase pathway

The GP consists of a series of sequential reactions involving GSH, Glo-1, Glo-2, and a dicarbonyl substrate, often MG (**FIG 1-1**).[1,21,22,34] MG spontaneously reacts with GSH to form a hemithioacetal, which is converted by Glo-1 into an intermediate compound S,D-lactoylglutathione.[1,21,22] Glo-2 participates in the final reaction producing D-lactate and recycling GSH into the pathway.[1,21,22] The GP is a dynamic system able to respond to changes in cellular redox states, with the rate of glyoxalase activity and expression varying based on the tissue type, location, and environment of the cell.[20,38,64] The GP is ubiquitously present in cells, and in addition to antioxidant defense it is implicated in cancer cell proliferation, maintenance

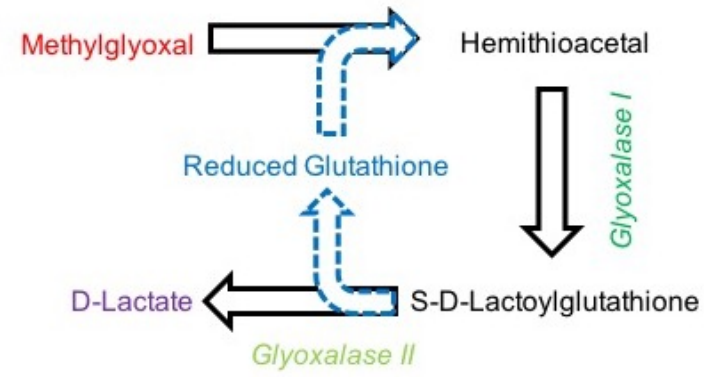

FIGURE 1-1: GLYOXALASE PATHWAY SCHEME.

Methylglyoxal spontaneously reacts with reduced glutathione forming a hemithioacetal adduct. Glyoxalase-1 uses hemithioacetal as a substrate to form S,D-lactoylglutathione. Glyoxalase-2 reacts with this intermediate compound to produce D-lactate, while also reducing glutathione to be recycled back into the pathway.

of blood glucose, liver enzymes, and cardiovascular and renal function.[3,38] While the primary function of the GP is antioxidant defense, the D-lactate produced can be converted to pyruvate and utilized as an energy substrate in the citric acid cycle.[1,22,34] In a functioning glyoxalase system MG is detoxified into D-lactate, however GP dysfunction results in decreased neutralization and accumulation of MG.[3,20,38,64] This can be caused by a reduction in GP constituent protein expression, dysregulated metabolism, or during states of disease, resulting in both elevated production of MG and a reduction in its neutralization.[21,24,25,34,64] MG is ubiquitously generated through both enzymatic and non-enzymatic mechanisms.[21,22,34] MG is generated through the metabolism of amino acids glycine, threonine, and tyrosine, protein catabolism, and lipid peroxidation.[21,65] Its primary production occurs through the glycolytic pathway, via elimination of glyceraldehyde-3-phosphate and dihydroxyacetone phosphate.[22,25,65] The glycolytic pathway is a ten step process of converting glucose into pyruvate, which is then utilized for ATP production in the citric acid cycle or Krebs cycle.[21,32,66] The D-lactate produced from the GP – also known as the glycolysis shunt - can be directly converted to pyruvate, thus bypassing glycolysis and providing energy substrates without the production of inflammatory glycolytic byproducts.[22,25,36,66] The GP directly detoxifies dicarbonyl compound and prevents their interaction with cellular macromolecules, and has an indirect influence on oxidation levels and metabolic energy production.[1,21,22,38,65]

MG is an electrophile and highly reactive glycating agent, able to irreversibly modify proteins, lipids, and nucleic acids, forming advanced glycation endproducts (AGEs).[27,30-32,65] These structural modifications to molecules can significantly reduce function, and may lead to degradation by immune cells.[21,25,30,67] AGEs influence the severity of disease pathology, with the pathological hallmarks of AD and PD are colocalized to AGEs.[30,31,33,35] MG is stable and membrane-permeable compound, capable of leaking into surrounding cells and tissues and impairing the function of cellular antioxidant systems.[21] MG is also able to modulate important signaling pathways, resulting in excessive ROS, inflammation, apoptosis, and ultimately chronic oxidative stress, leading to death of brain cells, tissues, and disease.[20,33-35,46] ROS can be directly produced during MG's formation and degradation, and MG can also deplete the concentration of antioxidant enzymes.[23,64] MG can neutralize enzymes that are able to catalyze reactions and scavenge substrates, including GSH.[23,68-70] MG can also increase NADPH oxidase activity, preventing the reduction of GSH and its recycling back into the GP.[22,23,52,69] GSH is one of the most important endogenous antioxidants involved in regulation of redox signaling, DNA and protein repair and synthesis, metal ion metabolism, and cellular survival.[68,69,71,72]

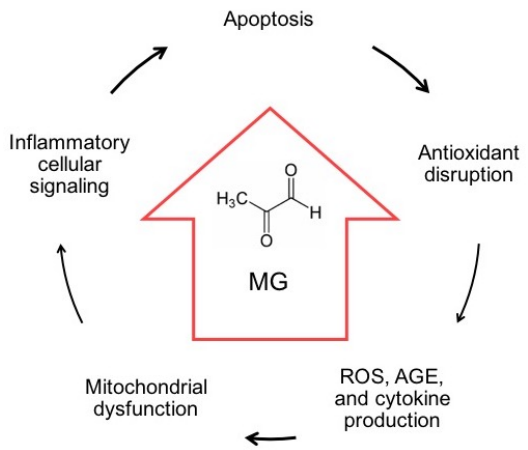

FIGURE 1-2: MG-MEDIATED INFLAMMATION IN DISEASES.

Elevated production and accumulation of MG contributes to the underlying inflammatory processes in neurodegenerative diseases, including disruption of endogenous antioxidant systems, mitochondrial dysfunction, production of ROS, AGEs, and cytokines, activation of apoptotic signaling pathways, and apoptosis.

The Glyoxalase Pathway in Neural Diseases

The GP exerts control over antioxidant mechanisms that are paramount to maintaining neural redox homeostasis and preventing the buildup of inflammatory compounds that drive ROS production.[1,21,48,57,58] While the GP is not a direct cause of neurological disorders, its impaired function can exacerbate the severity of accelerated aging, neurodegenerative disorders, and neuropsychiatric disorders (**Fig. 1-2**).[3,6,10,16,69] Accumulation of cytotoxic MG directly contributes to the elevated production of ROS, which drives and sustains apoptotic signaling pathways.[3,6,8,10,13,46,73,74] Chronic OS is a self-perpetuating cycle of damage to cells resulting from the corresponding immune response, which can be directly linked to elevated concentrations of MG.[21-24,32] Under normal cellular conditions, ROS are utilized in cell signaling and pathogen defense; during states of disease and metabolic dysfunction their unchecked production disrupts redox signaling and results in inflammation.[2,10,15] ROS and free radicals possess unpaired electrons and act as nucleophiles to target cellular macromolecules, irreversibly altering their structures and result in diminished cellular function - similar to the process of MG-mediated glycation that produces AGEs[19,75,76] Structural changes to proteins, lipids, or nucleic acids reduces the biological activity of macromolecules, and are often identified as misfolded or damaged and labeled for proteolytic degradation.[21,30,31,38] Lipids are easily oxidizable, and membranes - being rich in lipids - are prone to these modifications.[10,77,78] MG induces mutations in DNA and nucleic acids, culminating in telomere shortening, loss of heterochromatin, and altered gene expression patterns.[17,25,79] Metabolic defects and mitochondrial dysfunction can result from MG-mediated damage involving mitochondrial DNA strand breaks, mutations, and impaired repair mechanisms.[74,79,80] Mouse hippocampal neurons treated with MG exhibited altered neurogenesis, adversely impacting neural differentiation, survival, and

proliferation.[46] Mice treated with MG exhibited impaired locomotor activity, depressive-like behavior, and deficits in cognition and memory; these abnormalities were correlated to significantly decreased Glo-1 expression and dopamine levels in the prefrontal cortex.[65] While the specific mechanisms of MG in behavioral and psychiatric disorders remains to be elucidated, GP dysfunction is also implicated in psychiatric disorders including ASD, schizophrenia, anxiety, bipolar disorder, and depression.[73,81,82]

Aging

Aging is a progressive decline in physiological, structural, and metabolic functions of an organism, and decreased efficiency of homeostatic processes [3,13,15,20] While aging is a normal biological process, the presence of disease can exacerbate and accelerate the rate of aging.[15,83] The aging process is characterized by chronic, low-level inflammation which can be influenced by genetic, environmental, and pathological factors.[3,13,15,20,48,64,74,84-86] In the brain, accumulation of genetic and cellular damage occurs throughout the lifespan, resulting in functional losses in cognitive processes.[15,83] Proper and efficient functioning of endogenous antioxidant systems can reduce the severity of inflammatory processes that contribute to premature and pathological aging.[7,19,74] The GP exhibits dynamic activity in response to aging-related inflammatory insults, with glyoxalase activity differentially expressed based on age, cell type, and disease state.[64,86,87] Damage mediated by oxidation and glycation of cellular molecules steadily increases throughout aging, with a concomitant increase in the adaptive processes that offset inflammatory damage.[3,26,86] Glyoxalase activity increases during aging, however a progressive decline in Glo-1 expression occurs after age 55.[64,86]

This age-related reduction in GP efficiency results in production of dicarbonyl compounds, accumulation of AGEs, and elevated biomarkers of oxidative and inflammatory damage.[26,64,86,87]

Alzheimer's Disease

AD is the most common neurodegenerative disorder, and the leading cause of dementia in the elderly, and places a significant financial burden on the healthcare system.[6,88-90] While the underlying genetic causes for familial AD have been determined, over 90% of AD cases are classified as late onset or sporadic with no known cause for onset.[88-91] It is a multifactorial disease characterized by progressive neural loss of the hippocampus and cortex, memory and learning impairment, and changes in behavior and personality, exhibiting pathogenic hallmarks of beta amyloid (Aβ) plaques, neurofibrillary tangles (NFT), and loss of brain volume.[6,89,90,92] Cognitive impairment reflects synapse loss in dentate gyrus of hippocampus, and neuron loss in frontal and parietal lobe of cortex.[6,12,93-95] The amyloid precursor protein (APP) is a membrane protein involved in neural plasticity, synapse formation and repair, and export of metal ions.[88,89,93,96] The APP present in the brain can be cleaved by three different secretases: cleavage of APP first by α-secretase and then γ-secretase results in a functional protein; while cleavage by β-secretase results in insoluble Aβ peptides.[89,93,95] The product formed from this improper cleavage will aggregate into extracellular plaques, disrupt neural function, induce ROS production, and trigger apoptotic pathways.[6,12,93,95] Aβ plaques can be modified by MG and AGEs, forming crosslinks, affording them stability and defense against protease cleavage.[35,95] The Aβ plaques activate microglia, causing them to cluster and localize around the plaques, and release inflammatory cytokines.[77,95] The Aβ plaques cause additional production of AGEs, which reduce the activity of mitochondrial

enzymes and proteins causing increased production of ROS and improper APP cleavage.[21,30-32,35] Age is the biggest risk factor for development of AD, and is correlated with an increase in ROS formation, oxidized proteins and lipids, and apoptosis.[6,12,13] Impairment of the GP can directly impact the severity of AD, with Glo-1 and GSH expression inversely correlated to severity of disease pathology.[26,86,87] In a comparison of glyoxalase activity between healthy and AD brain tissue, the AD group had a significantly lowered Glo-1 activity and expression, at both the mRNA and protein level.[26,86,87] Tissue from AD brains exhibits high amounts of AGEs and oxidized lipids and proteins, with a positive relationship between MG and AGEs and severity of pathology.[31,97] Microtubule associated protein tau is responsible for promoting and stabilizing microtubule formation in cells, however hyperphosphorylation of tau destabilizes and disrupts the proper assembly of microtubules, resulting in intracellular NFT formation.[94,98,99] MG can also disrupt cell signaling pathways that control kinases and phosphatases used to regulate phosphorylation of tau.[33,88,97] MG derived AGEs aggregate in NFT and Aβ plaques, and Aβ plaques have an influential and contributing role in the production of NFT, and they are both stable molecules prone to glycation.[30,31,35,38] MG concentration can act as a biomarker of severity of disease, as AD patients were found to have increased MG in CSF compared to healthy aged controls.[26]

Parkinson's Disease

The second most common neurodegenerative disease, PD, is characterized by degeneration of dopamine producing neurons in the substantia nigra and deregulation in ganglion cell circuits, reducing dopamine levels in brain areas associated with movement.[5,14,79,100] The disease is characterized by motor deficiencies - including tremors, rigidity, and slowness of movement – pathological accumulation of α-synuclein (αS) into Lewy bodies, and cognitive deficiencies.[4,84,100] αS is a protein located in

presynaptic terminals of neurons that functions in recycling and storage of neurotransmitters.[5,101] Under conditions of inflammation and OS, αS proteins misfold and accumulate into aggregates which oligomerize into Lewy bodies; these aggregates are cytotoxic, disrupt connections between neurons, and deplete levels of neurotransmitters.[4,11,14,79] Accumulation of αS and Lewy bodies have a detrimental impact on mitochondria activity, causing an elevation of ROS production and deficit in metabolic activity. The degeneration of dopaminergic neurons and oxidation of dopamine causes altered mitochondrial respiration, inducing a state of OS in neural tissue.[47,79,101,102] αS also reacts with dopamine quinones leading to accumulation of toxic fibrils in the dopaminergic neurons, which have impaired activity and contribute to degeneration.[12,14,47,84] MG accumulation can lead to elevated production of ROS, oxidation of dopamine, and depletion of NADPH, which is critical for reducing glutathione for use in the GP.[5,17,71,84] Patients with PD have been found to have depleted levels of GSH, and disruption of GSH metabolism has been found to progress neurological disorders.[11,69,71,84,102] The decline in synthesis of dopamine also causes disruption in vesicle transport, and makes the cell prone to damage and mtDNA mutations.[78,102,103] There is a correlation between progression of disease and biomarkers of oxidative stress, and post-mortem studies of PD brains show high levels of oxidized substrates, and colocalization of AGEs to Lewy bodies.[7,14,17,79] AD and PD have different clinical pathologies but share similar causes and symptoms and Aβ plaques can be commonly found in PD brains.[12,13]

Autism Spectrum Disorder

ASD is a multifactorial neurodevelopmental disorder categorized by impairment in communication, language, social behaviors and relationships.[104-107] The basis for the

disease is still misunderstood, but it is known that mutations in over 100 genes can contribute to ASD, with the presence of genetic mutations in specific brain regions can affect emotional formation, learning and memory, cognitive control, and social orientation.[39,73,81,104,106,108,109] ASD exhibits mitochondria-mediated metabolic dysfunction and physical abnormalities and alterations in brain tissue.[109-112] DNA and mtDNA mutations and abnormalities are common in ASD, resulting in electron transport chain (ETC) dysfunction, low ratios of Bcl-2/Bak, elevated production of ROS, increased apoptosis, impairments in mitochondria membrane polarization, molecular transport, and protein translocation.[81,105,109,111-113] ASD is characterized by elevated OS and abnormal, chronic immune activation of microglia and astroglia which exert negative effects on the production of neurotransmitters, inflammatory cytokines, and brain specific growth factors.[73,81,110,111] Elevated levels of MG disrupted the function of GABA-producing Purkinje neurons through elevated production of ROS, high concentrations of AGEs, lipid peroxidation, and apoptosis, resulting in a disruption of the balance of inhibitory and excitatory neurotransmission.[39,43,104-106,110,114] Elevated MG concentrations in ASD brains was inversely correlated to levels of GSH; while it's unknown whether GSH deficits were due to reduced synthesis or regeneration, MG-mediated signaling pathway activation was determined to be the culprit.[43,69,72,104,108,114,115] The environment of OS present in ASD is driven in part by MG production, which could be alleviated and attenuated in a GP dependent manner.[1,39,43,69,104,108]

Flavonoid Function

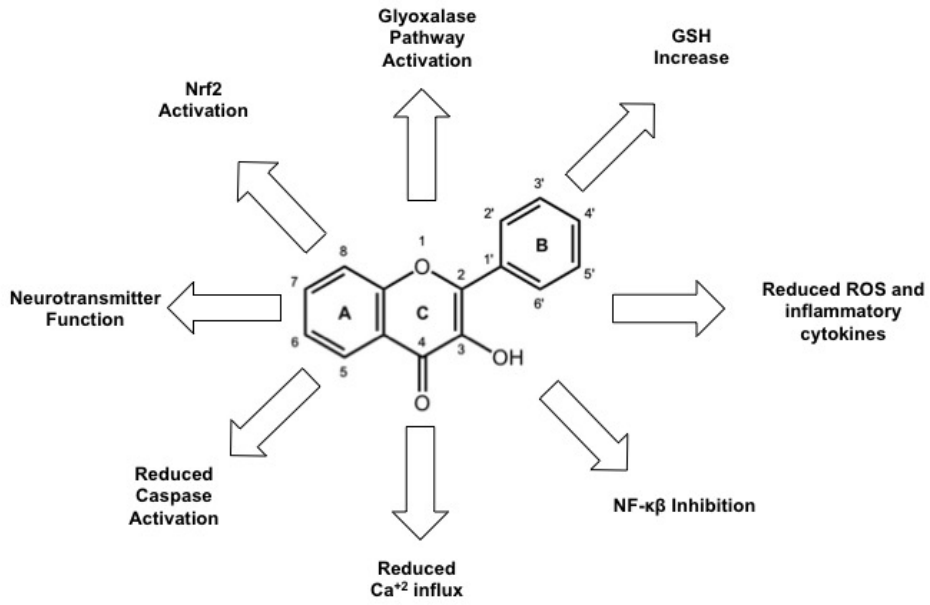

FIGURE 1-3: ANTIOXIDANT FUNCTION OF FLAVONOIDS.

Flavonols possess antioxidant and anti-inflammatory functions mediated through antioxidant action and modulation of cell signaling pathways.

Flavonoids belong to a family of polyphenol compounds and are secondary plant metabolites commonly found in fruits and vegetables, and have been found effective in combating elevated production of ROS.[1,51,116] Antioxidants are compounds - when present at a lower concentration compared oxidizable substrate - that delay or prevent oxidation of the substrate by acting as nucleophiles to prevent interaction with other molecules.[1,19,51,75,76,117] Endogenous antioxidants produced by the body function in prevention and neutralization of ROS and free radicals, repair of damaged macromolecules, and redox signaling; exogenous antioxidants consumed through food and drink also play an important role in cellular defense and survival, and have shown to aid the body in combating OS and inflammation.[15,19,51,55,75,76] Classes of flavonoids are distinguished by the presence of multiple phenol rings, C=C double bonds, and hydroxyl

groups.[1,37,118-120] These structural characteristics confer the antioxidant function of flavonoids, and the number and location of hydroxyl groups influence the biological activity of the flavonoids, and lipophilicity is also influenced by structure, which allows some flavonoids to favorably pass through the blood brain barrier.[1,37,50,51,119-121] This class of antioxidant molecules possesses several distinct mechanisms of protection from OS: flavonoids can directly scavenge and neutralize ROS and free radicals, increase intracellular GSH, prevent glutamate mediated Ca^{2+} influx, and modulation of signaling pathways involved in cellular survival.[1,51,52] Oxidative modification irreversibly changes structure and prevents normal function of macromolecules, however the presence of flavonoids is able to protect these molecules from MG mediated modification into AGEs through direct neutralization of ROS and prevents the oxidation of proteins, amino acids, lipids, metal ions, and other macromolecules.[37,51,55] Flavonoid treatments induce expression of antioxidant proteins and exhibit the ability to influence intracellular GSH concentrations, increase GSH protein expression, and increase mRNA transcript levels of both GSH constituent subunits.[68-71,114] GSH is a primary constituent of the GP, and one of the most important endogenous antioxidants for neutralization of dicarbonyl compounds and maintaining redox balance in cells.[52,68,69,71,122] GSH is also an essential substrate of astrocytic MG detoxification in the brain, and is critical for prevention of glutamate mediated apoptosis.[23-25,68,114] Elevated levels of glutamate are cytotoxic and deplete intracellular stores of GSH, leading to a decrease in activity of Glo-1 and induction of apoptosis via influx of Ca^{2+}, however flavonoids reduce intracellular Ca^{2+} influx in the presence of toxic levels of glutamate.[23,114]

Flavonoid molecules can regulate cellular, immune, and metabolic processes through modulation of cell signaling pathways including nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), mitogen activated protein kinases (MAPK), and

nuclear factor erythroid 2-related factor 2 (Nrf2).[37,123-127] Flavonoids can reduce gene expression of proapoptotic and proinflammatory products and also inhibit activation of kinases and phosphatases that contribute to apoptotic cell death.[128] ROS are used as signaling molecules during immune responses, and the presence of antioxidants can prevent ROS mediated phosphorylation of molecules and pathway targets, preventing their activation and transcription.[2,8,10,15,70] Flavonoids exhibit neuroprotective activity and can enhance the GP by modulating signaling pathways involved in cellular proliferation and survival, GSH synthesis, and expression of antioxidant proteins.[1,37,51,128]

After a flavonoid is oxidized by a free radical, the resulting quinones are involved in signaling pathways involved in cellular antioxidant and repair activities (**FIG 1-3**).[8,13,15,129] Flavonoids have shown effectiveness in modulation of GP and MG detoxification; our previous research (Chapter 2) has shown treatment with catechin, morin, and quercetin was able to attenuate the effects of MG toxicity while retaining cellular function.[1,37] The flavonoids increased Glo-1 activity and GSH concentration, while reducing the concentration of MG.[1,37] While a lack of flavonoids does not cause any disease, exogenous antioxidants can influence cellular health and offer protection against inflammatory and degenerative diseases.[18,19,75,76,117,130] A correlation exists between flavonoid consumption and low levels of dementia and neural pathology.[19,92,120,131,132] Silymarin and naringin are flavonoids that have shown efficacy in protection against excitotoxicity in dopaminergic neurons.[78,101,102,133] Silymarin protected mice against 1-methyl-4-phenylpyridinium (MPP+) induced toxicity by attenuating production of inflammatory cytokines, and prevented mitochondrial dysfunction.[78,101,102,133] Naringin protected neural cells from toxicity mediated by 6-Hydroxydopamine (6-OHDA), mediated by an increase in Nrf2 activation.[134] Morin has also shown to mitigate the damage caused by ischemia and stroke by downregulating expression and release of

proinflammatory cytokines.[135,136] A grape powder extract was shown to reduce anxiety-like behavior, depression, and memory impairments caused by elevated OS.[63]

Dietary habits are an influential factor in developing diseases, including neurological disorders, diabetes, and cardiovascular disease.[75,130] Several longitudinal, cohort studies determined an inverse relationship between flavonoid intake and all-cause mortality and cardiovascular disease associated deaths, and also determined that dietary flavonoid intake positively influenced markers of cardiovascular disease.[137] Intake of flavonoids catechin and quercetin were associated with lower all-cause mortality, and moderate to high intake of dietary flavonoids in groups with unhealthy lifestyle activities (tobacco use, alcohol consumption, obesity, and physical inactivity) significantly reduced the all-cause mortality in research subjects.[137]

Flavonoid compounds have shown tremendous potential *in vitro,* and *in vivo* with animal models, however their clinical effectiveness in humans has not exhibited the same successes.[131] Animal models utilizing flavonoid compounds as pharmacological agents are commonly administered through I.P. injection or dietary intake; however, the oral treatment concentrations are much higher than could be achieved through regular dietary intake.[131] While flavonoids are ubiquitously produced in plants, fruits, vegetables, nuts and seeds, the relative inefficiency of their breakdown and utilization by the human body results in circulatory concentrations below the therapeutic dose – no doubt a contributor to the less-than-stellar clinical results.[50,116,131] The bioavailability of flavonoids and concentrations in blood and tissue are dependent upon absorption, distribution, metabolism, and excretion, all of which are influenced by the chemical structure of the flavonoid compounds.[138] After ingestion, flavonoids are modified by enzymes in the digestive tract and liver, which can alter the flavonoids' biological functions; the breakdown and metabolism of flavonoids limits the physiological concentrations to levels

below a therapeutic threshold, preventing their efficient distribution and accumulation into critical tissues, including the brain.[50,138-140] The blood-brain-barrier (BBB) is a dynamic membrane that limits and prevents molecules in circulatory system from reaching the brain.[141] While flavonoids have lipophilic characteristics, they often exhibit the inability to permeate the BBB.[131,141,142] Extensive digestive and hepatic modifications - in addition to limited BBB permeability - contribute to the relatively low bioavailability and activity of dietary flavonoids in humans.[50,116,138] Thus, efforts have been made to improve the bioavailability of flavonoids through modification of chemical structures, and the use of enhanced drug delivery techniques including nanoparticle encapsulation (covered in further detail in Chapter 3).[50,138]

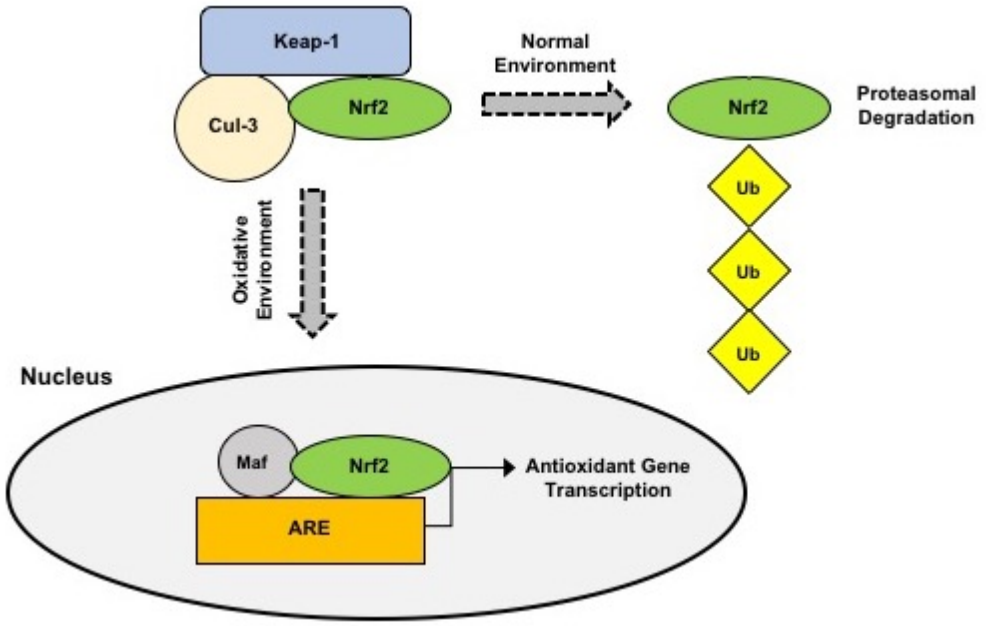

FIGURE 1-4: NRF2 SIGNALING PATHWAY.

The Nrf2 pathway is activated in response to ROS and oxidative compounds. Nrf2 is retained in the cytoplasm through interaction with Keap-1 and Cul-3. Under normal cellular environments, Nrf2 is ubiquitinated and degraded. During states of OS, ROS disrupt the Nrf2 complex and Nrf2 is translocated to the nucleus for gene transcription.

Nrf2 Activation by Flavonoids

Under states of inflammation and cytotoxicity, cells initiate protective mechanisms through activation of antioxidant signaling pathways; the Nrf2 pathway is one of the primary cytoprotective mechanisms utilized to counteract the damaging effects of OS.[56,70,129,143-146] Nrf2 function is dependent upon the cellular environment, making it highly responsive to changes in oxidative state: normal cellular conditions cause Nrf2 to be degraded in the cytoplasm, whereas oxidative conditions cause it to translocate to the nucleus.[53,56,70,144,147,148] Nrf2 forms a cytoplasmic complex with Kelch-like ECH-associated protein 1 (Keap1) and E3 ubiquitin ligase Cullin-3 (Cul-3).[53, 56, 72] Cul-3

facilitates ubiquitination and degradation, while Keap1 functions as an adaptor for both proteins, and a sensor of OS. [53, 56, 72] Under normal physiological conditions, unstimulated Nrf2 is sequestered in the cytoplasm through interaction with the Keap1-Cul3 complex.[53, 56, 72] Cul-3 is a protein in the E3 ubiquitin ligase family, and functions to ubiquitinate Nrf2 and cause it to be degraded in proteasomes.[53,56,70,144,147,148] In an oxidative environment, ROS enter the cell and phosphorylate cysteine residues located on Keap1, preventing interaction between components of the Nrf2 complex; this disruption of the Keap1-Cul-3 ubiquitination process prevents the degradation of Nrf2.[53,56,70,144,147,148] Nrf2 is released from the complex, revealing multiple nuclear localization signals and subsequently translocates to the nucleus.[70,129,144] Nrf2 heterodimerizes with transcription factor Maf, and binds to the antioxidant response element (ARE) in promoter regions of gene targets, initiating transcription of antioxidant proteins, including Glo-1, heme oxygenase-1 (HO-1), catalase (CAT), superoxide dismutase (SOD), NAD(P)H:quinone oxidoreductase 1 (NQO1), glutathione peroxidase (GPx) and enzymes involved in GSH function and synthesis, glutathione-S-transferase (GST), λ-glutamylcysteine ligase (GCL) (**FIG 1-4**).[58,132,144-147]

The Nrf2 pathway is highly responsive to changes in the redox state of cells, and is critical for mediating expression of cytoprotective proteins during states of OS.[58,129,144,145,147] Activation of Nrf2 in response to elevated MG concentrations can prevent cellular damage and cytotoxicity, and Nrf2-ARE interaction increased Glo-1 mRNA transcription, protein expression, and activity, resulting in decreases of both MG and AGEs.[70,129,143,144,147,148] Treatment with Nrf2 activators prevented apoptosis, and reduced the accumulation of levels MG-mediated protein carbonylation, while significantly elevating mRNA expression of GCL, an enzyme involved in GSH synthesis.[58,70,144,145] The observed cytoprotection against MG was a result of GSH

expression - in a GP dependent mechanism - as evidenced by the increase in D-lactate after Nrf2 activation.[1,37] Cells treated with Nrf2 inhibitor buthionine sulfoximine did not exhibit a protective effect.[1,37] Flavonoid curcumin demonstrated the ability to induce Nrf2 activation by inhibiting ubiquitination, through direct interaction with Keap1 and subsequent disruption of complex proteins.[149]

Flavonoids are able to indirectly induce Nrf2 expression by activation of kinase pathways resulting in phosphorylation of Nrf2 and induction of dependent genes.[37,135,147,150-152] Flavonoid mangiferrin upregulated Glo-1 via activating Nrf2/ARE signaling pathway.[67,123,152] Flavonoids are able to prevent ROS production and apoptosis through interaction with signaling pathways.[1-3,10,53,60,133,135] Flavonoids modulate proinflammatory signaling pathways, and can inhibit expression of TNF-α by modulating NF-κB, and reduce activation of the JNK/AP-1 pathway, inducing expression of antioxidant molecules like mitochondrial PNO_2, which exerts its neuroprotection primarily through protecting against mitochondrial-mediated OS.[129,132,144,146,151] Flavonoids were found to reduce MG, and also inhibit AGE formation by preventing formation/presence of dicarbonyl compounds, and enhance GP activity.[37,51,55,147] The GP is an integral part of the body's antioxidant system, and dysfunction can have deleterious and catastrophic events, resulting in elevated production of ROS which can further and exacerbate neurodegenerative disease.[5,45,46,48,49,69,86,97] This pathway found in all cells of our body can be responsible for production and formation of toxic intermediaries that alter a cell's normal function and contribute to the severity of aging and neurodegenerative diseases.[1,3,20,21,37,40,48,102,153] The GP is a promising drug target for inflammatory diseases, making it an attractive target for neurodegenerative disease.

CHAPTER 2: FLAVONOIDS ENHANCE NEURAL GLYOXALASE PATHWAY IN A METHYLGLYOXAL MODEL OF AGING AND ALZHEIMER'S DISEASE

INTRODUCTION

The GP involves sequential reactions – utilizing GSH, Glo-1 and Glo-2 - to prevent the buildup of MG and other carbonyl molecules, reducing the driving factors of ROS-mediated inflammation (**FIG 1-1**).[1,20-22,34,37,38] The resulting inflammation is fed by a perpetual cycle of elevated ROS production, inflammatory cytokine and chemokine signaling, and chronic immune activation.[2,8,10,13,15] Accumulation of MG results in disequilibrium of the body's redox environment, resulting in an inflammatory state of OS, known to drive the progression of neurodegenerative diseases and accelerate the aging process.[7,13,19] The GP antioxidant system is crucial in neural defense against OS and inflammation mediated by MG and other reactive dicarbonyl compounds. Pharmacological modulation of antioxidant pathways can bolster their effectiveness and prevent the accumulation and production of ROS and other inflammatory compounds.[19,75,76,117,129] Flavonoids are a class of polyphenol molecules with anti-inflammatory activity, and have emerged as viable candidates for enhancement of endogenous antioxidant systems.[1,37,51,55,122,130] This family of naturally-produced, secondary plant metabolites consists of over 4000 identified compounds, and possess a wide range of biological activity.[51,55,116] Their effects are contingent upon cell and tissue type, the surrounding microenvironment, and the composition and configuration of chemical structure.[37,51,55,116,130] For example, flavonoid treatment can increase the viability of normal cells, while also acting as inhibitors in some cancer cell lines.[55,67,123,126] Quercetin (3,3',4',5,7-pentahydroxy flavone) and morin (2',3,4',5,7-pentahydroxy flavone (**FIG 2-1**) treatments were found to be cytoprotective in primary cerebellar neurons, but promoted apoptosis in glioblastoma cells and colon cancer cells, respectively.[37,123,126]

The polyvalent, versatile activity of flavonoids and their ubiquitous production in nature has increasingly made these compounds the focus of drug related research. [28,54,61,62,147,155] The anti-inflammatory activity of some flavonoids, relatively efficient bioavailability, and ability to cross the BBB has resulted in their therapeutic use for neurodegenerative diseases.[1,9,116,154] Flavonoids' antioxidant activity allows them to directly bind and neutralize ROS, reactive nitrogen species (RNS), and metal ions, and are incredibly efficient at promoting cell viability in the presence of highly oxidizing agents, like H_2O_2 and MG.[28,54,61,62,147,155] While flavonoids can directly reduce ROS and free radicals, the mechanism of their protective function is mediated through modulation of antioxidant and apoptotic signaling pathways, chiefly Nrf2 and NF-κB.[58,62,123,127,143,144,146,147,152,156-161] MG plays a role in the progression of AD, and

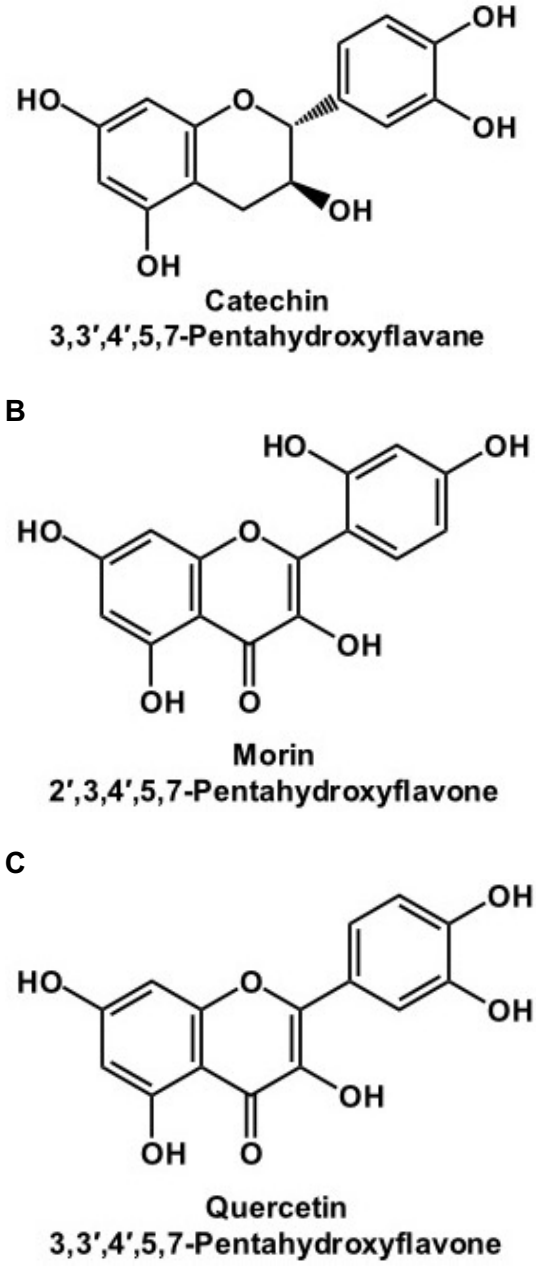

FIGURE 2-1: FLAVONOID STRUCTURES.

The chemical structures of flavanol catechin (**A**), and flavonols morin (**B**), and quercetin (**C**).

negatively contributes to disease pathological hallmarks Aβ, NFTs, and loss of neuronal viability.[26,32,33,35,87,97,162]

Quercetin is a widely distributed and common polyphenol flavonoid (found in high concentrations in capers, sorrel, radishes, red onions, and kale), with a wide range of bioactivity, and exhibits anti-inflammatory, anti-viral, anti-cancer activity, and has found to be cytoprotective in cells of the liver, kidneys, heart, and brain.[28,163,164] Its neuroprotective effects have been documented in a variety of disorders, including HD, MS, diabetes, PD, and AD, and has been shown to protect against cognitive deficits.[9,164,165] Quercetin was shown to be neuroprotective – through a GP-mediated mechanism - in a rat model of streptozotocin-induced diabetic cognitive decline.[57] Quercetin treatment reduced levels of blood glucose, and inflammatory signaling molecules COX-2, IL-1β, and TNF-α, while elevating antioxidant activity of superoxide dismutase (SOD).[57] Quercetin was found to directly modulate the GP by increasing Glo-1 activity and GSH levels, decreasing AGEs, and reducing expression of RAGE.[57] Quercetin has shown potent neuroprotective activity in AD.[9,53,124,166] Mouse hippocampal neurons insulted with Aβ and H_2O_2 showed markedly reduced neurotoxicity and ROS production when treated with quercetin.[167] It also prevented metabolic dysfunction by protecting mitochondrial membrane integrity and morphology.[167] Transgenic AD mice treated with quercetin exhibited attenuated disease pathology and neurobiological deficits.[166] Histological analysis of brain tissue from triple-transgenic AD mice (3xTg-AD) revealed a retention of cell morphology and cell density.[166] Quercetin treatment was remarkably efficacious against pathological hallmarks of AD, reducing levels of both Aβ and NFTs in the cerebellum and hippocampus of 3xTg-AD mice.[166] The neuroprotective effect of quercetin was evident through a reduction in activated astrocytes and microglia, mediators of neuroinflammation in neurodegenerative diseases.[166] The functional results

of these reduced pathologies were improvements in spatial learning, memory, and anxiety-like behavior.[166] Quercetin protected mouse hippocampal neuron morphology and viability in an okadaic acid-mediated OS model of AD.[124] The anti-inflammatory effects of quercetin were evidenced by an elevation in GSH and SOD, reductions of malondialdehyde (a marker of glycation) and ROS, retention of mitochondrial membrane permeability, and reduced caspase-mediated apoptosis.[124] Hyperphosphorylation of tau – the initiating factor in NFT formation – was significantly suppressed in neurons treated with quercetin.[124] Apoptotic signaling pathways commonly associated with AD – Akt, GSK-3β, and MAPK – were inhibited by quercetin treatment.[124] Hypoxia-induced mitochondrial dysfunction in rats was prevented by quercetin supplementation, with quercetin treated animals exhibiting drastically reduced memory impairments compared to the control cohorts.[159] Acrylamide is known neurotoxin and induces OS through the same mechanism as MG, the generation of reactive carbonyls through the Maillard reaction.[168] Quercetin treatment in rats insulted with acrylamide exhibited normalized expression of neurotransmitters serotonin and dopamine, and reduced concentrations 8-hydroxy-2-deoxyguanosine (8-OHdG), a marker of OS-mediated DNA damage.[168]

We hypothesize that treatment of neurons with exogenous antioxidant compounds reduces the oxidative burden of MG by enhancing the GP. To test this hypothesis, we utilized three well-known flavonoid compounds – flavanol catechin and flavonols morin and quercetin – and investigated their effects on GP function in mouse primary cerebellar neurons induced to a state of OS with MG. The purpose of this study is to provide evidence of flavonoids' ability to enhance the GP and elucidate the functional mechanisms. Through evaluation of GP substituents, signaling pathway activation, apoptosis, and neurotransmitter function, we determined all three flavonoids to be neuroprotective in an MG-mediated OS model of AD.[37] The specific mechanisms of

protection involved: Nrf2-mediated upregulation of Glo-1, Glo-2, GSH, and MG detoxification, retention of excitatory and inhibitory neurotransmission, reduction of caspase-mediated apoptosis, and inhibition of NF-κB signaling.[37]

MATERIALS AND METHODS

Care and use of animals

Animal studies were approved and performed in accordance with the UNMC Institutional Review Board (IRB) and Institutional Animal Care and Utilization Committee (IACUC). C57BL/6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME).

Chemicals and compounds

Morin was purchased from MP Biomedicals (Solon, OH). Quercetin dehydrate was purchased from Pfaltz & Bauer, (Waterbury, CT). Sodium D-lactate was purchased from Santa Cruz Biotechnology (Dallas, TX). Lactate dehydrogenase was purchased from US Biological (Salem, MA). Catechin Hydrate, Poly-D-lysine hydrobromide, β-nicotinamide adenine dinucleotide hydrate, methylglyoxal, and 2,4-Dinitrophenylhydrazine were purchased from Sigma Aldrich (St. Louis, MO). Unless otherwise noted, chemicals for this study were purchased from Thermo Fisher Scientific (Fair Lawn, NJ).

Cerebellar neuron culture

Primary cultures of cerebellar neurons were prepared from P3-5 C57/BL6 mice. Six well culture plates (Falcon, Indianapolis, IN) were coated with poly-D-lysine and seeded with cerebellar neurons at a density of 1.5×10^6 cells/well, and maintained with DMEM media supplemented with L-glutamine, pen-strep, 30% sucrose, B-27, and N-2. Ara-C was added 24 hours after seeding to produce a homogenous neuronal culture. Cells were grown to confluence for 6 days, with half media changes every 48 hours. The negative control received no treatment, the vehicle was treated with 500 μM MG. Antioxidant treatment groups were treated for 24 hours by adding 500 μM MG first and followed by 10 μM flavonoid.[37]

Western Blot Analysis.

Cerebellar neuron cultures were washed with ice cold PBS three times and lysed using RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X100, 1% Na deoxycholate 0.1% SDS, 1 mM EDTA) and a protease inhibitor cocktail (Thermo-Fisher). A BCA assay (Pierce) was used to ensure equal protein loading content. Samples were added to a loading buffer (LDS sample buffer 4X, β-mercaptoethanol, PBS) and denatured at 90 °C for 5 minutes. Proteins were resolved on a 4–20% tris-glycine gel (Bio-Rad) using SDS-PAGE, and transferred to a PVDF membrane (Immobilon). Membranes were blocked for 1 h in TBST and 5% fat free milk (BioRad), washed in TBST, and incubated overnight at 4 °C with antibodies directed against Glo-1 (Rabbit polyclonal, Santa Cruz), Glo-2 (Goat polyclonal, Santa Cruz), and p-Akt (Rabbit polyclonal, Cell signaling). Blots were washed and incubated with the appropriate HRP-conjugated secondary antibody (Santa Cruz). β-actin expression (Rabbit monoclonal, Cell Signaling) was assessed to ensure equal protein content.[37]

Determination of D-lactate concentration

Media samples from cerebellar cultures were collected prior to lysing the cells. D-Lactate released into the extracellular space following treatment with MG and antioxidant was measured spectrophotometrically. Culture media samples of 70 µL were loaded on a 96-well plate with 180 µL of glycine buffer (0.2 M glycine, 0.2 M semicarbazide, pH 10), containing 2 mg/mL NAD and 5 U/mL D-lactate dehydrogenase. Samples were incubated at room temperature for 2 hours. A spectrophotometer (340 nm excitation, 450 nm emission) was used to measure conversion of NAD to NADH. Absolute values were determined from a standard curve of D-Lactate concentrations.[34]

Determination of MG concentration

A 10 mM stock solution of 2,4-DNPH (Sigma-Aldrich, St. Louis, MO, USA) in 100% ethanol was used to create a working solution of 0.2 mM 2,4-DNPH with 12 mL HCl

(36%) per 100 mL ethanol. A working solution of 1 mM MG (Sigma) was prepared from a stock solution. The reaction consisted of 950 µL 0.2 mM 2,4-DNPH with different volumes of 1 mM MG, filled to 1 mL with media for the blank, or 50 mL of media from cerebellar neuron culture. The samples and blanks were heated in an Eppendorf Thermomixer at 42 °C for 45 mins and 600 rpm, and were incubated at room temperature for 5 minutes. Spectrophotometer measurements were performed at 432 nm, according to absorbance of MG-bis- 2,4-DNPH-hydrazone for calculating concentration of MG.[169]

Glo-1 activity

Neurons were plated in 12 well plates at 5×10^5, and grown to maturity at 6 days. Cells were treated with 500 µM MG and 10 µM antioxidant for 24 hours. Media was removed, and cells were rinsed with ice cold PBS. Cells were lysed with buffer containing: 10 mM HEPES (pH 7.0), 0.02% Triton X-100, and 100 µg/ mL BSA. Samples were briefly sonicated and centrifuged at 16,000 g for 30 minutes at 4 °C. Cellular lysates were added to a 96 well plate at 50 µL per well. Reaction mix consisted of 60 mM sodium phosphate pH 6.6 containing 4 mM GSH and 4 mM MG. Two hundred µL reaction mix was added to the 96 well plate and incubated for 10 minutes at 37 °C, and 50 µL of sample lysate was added to the plate. S-lactoylglutathione synthesis was determined by measuring absorbance at 240 nm every 15 seconds for 5 minutes. Protein concentration was determined using a BCA protein assay reagent kit (Thermo Scientific).[37]

Determination of GSH concentration

Total intracellular glutathione levels were determined spectrophotometrically. Levels of total glutathione and oxidized glutathione (GSSG) were determined and used to calculate levels of GSH. Values were calculated from a standard curve of GSSG.[37]

Immunocytochemistry

Primary cerebellar neurons were harvested from P5 C57/BL6 mice. Neurons were plated in 8 well imaging plates at 300,000 cells/well. After growing for 6 days, cells were treated with 500 μM MG and 10 μM flavonoids for 24 hours. Media was removed and cells were fixed with 4% PFA/30% sucrose solution in PBS for 10 mins. Wells were washed with PBS (0.1% Triton 100-X) in PBS for 10 mins. Wells were again washed with PBS, and blocked with 5% BSA in PBS at room temperature for 1 hour. Primary antibodies for NeuN (mouse monoclonal) and cleaved caspase-3 (mouse monoclonal), and VGLUT1 (guinea pig polyclonal) and GAD65 (rabbit monoclonal) were added in 1% BSA in PBS overnight. Wells were washed with PBS, and incubated with the appropriate IgG (H + L) conjugated secondary antibodies at room temperature for 1 hour. Wells were washed, and covered with DAPI stain for 30 seconds. DAPI stained cells were washed and aspirated, and Prolong Gold Antifade (Thermo Fisher Scientific, MO) was added directly to each well. Plates were imaged on a confocal microscope (LSM 710 Zeiss Confocal Microscope) at 20-40x.[37]

Statistics

Statistics were performed using Excel (Microsoft) and SPSS (IBM Corporation). Data were expressed as the mean +/− SEM of multiple experiments performed in triplicate. Statistical differences between means were analyzed using a student's t-test or one-way analysis of variance (ANOVA). Data with at least $p<0.05$ was considered statistically significant.[37]

RESULTS

MG and Flavonoid Cytotoxicity

Accumulation of MG is commonly present during aging and in neurodegenerative disorders, and mediates an inflammatory cellular environment.[24,25,64] In this study, we utilized MG as a means to induce the OS that contributes to the pathogenesis of AD. To determine the appropriate concentration of MG for induction of OS, we treated cerebellar neuron cultures with a range of MG concentrations to determine the most appropriate concentration for induction of OS. Through an MTT assay, we determined 500 μM was the optimal concentration to induce OS without excessive cytotoxicity.[37]

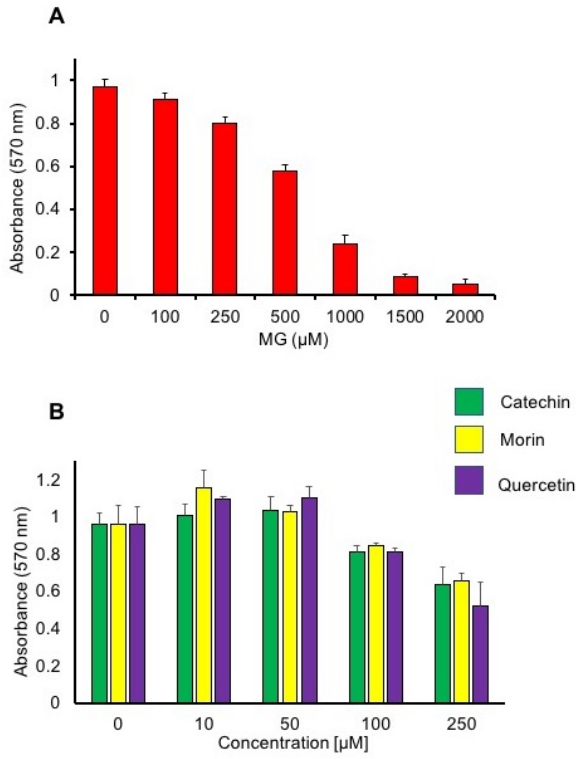

FIGURE 2-2: CYTOTOXICITY OF MG AND FLAVONOIDS.

Determination of treatment dosage level for cerebellar neurons was determined through an MTT assay using various concentrations of MG (**A**) and flavonoids (**B**).

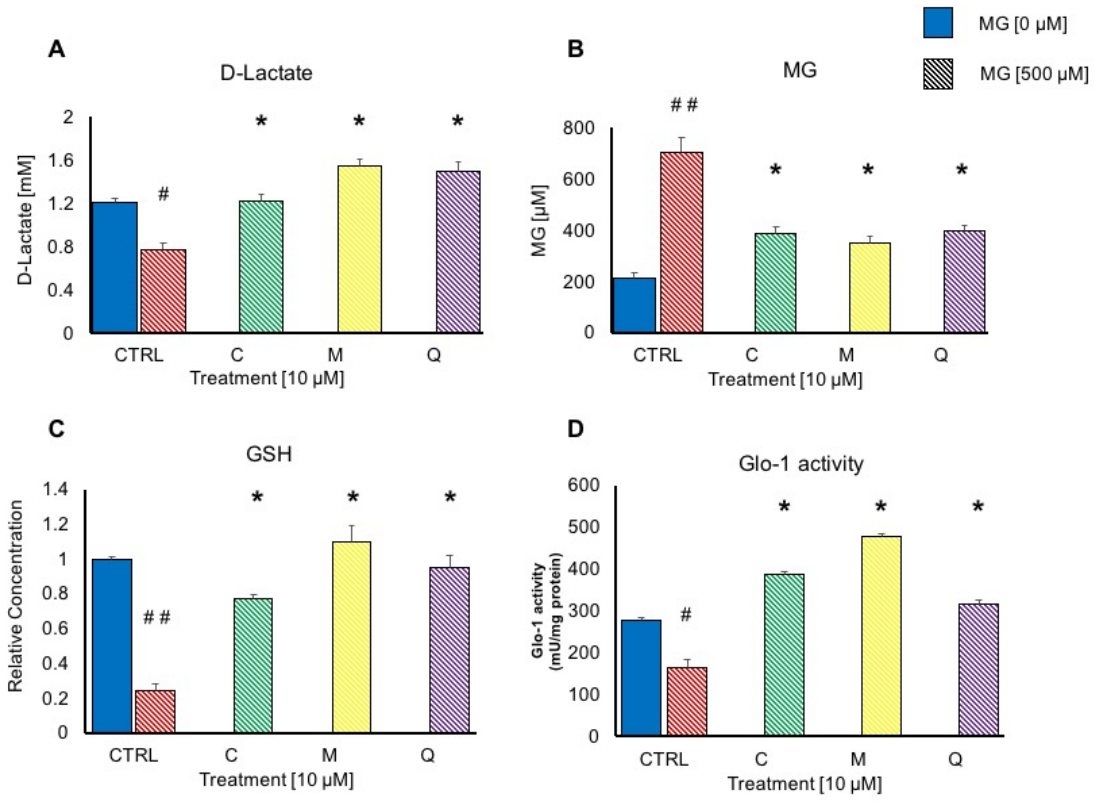

FIGURE 2-3: ANALYSIS OF GP FUNCTION.

Cultured cerebellar neurons were induced to a state of OS with MG [500 µM], treated with flavonoids [10 µM] (Control = no MG, Vehicle = only MG) and markers of GP function were measured. (**A**) D-lactate release into extracellular space #P < 0.05 (vs Control), *P < 0.05 (vs Vehicle). (**B**) Concentration of MG [µM/mL] #P < 0.05 (vs Control), *P < 0.05 (vs Vehicle). (**C**) Concentration of GSH ##P < 0.01 (vs Control), *P < 0.05 (vs Vehicle), **P < 0.01 (vs Vehicle). (**D**) Glo-1 activity #P < 0.05 (vs Control), *P < 0.05 (vs Vehicle). Values are means +/− standard error of samples performed in triplicate from several independent experiments.

Flavonoids Increase MG Detoxification

GP function can be assessed by measuring the concentration and activity of substituent proteins. The concentration of D-lactate in the extracellular space provides a means to measure the detoxification of MG by the GP. D-lactate concentration was determined by spectrophotometrically measuring the conversion of NAD to NADH. To induce a state of OS, cerebellar neuron cultures were insulted with MG [500 µM] for 24 hours, and treated with flavonoids [10 µM] to determine its effect on GP function. Catechin, morin, and

quercetin treated cerebellar neurons had a significantly higher concentration of D-lactate than the MG treated control (**FIG 2-3A**). The media from MG treated cells contained 0.7 mM of D-lactate, while the flavonoid treated groups had D-lactate concentrations in excess of 1 mM. The increase in D-lactate concentration in the presence of flavonoids indicates that the GP is enhanced and MG has been detoxified in high amount. The reaction of MG with 2,4-DNPH produces dinitrophenylhydrazone, which was measured spectrophotometrically to determine the MG content. Catechin, morin, and quercetin were able to significantly decrease the concentration of MG compared to the MG-treated control (**FIG 2-3B**). Flavonoid treatment attenuated MG accumulation, with MG concentration in flavonoid treated cells similar to levels of the non-treated control. This result matches when correlated with increase in lactate concentration.

Flavonoids Increase GSH Concentration and Glo-1 Activity

GSH is a protein utilized for antioxidant defense. In the GP, it catalyzes the first step in the conversion of MG to D-lactate. High concentrations of MG deplete the intracellular concentration of GSH, preventing efficient functioning of the GP. MG treatment drastically decreased the concentration of GSH in neural cell cultures, however this effect was prevented significantly by flavonoid treatments (**FIG 2-3C**). Catechin resulted in a significant increase in GSH, and morin and quercetin were able to increase GSH levels to that of the untreated control. The glyoxalase activity assay evaluated the capacity of the cerebellar neurons to detoxify MG. Glyoxalase activity measures the mU/mg protein of Glo-1 needed to catalyze the conversion of the GSH-MG hemithioacetal into S-D-lactoylglutathione. MG treatment of 500 μM significantly reduced the activity of Glo-1 in cerebellar neurons, while flavonoid treatment decreased the Glo-1 activity inhibition caused by MG (**FIG 2-3D**). Catechin, morin, and quercetin were found to significantly increase Glo-1 activity compared to the MG treated control.

Flavonoids Prevent Cleaved Caspase-Mediated Apoptosis

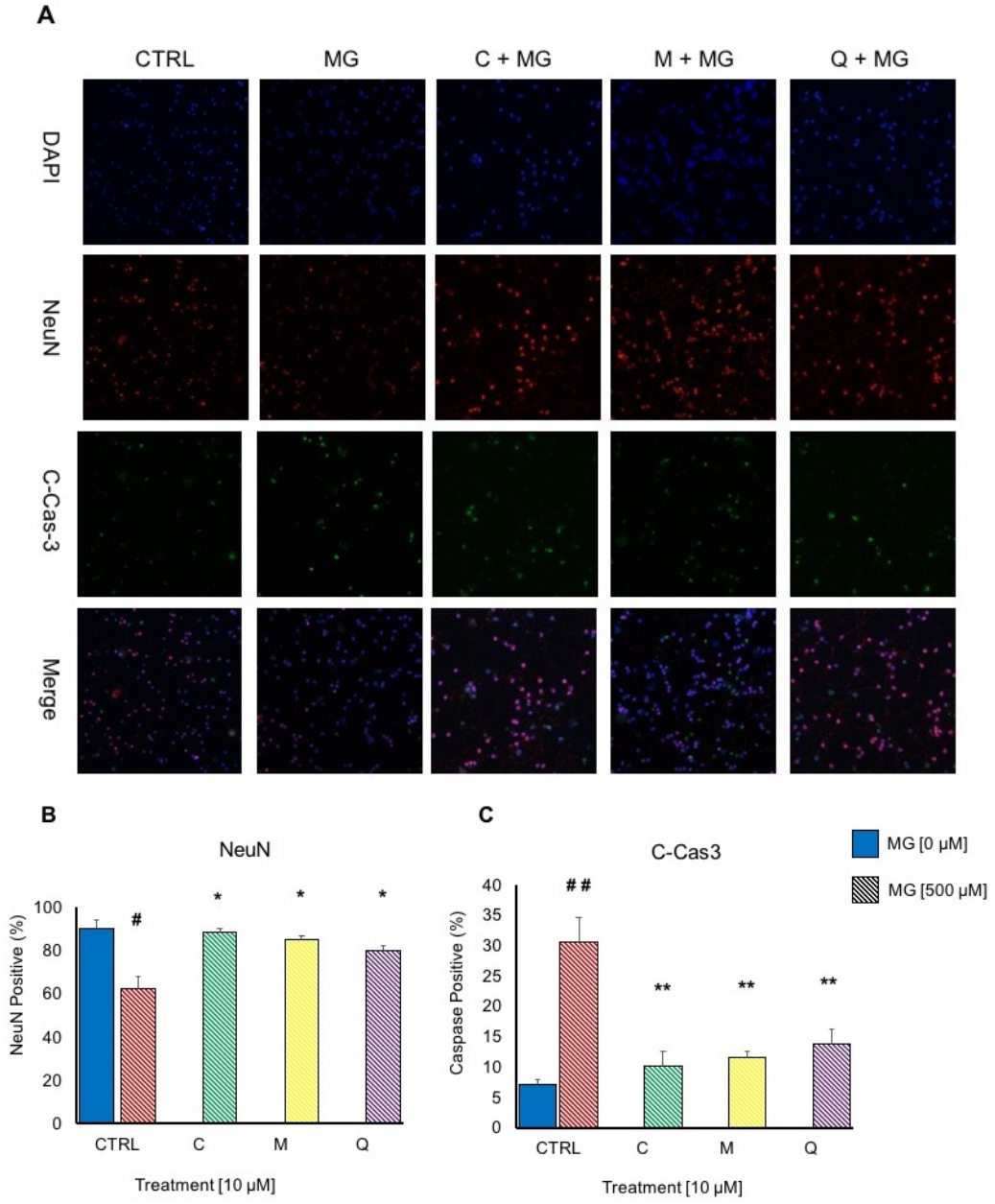

FIGURE 2-4: EFFECTS OF FLAVONOID TREATMENT ON CELL VIABILITY AND APOPTOSIS.

Cerebellar neurons were treated with flavonoids [10 µM] and MG [500 µM]. Data was determined via immunocytochemistry (A). Viability was assessed through detection of cleaved caspase-3 (C-Cas-3) (B) and neuronal specific nuclear protein (NeuN) (C). Control = no MG, Vehicle = only MG. Values are means +/− SEM of samples performed in triplicate from several independent experiments, n = 3–4. #P < 0.05 (vs Control), *P < 0.05 (vs Vehicle). Images were quantified with ImageJ, and resulting data were analyzed using an ANOVA. Morin treatment significantly decreased the presence of apoptosis, and catechin and morin significantly increased cell viability.

MG promotes the activation and subsequent cleavage of caspase-3 (Cas-3), which is a regulator of apoptosis and triggers cell death through a mitochondrial-mediated pathway.[34,46] Cerebellar neuron cultures underwent a 24 hour treatment with MG and flavonoids, and were fixed for immunocytochemistry. Cells were conjugated with antibodies directed towards cleaved caspase-3 (C-Cas-3) and neuronal specific nuclear protein (NeuN) to determine apoptosis and survival, respectively. Relative fluorescence was determined through confocal microscopy imaging. MG treatment significantly increased the amount of Cas-3 positive cells, and decreased the amount of NeuN positive cells compared to the non-treated control. This increase in apoptotic cells was prevented by flavonoid treatment. A significant decrease in the amount of Cas-3 positive cells was found in the catechin, morin, and quercetin treated conditions (**FIG 2-4B**). A significant increase in the amount of NeuN positive cells compared to the MG-treated control was found in the catechin and morin treated conditions (**FIG 2-4C**). These results suggest flavonoid treatments increase survivability of cerebellar neurons in the presence of cytotoxic MG.

Flavonoids Protect Excitatory and Inhibitory Neurotransmission

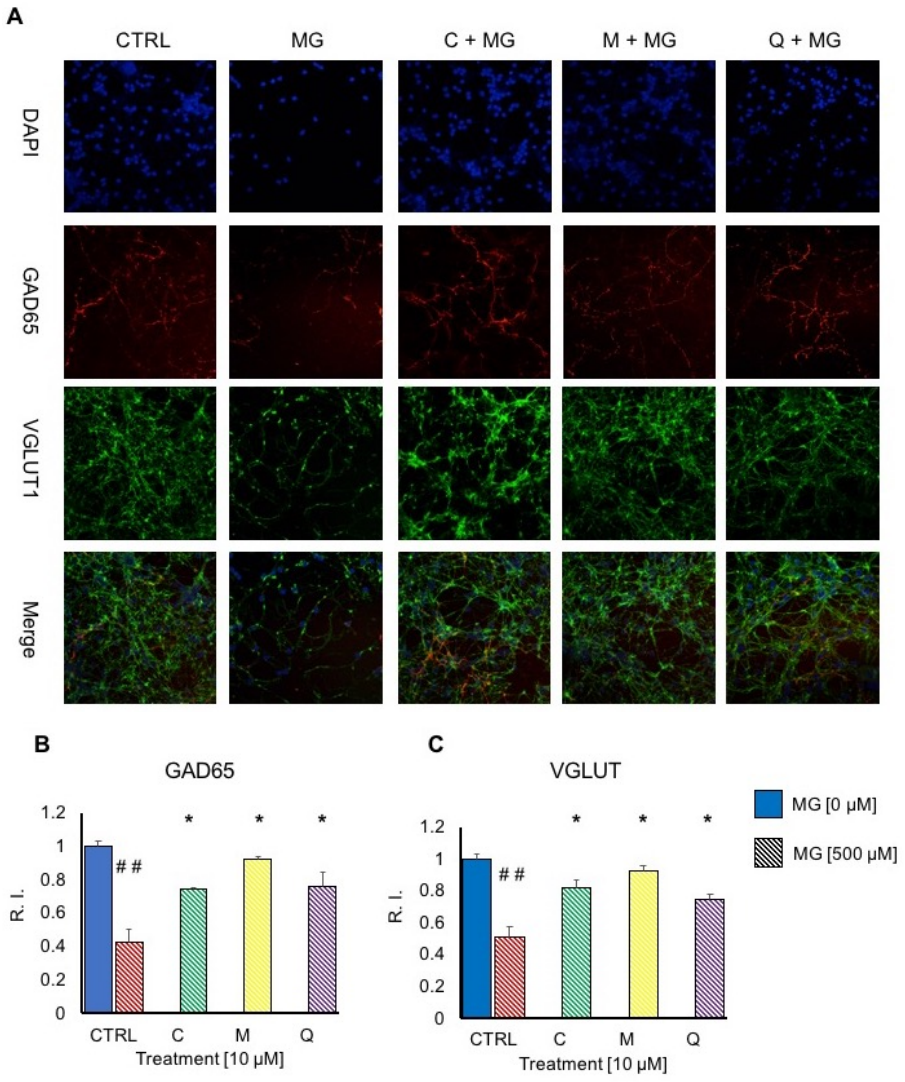

FIGURE 2-5: FLAVONOID TREATMENT ON EXCITATORY AND INHIBITORY SYNAPTIC FUNCTIONS.

Functions were assessed through immunocytochemistry. Representative images showing DAPI, VGLUT1, and GAD65 in flavonoid (10 μM) and MG (500 μM) treated cerebellar neurons (A). Excitatory and inhibitory synapse function was assessed through detection of VGLUT1 (B) and GAD65 (C). Control = no MG, Vehicle = only MG. Values are means +/− SEM of samples performed in triplicate from several independent experiments, n = 3–4. #P < 0.05 (vs Control), *P < 0.05 (vs Vehicle).

Glutamate and γ-amino butyric acid (GABA) are the principal excitatory and inhibitory neurotransmitters (respectively) utilized by neurons for cell-to-cell communication and signal propagation; the balance of both excitatory and inhibitory neurotransmission is

critical for neurological function and environmental homeostasis.[170] Excitatory neurotransmission was determined through evaluation of vesicular glutamate transporter-1 (VGLUT), involved in transport of glutamate into the synaptic cleft; inhibitory neurotransmission was determined through evaluation of glutamic acid decarboxylase-65 (GAD65), involved in conversion of glutamate to GABA.[170] The expression of both GAD65 (**FIG 2-5B**) and VGLUT1 (**FIG 2-5C**) in DAPI-positive cells were significantly reduced with the addition of MG compared to the control, indicative of drastic deficits in neurotransmission. However, MG insulted cells treated with catechin, morin, or quercetin exhibited significantly higher expression of GAD65 and VGLUT1 in DAPI-positive cells compared to the MG treated cells alone. Flavonoids prevented MG-mediated decreases in neurotransmission, and neurons retained both excitatory and inhibitory neurotransmitter function. While all tested flavonoids significantly elevated neurotransmitter function in the presence of MG, morin treatment resulted in expression levels similar to the control group.

Flavonoids Increase Glo-1 and Glo-2 Expression and Prevent Akt Phosphorylation

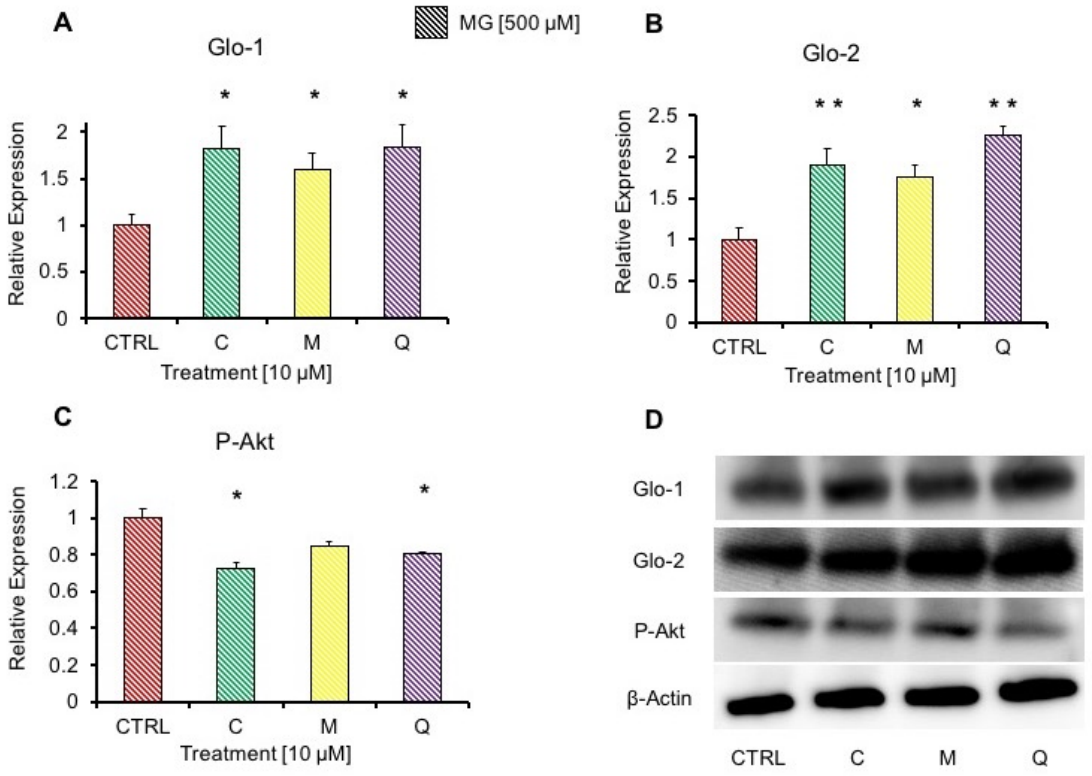

FIGURE 2-6: FLAVONOID TREATMENT ON GLYOXALASE PROTEIN EXPRESSION.

Proteins Glo-1 (B), Glo-2 (C), and P-Akt (D). Representative Western Blot images (A). Values are means +/− SEM of samples performed in triplicate from several independent experiments, n = 3–4. *P < 0.05 (vs Control). Expression of P-Akt in cerebellar neurons was assessed via Western Blotting. Cerebellar neurons were treated with MG (500 μM) and flavonoids (10 μM). Blots were conjugated with β-actin to ensure equal protein loading. Control = only MG. Values were determined using ImageJ. Resulting data were analyzed using an ANOVA. A significant decrease (*p < 0.05) in P-Akt was found in the catechin and quercetin treated groups (10 μM) compared to the MG treated control.

The efficiency of the GP to detoxify MG is predicated upon the presence and activity of Glo-1 and Glo-2 proteins, with positive correlations between protein expression and MG detoxification.[1,20-22,86] Protein expression of Glo-1, Glo-2, and P-Akt in cerebellar neurons was determined through western blotting (**FIG 2-6D**). Flavonoid treatment in MG-insulted cells significantly increased the expression of both Glo-1 (**FIG 2-6A**) and Glo-2 (**FIG 2-6B**) compared to cells treated only with MG.

The PI3K-Akt pathway influences NF-κB signaling, with activation of Akt (protein kinase B) causing phosphorylation of IKKα, degradation of IκB, and subsequent NF-κB nuclear translocation.[124,126] MG treatment resulted in elevated expression of P-Akt, which was abolished in neurons also treated with flavonoids treatment in MG-insulted cells reduced P-Akt (**FIG 2-6C**). Catechin and quercetin treatments in MG-insulted neurons significantly reduced phosphorylation of Akt compared to the MG treated cells alone.

DISCUSSION

This study investigated the effects of flavonoids on GP function. Here we provide evidence that catechin, morin, and quercetin can enhance the GP and attenuate MG-mediated toxicity in neurons.[1,37] Our *in vitro* experiments utilized primary cerebellar neurons insulted with MG to recreate the cellular environment commonly found in pathological aging, AD, and neurodegenerative diseases.[13,33,35,60,77,92,171] MG accumulation and GP dysfunction are implicated in accelerated aging of neural cells and progression of AD pathology, and attenuating the driving factors of ROS-mediated inflammation can reduce disease severity.[26,33-35,64,87,97,162] At relatively low concentrations, flavonoid treatments had robust influences on GP function, nearly every aspect of the GP was improved with flavonoid treatments: elevated expression of Glo-1 and Glo-2 proteins, increased concentrations of GSH, elevated Glo-1 activity, and increased detoxification of MG to D-lactate.[37] Even under conditions of extreme cytotoxicity, flavonoids exhibited the ability to attenuate MG-mediated cellular damages. Cerebellar neurons increased the rate of their endogenous antioxidant system through flavonoid supplementation. In accordance with this, we observed a decreased concentration of MG in the media. Reducing the concentration of MG will reduce the production of ROS, proinflammatory cytokines, AGEs, and oxidized molecules, which provoke a chronic immune response and directly lead to conditions of OS.[21,31-35] Reducing the oxidative burden on the GP allows its antioxidant functions to be more efficient and prevent the exponential rise of toxic molecules in the cellular milieu. Reduced expression of the constituent pathway proteins has been linked to premature aging, and increased severity of neurodegenerative disease.[3,20,21,64,86,87] Increased expression of glyoxalase proteins has been shown to lower formation of ROS and free radicals, and elevated levels corresponded to a decrease in concentration of α-

oxoaldehydes.[38,39,57,58,172] MG detoxification requires the presence of both Glo-1 and Glo-2, and a decrease in either pathway protein would prevent efficient pathway function and have a dramatic negative impact on neuronal viability.[3,20,21,38,48,58] MG-induced toxicity can alter synaptic morphology through disruption of uptake and release of neurotransmitters, negatively impacting the neural communication and signaling.[10,170] Flavonoid treated cerebellar neurons were able to effectively retain the structure and function of both excitatory and inhibitory synapses.[37] Accumulation of glutamate in the synapses is a trigger for a cellular influx of Ca^{+2}, which initiates an apoptotic signaling cascade.[11,55,116,136] We found flavonoid treatment positively increased markers of glutamate and GABA receptor function in the presence of MG.[37] These neurons were able to more efficiently convert glutamate to GABA, and clear glutamate from the synaptic cleft. Flavonoids also had a significant impact on cell viability by decreasing the activated form of caspase-3. Cleavage of this protein initiates mitochondrial mediated apoptosis, releasing other pro-apoptotic proteins and resulting in destruction of the mitochondria and death of the cell.[11,17,135,173] NF-κB signaling is initiated in response to cellular damage, inflammation, and cytokine release, which can be directly and indirectly influenced by MG concentrations.[123,152,161] The NF-κB heterodimer has two subunits (p65, p50) and is sequestered in the cytoplasm when bound to its inhibitory subunit, IκBα.[123,129,152,174] Phosphorylation degrades the inhibitory subunit - IKKβ - and NF-κB is translocated to the nucleus for rapid initiation of apoptotic gene transcription. [123,129,152,174] MG and ROS mediate inhibitory subunit phosphorylation, thus regulating the transcription of pro-apoptotic protein products. [123,129,152,174] Decreasing MG and ROS in the cell directly reduces the amounts of molecules able to modulate and activate this signaling pathway.[123,174,175] Flavonoids were found to modulate NF-κB signaling through a reduction in phosphorylation of Akt.[51,123,152,161] Reduced P-Akt prevents the activation

and nuclear translocation of NF-κB, causing it to undergo cytoplasmic degradation.[51,123,152,161]

We observed that flavonoids offered protection against MG-mediated OS in cerebellar neurons, preventing progression of neurodegenerative disorders. These compounds offer an attractive solution against ROS mediated damage to cells, as they have very low cytotoxicity, and function to increase the efficiency of the GP. Proper function of the GP decreases the amounts of ROS, free radicals, and damaging reactive compounds, and allows for retention of neuronal function. Based on the results of this study, flavonoids compounds may prove to be an effective treatment for GP-mediated prevention of aging and neurodegenerative disease.

CHAPTER 3: EFFECT OF MORIN DERIVATIVES ON NEURAL GLYOXALASE PATHWAY

INTRODUCTION

The brain's high metabolic requirements combined with limited antioxidant capacity render the organ highly vulnerable to ROS-mediated by damage. [3,6,12,13,19] Ubiquitously produced MG is highly reactive and alters the structure and function of proteins, lipids, and nucleic acids through formation of AGEs. [5-17] The GP performs indispensable functions in the brain by neutralizing and preventing accumulation of MG and producing an alternate energy source for neurons.[22,34,64] Increasing the activity of the GP can prevent elevated levels of MG in neural cells.[1,37,57,58] Thus, the GP is a valid target for pharmacological intervention in AD and other neurodegenerative disorders, and plant-produced compounds have been widely investigated and utilized for their biological activity.[19,116] Morin is a flavonoid present in a variety of fruits, vegetables, and nuts, with high concentrations found in plants in the moraceae family (e.g. mulberry and fig). [123,127,133,147,152,161] Morin exhibits antioxidant, anti-inflammatory, and anti-cancer activity, mediated through modulation of cellular signaling pathways .[7,20,24,26] Morin has previously been characterized as cytoprotective in liver, pancreas, lung, cardiac, and neural cells.[20-24] Evidence shows morin possesses protective effects in neurodegenerative conditions including ischemia, MS, PD, and AD. [1,37,55,123,133,135,136,151,152,158,161,173,176] Morin exerted its control over the NF-κB signaling pathway by attenuating TNF-α induced activation by inhibiting degradation of IκBα, and was also able to inhibit TNF-α mediated p65 nuclear translocation.[123,127,152,161,177] Morin was shown to decrease survival of cancer cells, while increasing viability of normal endothelial cells, for example morin inhibited phosphorylation of Akt in a breast cancer cell line, preventing metastases and tumor proliferation.[174,177]

While flavonoid treatments have shown efficacy against AD *in vitro* and animal models *in vivo*, no substantial benefit has been exhibited in clinical trials in AD patients.[9,116,131] The

ineffective results stem from flavonoids not reaching critical areas of brain tissue, which could be due to premature metabolic degradation, and poor permeability through the blood brain barrier (BBB).[131] Plant compounds are commonly utilized in drug design, as modification of chemical structural can influence and improve chemical activity.[178] Halogens are versatile atoms with a wide variety of chemical functions and commonly utilized in drug discovery.[179] Also, the addition of halogens to compounds is frequently used in drug design to increase the compound's lipophilicity, membrane permeability, oral absorption, and resistance to metabolic degradation, while generally not increasing toxicity.[179]

We have previously demonstrated the ability of flavonoids catechin, morin, and quercetin to enhance the GP to prevent MG mediated OS in neural cells.[1,37] As an extension to this, our current research is focused on creating novel morin structural variations to improve its inherent antioxidant ability. We synthesized a morin analogue with the addition of two bromine molecules – Di-bromomorin (DBM) - and a morin encapsulated nanoparticle (MNP). We hypothesize structural modifications to morin will increase its ability to enhance the GP in a MG-mediated model of AD. Cultured mouse cerebellar neurons and strains of *C. elegans* were induced to a state of OS with MG, and treated with morin and its derivatives to evaluate the effect on GP function. Through evaluation of constituent pathway proteins, MG, D-lactate, and imaging of neural structures, we provide evidence of the GP efficiency increase by structurally modified compounds. Our research shows the modified morin compounds – DBM and MNP – were more effective in GP enhancement than the unmodified flavonoid itself.

MATERIALS AND METHODS

Care and Use of Animals

Animal studies were approved and performed in accordance with the UNMC Institutional Animal Care and Utilization Committee (IACUC). C57BL/6 mice breeding pairs were obtained from The Jackson Laboratory (Bar Harbor, ME). *C. elegans* strains N2 (Bristol), VC343 (glod-4(gk189)), and CL2006 (dvls2), and *Escherichia coli* OP50 were purchased from the Caenorhabditis Genetics Center (CGC) at the University of Minnesota (MN, USA).

Chemicals and Compounds

Morin was purchased from MP Biomedicals (Solon, OH). Sodium D-lactate was purchased from Santa Cruz Biotechnology (Dallas, TX). Lactate dehydrogenase was purchased from US Biological (Salem, MA). Poly-D-lysine hydrobromide, β-nicotinamide adenine dinucleotide hydrate, methylglyoxal, 5-fluorodeoxyuridine, and 2,4-Dinitrophenylhydrazine were purchased from Sigma Aldrich (St. Louis, MO). Antibodies used were B-Actin (sc-47778), MAP-2 (sc-32791), caspase-3 p17 (sc-373730), HO-1 (sc-390991), GAD-65 (sc-377145), Nrf2 (sc-81342), Glyoxalase I (sc-133214), Glyoxalase II (sc-166781), m-IgGκ BP-HRP (sc-516102), m-IgGκ BP-CFL 488 (sc-516176), m-IgGκ BP-CFL 555 (sc-516177) from Santa Cruz Biotechnology (Dallas, TX); DJ-1 (5933), P-Akt (4060), IKKα (11930S), IκBα (4812S), P- IKKα (2697S) from Cell Signaling Technology (Danvers, MA); VGLUT1 (AB5905), NeuN (MAB377) EMD Millipore (Temecula, CA). Unless otherwise noted, chemicals for this study were purchased from Thermo Fisher Scientific (Fair Lawn, NJ).

Primary Cell Culture

Cerebellar neurons were harvested from P3 C57/BL6 (Jackson Labs) as previously described.[34,37] Corning plates were left under UV light for 30 mins, and poly-D-lysine HBr (MP Biomedicals) was added to wells (150 µg/mL) for 3 hours. Wells were washed three times with endonuclease-free water. The brains were removed via cervical dissection. The cerebellum was isolated, and the veins and meninges were removed. The tissue was treated with 2.5% trypsin for 15 minutes. The trypsin was removed, and 1% deoxyribonuclease was added and tissue was pipetted gently to form a homogenous mixture. Cells were centrifuged at 700 x g for 5 minutes. The supernatant was removed, and 1% DNase was added and resuspended. The solution was then filtered through a 40 µm nylon screen and centrifuged at 700 x g for 5 minutes. The supernatant was removed, and the pellet resuspended in BME serum media (Fetal Bovine serum, horse serum, glucose, glutamine). Cells were counted using a hemocytometer and seeded onto 6-well plates ($1.5x10^6$ cells/well), 12-well plates ($5×10^5$ cells/well), or 96-well plates ($5x10^4$ cells/well). Media were changed into serum-free DMEM (B27, N2, sucrose, glutamine, PS) after 4 hours. AraC was added (5 µM) after 24 hours to ensure a homogenous neural culture. Cells were incubated at 37˚C (5% CO_2) with half media changes every 2 days. Confluent cultures on day 5 were treated with MG (500 µM) and flavonoid (10 µM), or vehicle (0.1% DMSO) for 24 hours. Media and lysates were collected and stored at -80˚C.[128]

Synthesis of Di-bromomorin

2-(2,4-dimethoxyphenyl)-5-hydroxy-3,7-dimethoxy-4H-chromen-4-one (1). To a mixture of morin (3.3 mmol) and K_2CO_3 (33 mmol) in acetone (20% DMF,100 mL) was slowly added dimethyl sulfate (33 mmol) at room temperature. The mixture was stirred at room temperature for 24 h. K_2CO_3 was filtered off and acetone was evaporated. The residue was dissolved in EtOAc and washed with H_2O and brine and dried over $MgSO_4$. The

product was purified by column chromatography (20 % EtOAc in hexane) to give a pale yellow solid (90%). ^{1}H NMR (500 MHz, $CDCl_3$); δ 12.67 (s, 1H), 7.36 (d, 1H, *J* = 8.5), 6.58 (m, 2H), 6.34 (m, 2H), 3.87 (s, 3H), 3. 84 (s, 3H), 3.83 (s, 3H), 3.77 (s, 3H). 13C NMR; δ 178.7, 165.2, 162.9, 162.0, 158.7, 157.4, 156.4, 140.0, 131.6, 112.4, 106.5, 104.7, 98.8, 97.7, 92.1, 60.5, 55.7, 55.5.

6,8-dibromo-2-(2,4-dimethoxyphenyl)-5-hydroxy-3,7-dimethoxy-4H-chromen-4-one (2). To a solution of a methylated morin (0.28 mmol) in CH_2Cl_2 was added NBS (0.28 mmol) at room temperature. The mixture was stirred at room temperature for 18 h. Additional NBS (0.28 mmol) was added and stirred for 5 h. The reaction was washed with water and brine. The organic phase was dried over $MgSO_4$ and concentrated. Flash chromatography (25 % EtOAc in hexane) was performed on silica gel to give a yellow solid (41%). ^{1}H NMR (500 MHz, $CDCl_3$); δ 13.59 (s, 1H), 7.47 (d, 1H, *J* = 8.0), 6.58 (m, 2H), 3.96 (s, 3H), 3. 87 (s, 3H), 3.86 (s, 3H), 3.80 (s, 3H). ^{13}C NMR; δ 178.2, 163.4, 159.5, 159.0, 157.77, 157.7, 151.9, 139.9, 131.9, 111.4, 109.3, 104.9, 100.1, 98.8, 95.1, 61.0, 60.4, 55.6, 55.5.

6,8-dibromo-2-(2,4-dihydroxyphenyl)-3,5,7-trihydroxy-4H-chromen-4-one (Dibromo-morin). The mixture of pyridine hydrochloride (30 eq.) and a compound 2 (0.096 mmol) was heated to 150-160 °C for 4 h with stirring. The reaction was cooled to room temperature, acidified by 1 M HCl and extracted with EtOAc. The organic phase was washed with water and brine and dried over $MgSO_4$. The crude product was purified by column chromatography (silica gel, 10-20% MeOH in CH_2Cl_2) to give a yellow solid (32%). LC-MS ($C_{15}H_8Br_2O_7$); [M+2] calc. 460, found 460. ^{1}H NMR (500 MHz, DMSO-d_6); δ 13.49 (s, 1H), 12.75 (s, 1H), 7.63 (d, 1H, *J* = 2.0), 6.68 (m, 2H). ^{13}C NMR; δ 176.8, 163.2, 159.9, 159.2, 157.7, 157.2, 150.8, 138.7, 132.4, 110.4, 108.3, 103.9, 99.1, 98.2, 94.9.[128]

Morin Nanoparticle Synthesis and Concentration Quantitation

MNP was synthesized through the use of a synthetic polymer to surround and encapsulate morin. Morin in water was intimately mixed with p407 polymer. The solution was sonicated and put through a high pressure homogenizer to produce a homogenous mixture of particle sizes. MNP size was analyzed via Dynamic Light Scattering. Morin (MP biomedicals) used as standard, dissolved in methanol. MNP was centrifuged for 20 minutes at 20,000 RPM 4˚C, supernatant was removed, and methanol was used to resuspend. The concentration of morin in the MNP was determined by a morin standard dissolved in methanol. Serial dilutions were added to 96 well plate and absorbance (360 nm) measured using a BioTek scanner.[128]

MTT Cellular Toxicity

Cerebellar neurons were cultured in 96-well plates as previously described.[34,37] At day 5, neurons were treated for 24 hours with varying concentrations of morin derivatives (10 μM – 250 μM) to determine cellular toxicity of the compounds. After 24 hours, media was removed and wells were washed with PBS. MTT was added to each well (0.5 mg/mL) and incubated at 37˚C for one hour. DMSO was added to the wells, and plate was placed on a shaking incubator for 30 minutes. Absorbance (570 nm) of MTT was measured using BioTek scanner (Winooski, VT).[128]

SDS-PAGE Western Blot

Media was removed from 6-well plates and wells were washed with ice cold PBS. RIPA buffer (25 mM tris, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Na deoxycholate, 1% triton x-100, 3% glycerol) with protease and phosphatase inhibitors (Thermo-Fisher) were added to each well and rocked on ice for 15 minutes. Cells were scraped from the well, briefly sonicated, and centrifuged at 15,000 RPM for 10 minutes. The supernatant

was removed. Protein concentration was quantified using a Pierce BCA assay (Thermo Fisher) to ensure equal loading. Cell lysate, β-Me, and lamelli dye were heated at 90 C for 5 minutes. Lysates were loaded into a 4-20% PAGE SDS gel (BioRad), and run at 100 V for 40 minutes. The gels were removed and washed, and transferred onto a PVDF membrane at 75 V for 2 hours. Blots were washed with TBST, and blocked for one hour at RT in TBST containing 5% bovine serum albumin. Blots were washed and conjugated with primary antibody (directed towards: Glo-1, Glo-2, Nrf2, HO-1, IκB-α, P-IKK, IKKα/ß, ß-actin) in 5% BSA, rocking overnight at 4˚C. Blots were washed with TBST, and conjugated with appropriate secondary antibodies in TBST containing 5% BSA for one hour at room temperature. Blots were washed and imaged using Western Dura Super Signal (Thermo Fisher) on an Azure C600 imager (Azure Biosystems, Dublin, CA). Densiometric analysis was performed with ImageJ (Madison, WI).[128]

ROS Detection

Cerebellar primary neurons were cultured in 96-well plates as previously described.[180] Cells were treated with morin, DBM, or MNP [10 μM] for 24 hours, and then additionally treated with MG [500 μM] for 24 hours. Media was removed, cells were washed with PBS, and incubated with 1 μM CM-H_2DCF-DA (Life Technology) in EBSS in dark at 37 ˚C for 30 minutes. Fluorescence was recorded at 485 nm excitation and 520 nm emission on a BioTek scanner.[128]

Immunocytochemistry

Cerebellar primary neurons were cultured in 96-well plates as previously described,[34,37] Cells were treated with MG [500 μM] for 24 hours, and then additionally treated with morin, DBM, or MNP [10 μM] for 24 hours,. Media was removed, wells were washed with PBS, cells were fixed for 30 minutes with a 30% sucrose solution containing 4% PFA, and washed. Cells were solubilized for 10 minutes with PBS containing 0.1% Triton X-

100, and washed with PBS. Cells were blocked for 1 hour at room temperature with PBS containing 2% BSA. Blocking solution was removed, and cells incubated overnight at 4˚C with PBS containing 2% BSA and primary antibodies directed towards NeuN, cleaved caspase-3, VGLUT1, GAD65, and MAP2. Wells were washed with PBS and incubated with the appropriate fluorescent conjugated secondary antibodies at room temperature for 1 hour. Wells were washed, covered with DAPI stain [1 µg/mL] for 10 seconds, washed and aspirated. Prolong Gold Antifade (Thermo Fisher Scientific, MO) was added directly to each well, and allowed to cure in the dark overnight. Plates were imaged on CLS Operetta confocal microscope (PerkinElmer, Waltham MA). Statistical analysis was performed through quantification of fluorescence normalized to the number of DAPI positive cells.[128]

Glyoxalase Activity

Cerebellar primary neurons were cultured in 12-well plates as previously described, [34,37] After MG and morin derivative treatment, media was removed, and cells rinsed with PBS. Cells were lysed with buffer (10 mM HEPES, 0.02% Triton X-100, and 100 µg/mL BSA), briefly sonicated, and centrifuged. Reaction solution (60 mM sodium phosphate, 4 mM GSH, and 4 mM MG) in a 96 well plate was briefly incubated, followed by addition of cell lysates. S-lactoylglutathione synthesis was determined by measuring absorbance (240 nm) on a BioTek scanner. Protein concentration was determined using a BCA protein assay reagent kit.[128]

D-Lactate Concentration

D-Lactate released into the extracellular space was measured spectrophotometrically using collected cell media.[34,37] Culture media samples were loaded on a 96-well plate with 0.2 M glycine and semicarbazide buffer containing 2 mg/mL NAD and 40 U/mL D-lactate dehydrogenase. Samples were incubated at room temperature for 2 hours. A

spectrophotometer (340 nm excitation, 450 nm emission) was used to measure conversion of NAD to NADH. Absolute values were determined from a standard curve of D-lactate concentrations.[128]

Methylglyoxal Concentration

MG concentration in cerebellar neuron cultures was determined using dinitrophenylhydrazine (2,4-DMNPH).[34,169] The reaction consisted of 0.2 mM 2,4-DMNPH with 1 mM MG and previously collected culture media. Samples were heated in a thermomixer at 42 °C for 45 mins and 600 rpm. Spectrophotometer measurements were performed at 432 nm, according to absorbance of MG-bis- 2,4-DMNPH-hydrazone for calculating concentration of MG.[128]

***Caenorhabditis Elegans* Strains and Maintenance**

Caenorhabditis elegans (*C. elegans*) were cultured as previously described.[181] *C. elegans* were maintained on nematode growth medium (NGM) plates [Bacto Agar 1.7%, Bacto Tryptone 0.25%, NaCl 50 mM, KPO_4 25 mM, $CaCl_2$ 1 mM, $MgSO_4$ 1 mM, and cholesterol 5 µg/mL], or in liquid S media [5.85 g NaCl, 1 g K_2HPO_4, 6 g KH_2PO_4, 1 ml cholesterol (5 mg/ml), 10 ml 1 M potassium citrate pH 6, 10 ml trace metals solution, 3 ml 1 M $CaCl_2$, 3 ml 1 M $MgSO_4$] at 20 Cº. A single colony of *E. coli* OP50 was cultured in LB media to OD 0.1, and 100 µL of was spread on NGM plates and incubated overnight at 37 ºC. Synchronous L1 nematodes were added to NGM plates for experiments. *C. elegans* cultures were washed and collected from starved NGM plates, and added biweekly to fresh NGM plates with OP50.[128]

Culture Synchronicity

Synchronous populations of L1 *C. elegans* were obtained by bleaching as previously described.[181] NGM plates were washed with M9 media, and cultures collected for

centrifugation (200 g, 2 min at 25ºC). The supernatant was removed, pellet washed with M9 media, and recentrifuged. After removing supernatant, 2 mL of a bleaching solution (2 mL of 8% bleach, 200 µL of 10M NaOH , and 8 mL H_2O) was added for 7 minutes, with gentle agitation every minute. Cultures were checked under a microscope to ensure all adult worms died. M9 media was added to cultures to stop the reaction. The solution was centrifuged (400 gx3 mins at 25º), supernatant removed, pellet washed, and recentrifuged. The eggs were resuspended in S media, and allowed to gently rock for 24 hours until eggs hatched. An equal amount of L1 nematodes were added to plates with M9 media for culturing. 5-Fluoro-2'-deoxyuridine (FUdR) was added to cultures on the first day of adulthood to sterilize and prevent egg laying of gravid adults.[128]

Morin Derivative Treatment

Morin derivatives were dissolved in DMSO at a final concentration of 0.1%, and added to freshly poured NGM plates at a concentration of 100 µM. MG was added directly to freshly poured NGM plates at a concentration of 1 mM. All NGM plates contained FUdR [250 µM] to prevent egg laying. Age synchronized L4 *C. elegans* were added to NGM drug treatment plates that were changed biweekly.[128]

Statistics

All experiments were performed in triplicate, with values are presented as the mean +/- SEM. Significance was determined by Student t-test, with $p<0.05$ being statistically significant. # denotes statistical significance between the non MG-treated control and MG-treated control; * denotes statistical significance between the MG-treated morin derivatives and MG-treated control; + denotes statistical significance between non MG-treated control and non MG-treated morin derivatives; & denotes statistical significance between morin and morin derivatives. Data were evaluated using Excel and SPSS.[128]

RESULTS

Synthesis of DBM and MNP

DBM was synthesized through a series of reactions involving protection, bromination and deprotection of morin (**FIG 3-1A**). Molecular structure was confirmed through ^{1}H-NMR and MS/MS analysis (**FIG 3-1B**). Nanoparticles are small particles (less than 1000 nm) composed of an active chemical compound encapsulated in a polymer in a colloid solution.[182,183] Characterization of the MNP occurred through dynamic light scattering (DLS) to determine the size, polar surface area, and charge distribution. The size was determined to be ~415 nm with PDI 0.4, indicating a homogenous concentration of nanoparticles. The concentration of morin encapsulated in the nanoparticle was determined to be 3 mM, or 80%, by comparison with a standard curve of morin concentrations.

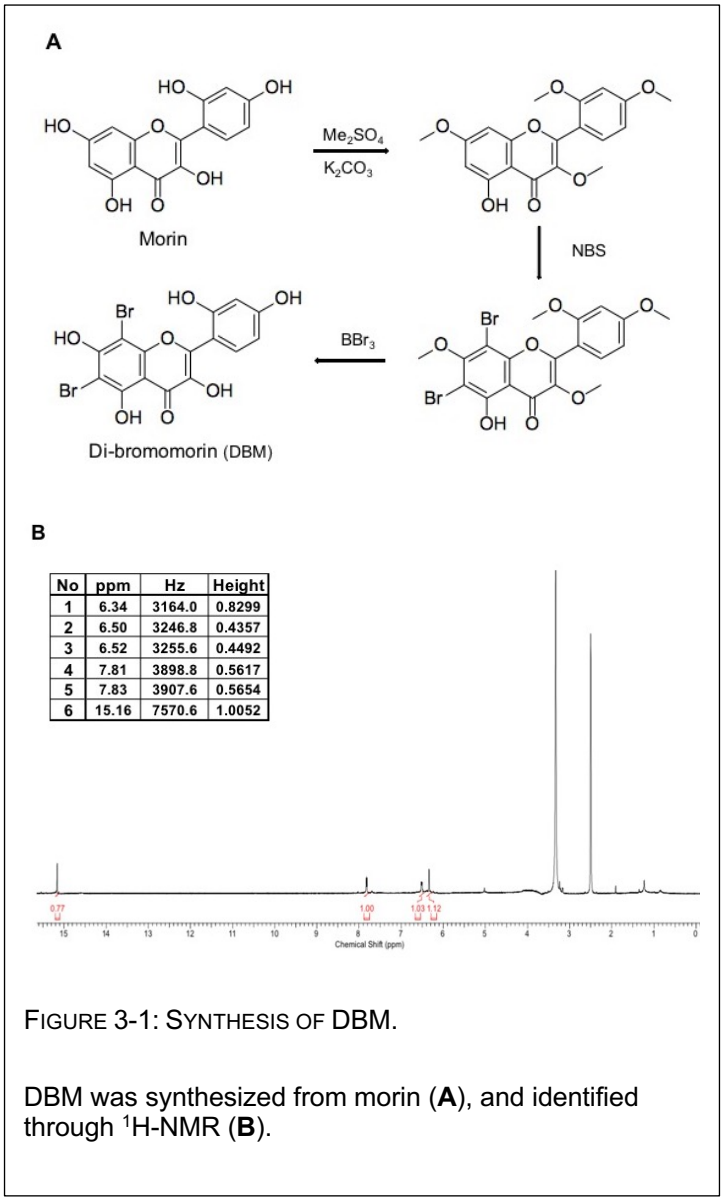

FIGURE 3-1: SYNTHESIS OF DBM.

DBM was synthesized from morin (**A**), and identified through ^{1}H-NMR (**B**).

Toxicity of Morin Derivatives

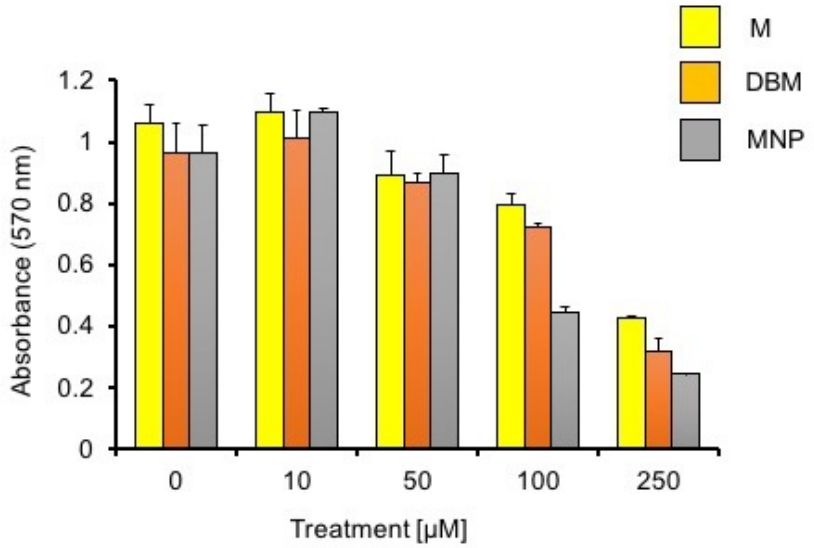

FIGURE 3-2: TOXICITY OF MORIN DERIVATIVES.

The toxicity of morin derivatives were determined through an MTT assay. Cultured neurons were treated with a range of doses [0-250 μM] of morin, DBM, and MNP. Survival was determined 24 hours after treatment.

An MTT assay was performed in cerebellar neurons to determine the toxicity of morin, DBM, MNP (**FIG 3-2**). All morin compounds exhibited low toxicity at concentrations up to 100 μM, and only the MNP exhibited significant toxicity only at extremely high doses [250 μM].[128] We determined the appropriate treatment condition to be 10 μM.

Morin derivatives increase GP function and detoxification of MG

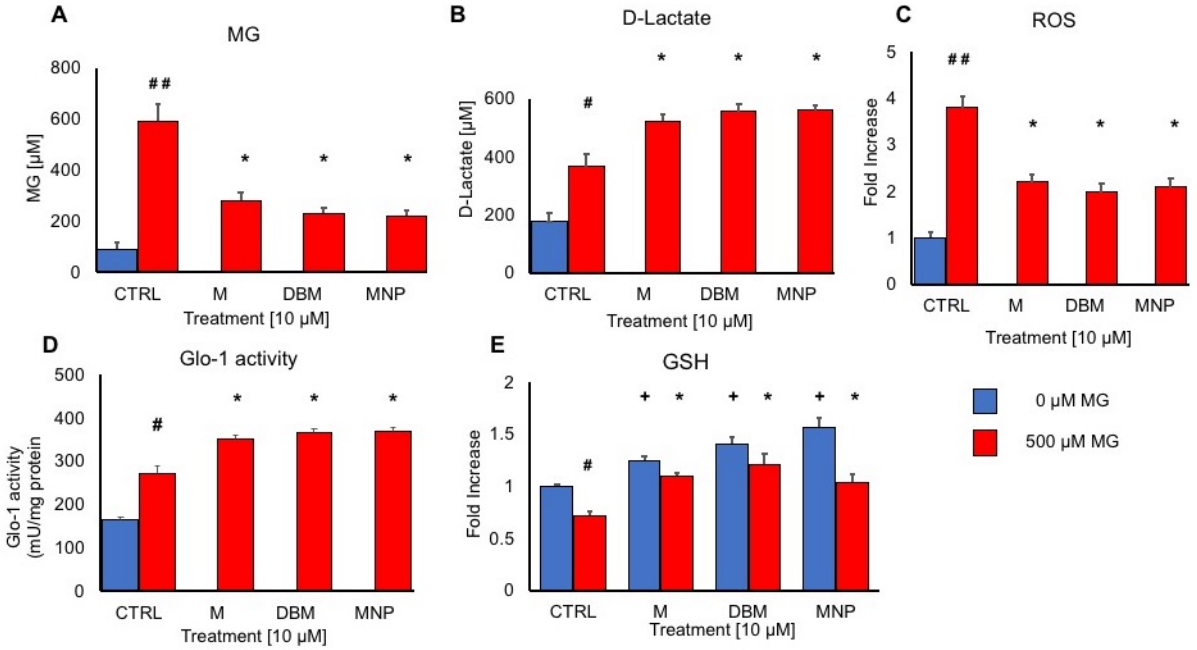

FIGURE 3-3: GP AND ANTIOXIDANT FUNCTION IN CEREBELLAR NEURONS.

Morin derivative treatments on MG (**A**), D-lactate (**B**), Glo-1 protein activity (measured in mU/µg protein) (**C**), ROS production (**D**), and GSH (**E**). Results are the mean +/- SEM of experiments performed in triplicate. #p<0.05, ##p<0.01, control compared to the MG-treated control; *p<0.05, **p<0.01 morin derivative and MG-treated compared to MG treated control; +p<0.05 morin derivative treated compared to non MG-treated control.

Addition of MG to cerebellar neuron cultures resulted in significant elevation of MG (#p<0.05) compared to the control, however morin, DBM, and MNP significantly (*p<0.05) decreased the concentration of MG compared to the MG treated control (**Figure 3-3A**). Treatment with MG significantly (#<0.05) elevated D-lactate concentration compared to the control, similarly morin, DBM, and MNP significantly (*p<0.05) elevated the concentration of D-lactate compared to the MG treated control **(Figure 3-3B**). The production of ROS was significantly (##p<0.01) increased by treatment with MG compared to the control, but morin, DBM, and MNP significantly (**p<0.01) reduced the production of ROS compared to the MG treated control (**Figure 3-3C**). The activity of Glo-1 (mU/µg)

was significantly (*p<0.05) elevated in MG insulted cells treated with morin, DBM, and MNP compared to the MG treated control (**Figure 3-3D**). Cerebellar neurons insulted with MG exhibited significantly decreased GSH (#p<0.05) compared to the control, and treatment with morin, DBM, and MNP significantly (*p<0.05) elevated the concentration of GSH compared to the MG-treated control (**Figure 3-3E**). In the non-MG treated conditions, morin, DBM, and MNP significantly (*p<0.05) elevated GSH compared to the control.

Morin derivatives increase expression of glyoxalase pathway proteins

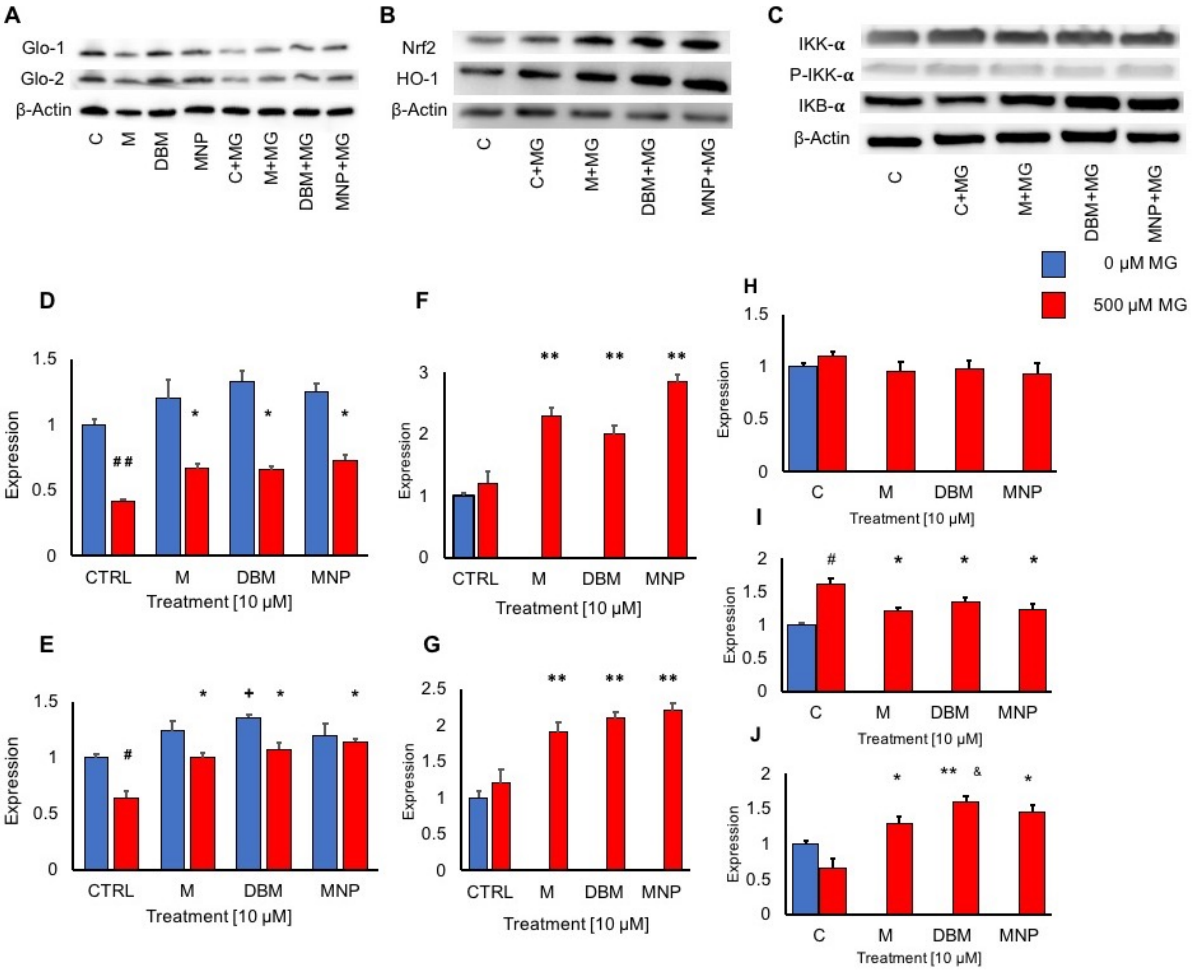

FIGURE 3-4: PROTEIN EXPRESSION IN CEREBELLAR NEURONS.

Morin derivative treatments in expression of Glo-1 (**A**) and Glo-2 (**B**); Nrf2 (**C**) and HO-1 (**D**); IKKα (**E**), p-IKKα (**F**), and IκBα (**G**). Images are from representative Western blots. Results are the mean +/- SEM of experiments performed in triplicate. #p<0.05, ##p<0.01, control compared to MG treated control; *p<0.05, **p<0.01 morin derivative and MG treated compared to MG treated control; +p<0.05 morin derivative treated compared to control. &p<0.05 DBM compared to morin.

Cerebellar neurons insulted with MG had significantly (#p<0.05) lowered expression of Glo-1 compared to the control, while treatment with morin, DBM, and MNP significantly (*p<0.05) elevated expression of Glo-1 in MG insulted cells (**Figure 3-4A**). MG treatment significantly (#p<0.05) reduced expression of Glo-2 compared to the control, while

treatment with morin, DBM, and MNP significantly (*p<0.05) elevated expression of Glo-2 in MG insulted cells (**Figure 3-4B**).

Morin derivatives increase expression of antioxidant proteins

Cells produce cytoprotective proteins in response to elevated OS to counteract the presence of inflammatory compounds. Nrf2 is a primary protein involved in antioxidant response, and It functions as a transcription factor interacting with the antioxidant response element (ARE) to induce the expression of other cytoprotective proteins. Heme-oxygenase-1 (HO-1) is another protein involved in antioxidant response that is expressed under states of OS to prevent the accumulation and damaging effects of inflammatory compounds. In MG insulted cells, treatment with morin, DBM, and MNP significantly (**p<0.01) elevated the expression of both Nrf2 (**Figure 3-4C**) and HO-1 (**Figure 3-4D**). Cells insulted with MG exhibit activation of antioxidant response, including expression of Nrf2 (**Figure 3-4C**). Morin, DBM, and MNP significantly (*p<0.05, **p<0.01) elevated the expression of Nrf2 and HO-1.

Morin derivatives inhibited activation of NF-κB signaling pathway

Activation of NF-κB causes its translocation to the nucleus, where it functions as a transcription factor for the expression of apoptotic proteins.[123,152,161] In its inactive form, it is bound in the cytoplasm to the IκBα complex.[123,152,161] Continued interaction with IκBα protein IKKα causes NF-κB to be retained in the cytoplasm and degraded. [123,152,161] MG treatment significantly elevated P-IKKα (*p<0.05) compared to the control (**Figure 3-4F**). We observed a significant decrease in P-IKKα in MG insulted cells upon treatment with morin(*p<0.05), DBM(*p<0.05), and MNP(*p<0.05). There was a significant increase in IκBα in MG insulted cells treated with morin(*p<0.05), DBM(*p<0.01), and MNP(*p<0.05)

compared to the MG treated control, and a significant increase upon treatment with DBM (&<0.05) compared to morin (**Figure 3-4G**).

Morin derivatives prevent MG-mediated caspase activation and apoptosis

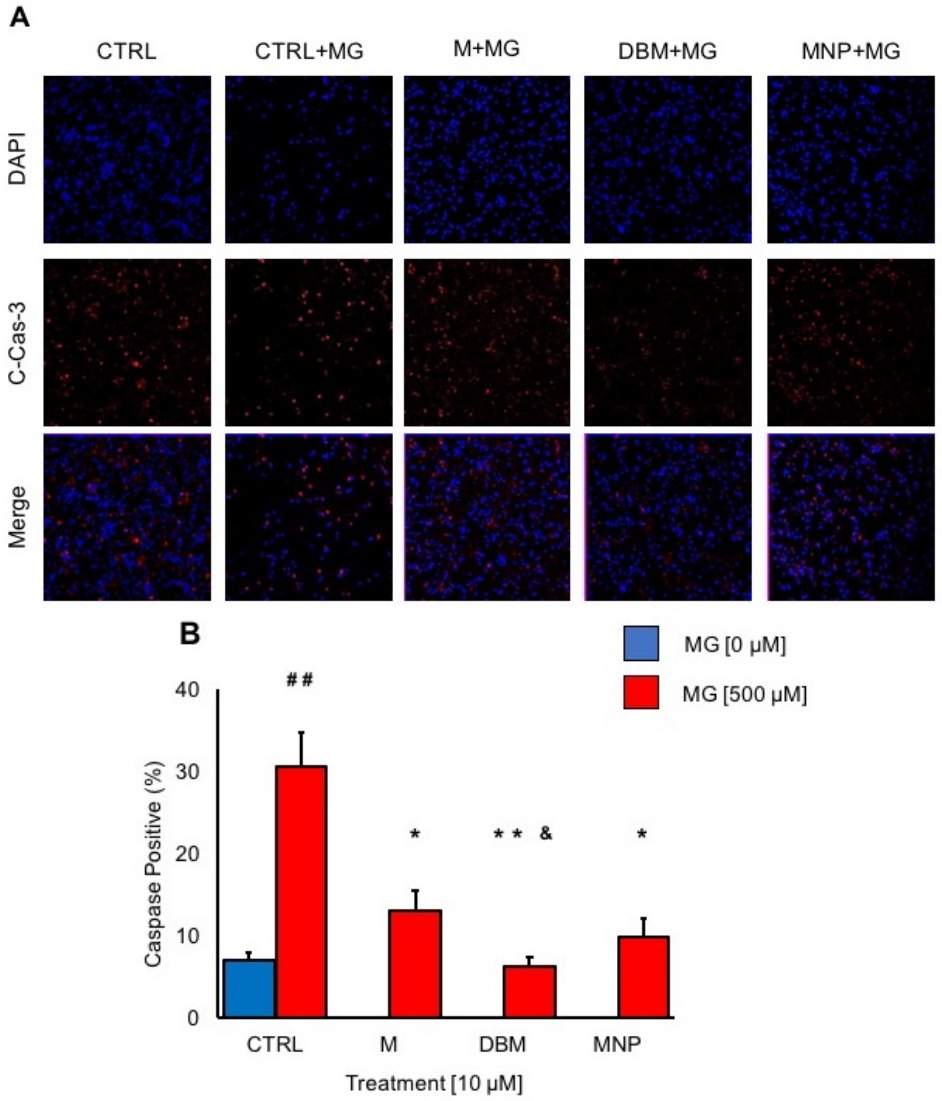

FIGURE 3-5: APOPTOSIS IN CEREBELLAR NEURONS.

Morin derivative treatments in MG insulted cells measured by the percentage of cleaved caspase-3 positive cells (**B**). Representative images are shown (**A**). Results are the mean +/- SEM of experiments performed in triplicate. #p<0.05, ##p<0.01, control compared to MG-treated control; *p<0.05, **p<0.01 MG treated control compared to MG treated morin derivatives, &p<0.05 DBM compared to morin.

NF-κB pathway mediated apoptosis occurs with the activation of cell death caspases. Activation of caspase-3 causes it to be cleaved, inducing a signaling cascade that results in apoptosis. Addition of MG to cerebellar neuron cultures lead to a significant (##p<0.01) increase in the amount of cleaved caspase-3 positive cells (**FIG 3-5**). In MG insulted cells,

we witnessed significant decrease in caspase positive cells with treatment of morin (*p<0.05), DBM (**p<0.01), and MNP (*p<0.05). There was a significant decrease in cleaved caspase-3 positive cells in treatment with DBM (&<0.05) compared to morin.

Morin derivative treated neurons retained inhibitory neurotransmitter function

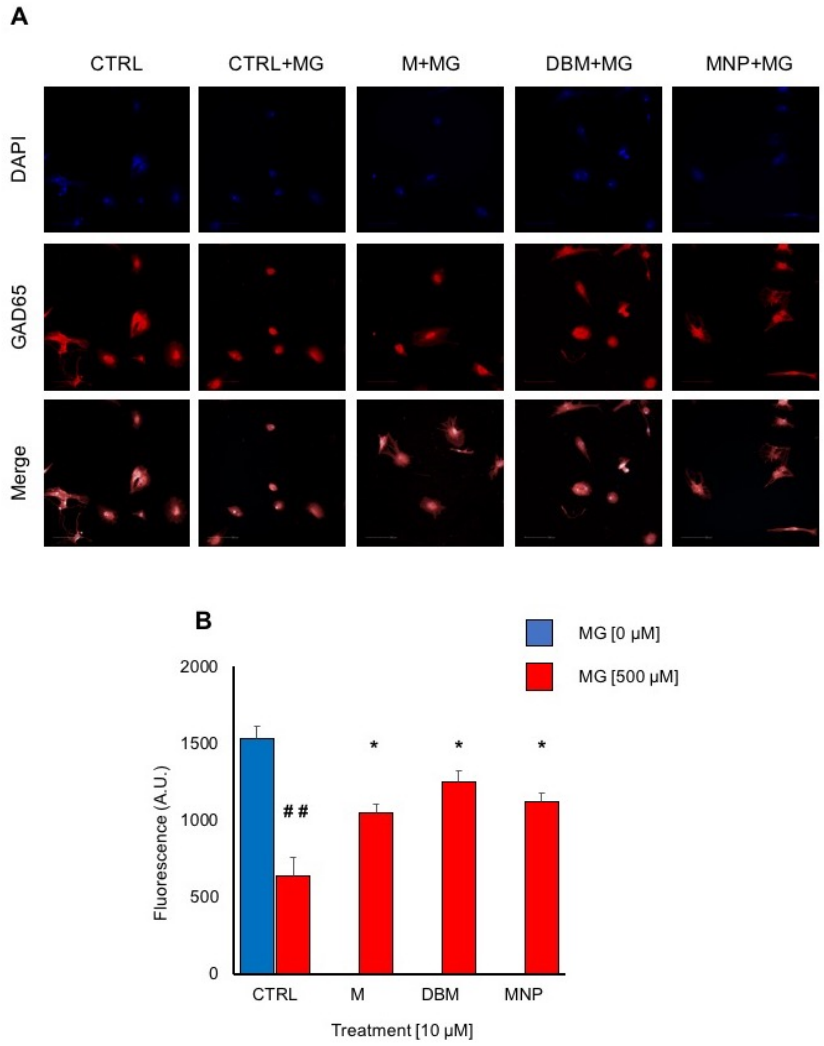

FIGURE 3-6: INHIBITORY NEUROTRANSMITTER FUNCTION.

Representative confocal images (A) and graphs of morin derivative treatments in MG insulted neurons of GAD65 (B). Results are the mean +/- SEM of experiments performed in triplicate. Fluorescence was normalized to amount of DAPI positive cells, with representative images shown. #p<0.05, ##p<0.01, control compared to MG treated control; *p<0.05 significant difference between MG treated control and MG treated morin derivatives.

GAD65 is the protein responsible for synthesis of GABA - the primary inhibitory neurotransmitter- through decarboxylation of glutamic acid or glutamate. Cerebellar

neurons insulted with MG exhibited significant decrease in GAD65 compared to the control. MG insulted cells treated with morin exhibited significant elevation in GAD65 (*$p<0.05$), DBM significantly elevated GAD65 (*$p<0.05$), MNP significantly elevated GAD65 (*$p<0.05$) (**FIG 3-6B**). Representative confocal images are shown (**FIG 3-6A**).

Morin derivative treated neurons retained excitatory neurotransmitter function.

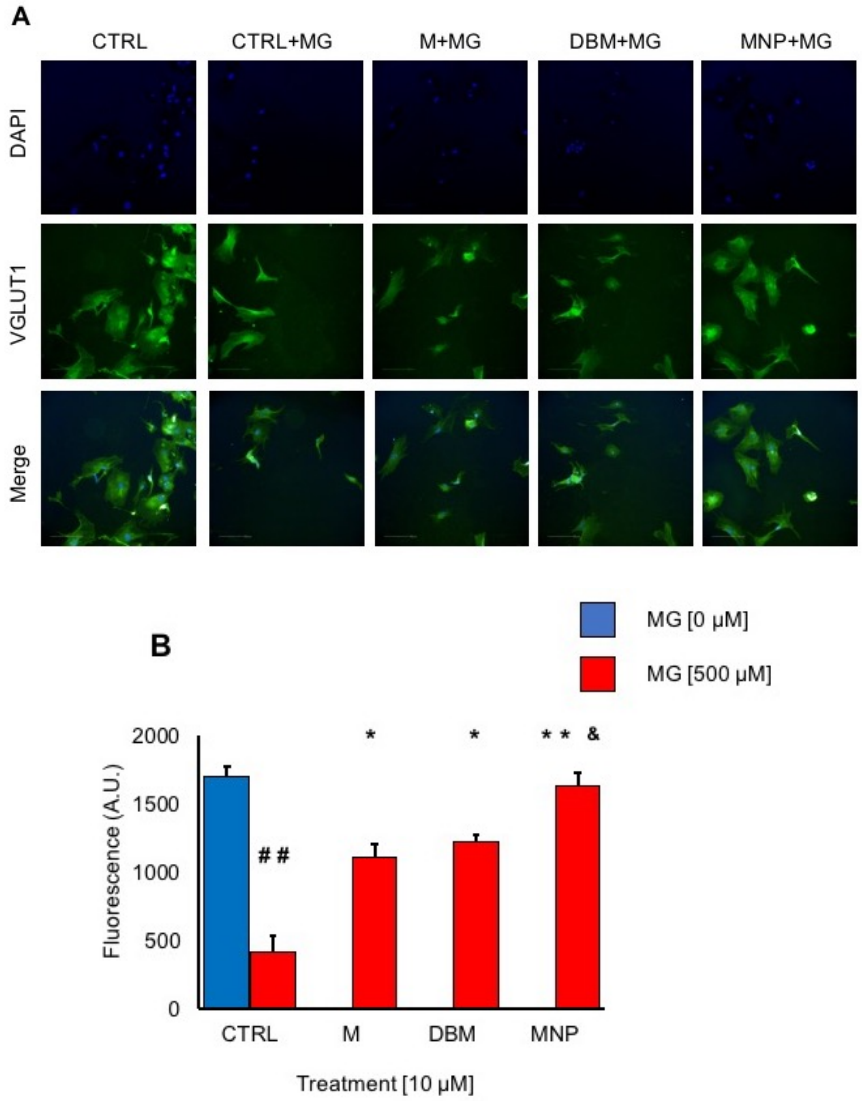

FIGURE 3-7: EXCITATORY NEUROTRANSMITTER FUNCTION.

Representative confocal images and graphs of morin derivative treatments in MG insulted neurons of VGLUT. Results are the mean +/- SEM of experiments performed in triplicate. Fluorescence was normalized to amount of DAPI positive cells, with representative images shown. #p<0.05, ##p<0.01, control compared to MG treated control; *p<0.05, **p<0.01 MG treated control compared to MG treated morin derivatives, &p<0.05 MNP compared to morin.

VGLUT is the protein involved in glutamate transport from neural vesicles, it is responsible for shuttling glutamate from neural synapses. Buildup of glutamate in

neurons is cytotoxic and induces apoptosis, and expression of VGLUT allows for glutamate to be transported from neurons. Cerebellar neurons insulted with MG exhibited significant decrease in VGLUT (**FIG 3-7B**) compared to the control. There was a significant increase in VGLUT expression in MNP (&p<0.05) treated cells compared to morin. Representative confocal images are shown (**FIG 3-7A**).

Morin derivatives elevate expression of synaptic structural proteins.

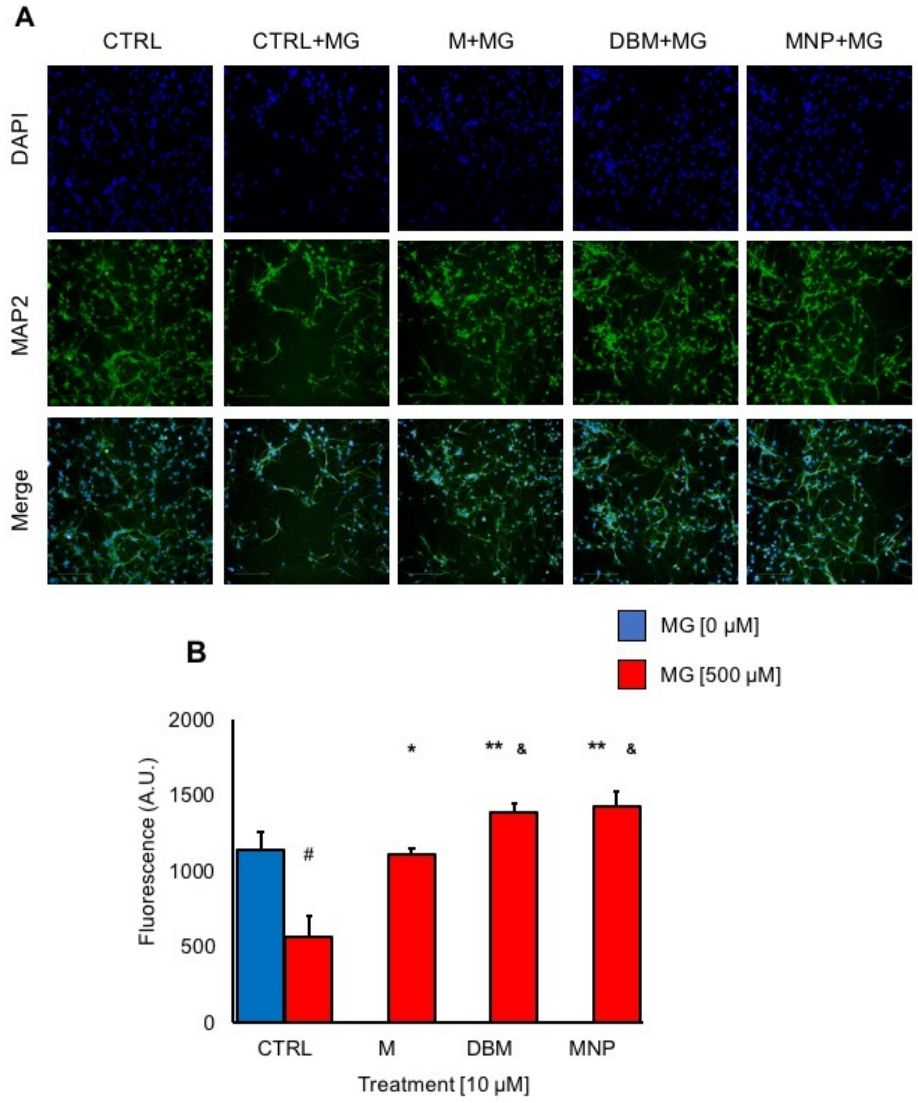

FIGURE 3-8: SYNAPTIC STRUCTURAL PROTEIN EXPRESSION.

Representative confocal images (A) and graphs (B) of MAP2 in morin derivative treatments in MG insulted neurons. Results are the mean +/- SEM of experiments performed in triplicate. Fluorescence was normalized to amount of DAPI positive cells, with representative images shown. #p<0.05, control compared to MG treated control; *p<0.05, **p<0.01 MG treated control compared to MG treated morin derivatives, &p<0.05 DBM and MNP compared to morin.

Microtubule-associated protein 2 (MAP2) is a structural protein involved in neurite and dendritic branching, and is involved in interneuron signaling. Cerebellar neurons insulted

with MG exhibited significant decrease in MAP2 (**FIG 3-8B**) compared to the control. MG insulted cells treated with morin exhibited significant elevation in MAP2 (*p<0.05); DBM significantly elevated MAP2 (*p<0.05); MNP significantly elevated MAP2 (*p<0.05). DBM (&p<0.05) and MNP (&p<0.05) significantly increased MAP2 expression compared to morin. Representative confocal images shown (**FIG 3-8A**) .

Morin derivatives extend lifespan of *C. elegans*

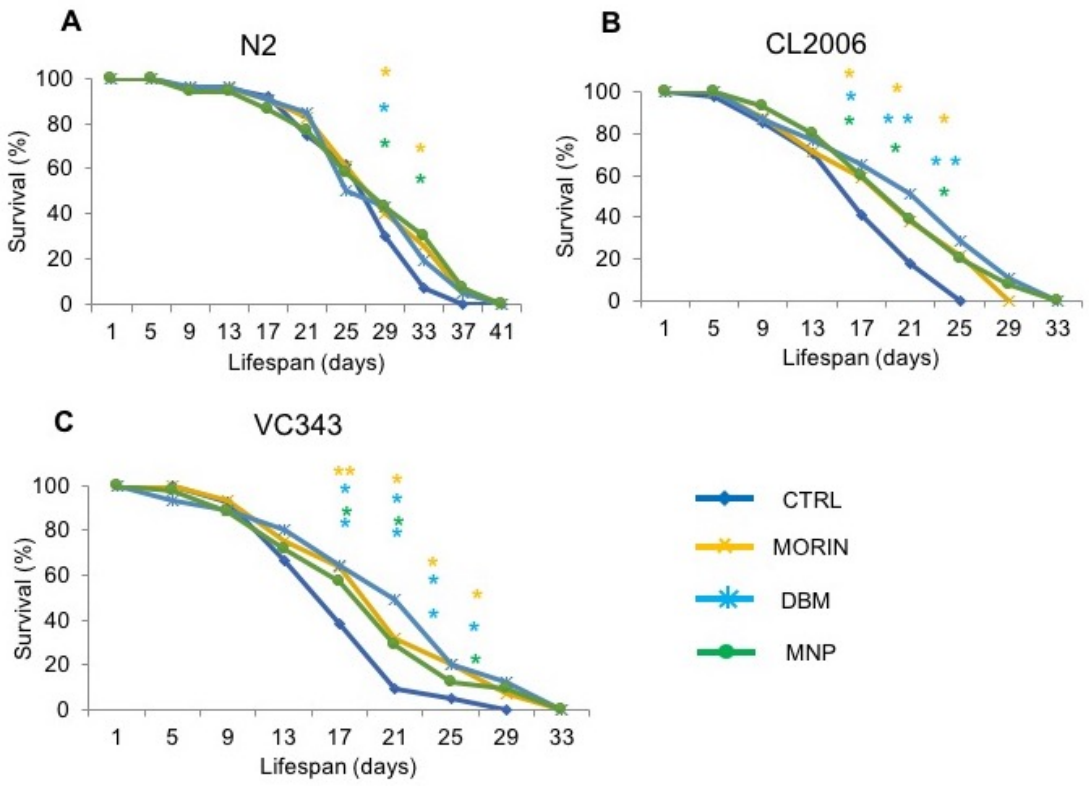

FIGURE 3-9: C. ELEGANS LIFESPAN EXTENSION.

Morin, DBM, and MNP were utilized to treat N2 (B), CL2006 (C), and VC343 (D) strains of *C. elegans*, to determine the effect on lifespan. Morin, DBM, and MNP resulted in significantly extended lifespans compared to the controls. Results are the mean +/- SEM of experiments performed in at least triplicate. *p<0.05, **p<0.01 compared to control.

Morin, DBM, and MNP were utilized to treat *C. elegans* strains N2 (**FIG 3-9B**), CL2006 (AD model overexpressing Aβ, (**FIG 3-9C**), and VC343 (Glo-1 knockdown) (**FIG 3-9D**). The average lifespan of morin treated *C. elegans* was significantly higher than the control at days 17 (**p<0.01), 21 (**p<0.01), 25 (**p<0.01), and 29 (*p<0.05); The lifespan of DBM treated *C. elegans* was significantly higher than the control at days 17(**p<0.01), 21(**p<0.01), 25(**p<0.01), and 29(**p<0.01); The lifespan of MNP treated *C. elegans* was significantly higher than the control at days 17(**p<0.01), 21(**p<0.01), 25(**p<0.01), and 29(*p<0.05).

DISCUSSION

Proper functions of neural cells are heavily reliant upon GP detoxification of MG, to prevent production of AGEs, ROS, and pro-inflammatory signaling molecules.[20,21,97]

Conversion of hemithioacetal to S,D-lactoylglutathione by Glo-1 is the rate limiting step in the GP, and Glo-1 function is influenced by the expression levels of the protein and its enzymatic activity.[20,21,97] Therefore, modulating Glo-1 expression influences the activity of the GP and impacts the levels of cellular OS and inflammation.[20,21,97] Reducing Glo-1 expression results in elevated OS-mediated damage to cells and tissues. [44] Mice transduced with Glo-1 siRNA showed accumulation of MG and AGEs in neural tissue, and exhibited cognitive and behavioral dysfunction, and Glo-1 KO mice showed significantly elevated concentrations of MG and AGEs.[153] Glo-1 KO in mammalian Schwann cells exhibited increased toxicity to MG and elevated levels of GSSG, with the decrease in GSH/GSSH ratio corresponding to a decrease in Glo-1 activity, and elevated MG and AGEs.[45] Elevating Glo-1 through gene transduction reduced protein and lipid glycation, and attenuated cognitive dysfunction in rats, and reduced markers of apoptosis in H_2O_2 mediated OS in mouse hippocampal cells.[48] [42]

The activity of flavonoids is influenced by structural characteristics, and antioxidant activity was found to be highest in structures that contained: di-hydroxylated B ring, a C ring containing a C2=C3 double bond and C4=O, and hydroxyl groups at C3 and C5.[118] Morin and quercetin both contain all these structures, and performed better as antioxidants than flavonoids naringenin and pinocembrin, which lack a C2=C3 bond.[118] The only structural variation between quercetin and morin is the configuration of -OH groups on the C ring, with quercetin possessing a 3', 4' configuration instead of the 2', 4' in morin.[118] Flavonols are distinguished from other flavonoids by containing a C2=C3

bond, 4C=O keto group, and a hydroxyl group at C3.[184] A critical aspect of antioxidant activity is influenced by the number and position of -OH groups; flavonol -OH groups aid in binding interactions and stabilization with proteins and nucleic acids.[185] Flavonoids function as direct anti-AD agents through their ability to bind and prevent the fibrillation of Aβ proteins.[119] Destabilizing the amyloid fibrils can disrupt their aggregation into Aβ plaques, leading to decreased toxicity and elevated clearance.[119] The effectiveness of Aβ fibrilization inhibition was influenced by the ketone structure, and the presence of hydroxyl groups - with dihydroxy structures more effective than monohydroxy.[184184,185] Chemical substitutions at the site of -OH drastically reduced inhibition activity. [184184,185] Flavonol morin exhibited anti-Aβ activity by binding to amyloid fibrils and reducing the rate of polymerization.[119] The bioavailability of flavonols – mediated by the presence and position of hydroxyl groups - allows for penetration through the BBB to reach critical areas of the brain where Aβ formation occurs.[119] The mechanism of flavonol interaction with Aβ occurs through hydrogen bonding of flavonol hydroxyl and carbonyl groups with the amine groups present on Aβ peptides.[119] These interactions disrupt the peptide structure of Aβ, preventing the formation of α-helices and β-sheets present in Aβ plaques.[119] Therefore, the presence of multiple hydroxyl groups on flavonols makes them more adept at interaction and disruption of the Aβ structure.[119,184] An increasing molar ratio of flavonol morin to Aβ resulted in a change of the tertiary structure of Aβ, caused by the intercalation of flavonols into the Aβ structure.[119]

In this report we show that morin derivatives are capable of increasing the activity of the GP. During states of OS, the GP is activated and elevates the transcription and expression of its constituent proteins.[22,64] Cerebellar neurons insulted with MG and treated with morin, DBM, and MNP exhibited significantly elevated levels of GSH, Glo-1, and Glo-2 compared to the MG treated controls. Treatment with morin derivatives also significantly elevated Glo-1 activity, indicative of efficient and continuous GP activation. In agreement with this, morin derivative treated cells had low levels of MG and ROS, and elevated D-lactate, evidence of robust GP activity. Accumulation of MG induces an inflammatory response and apoptosis, causing the death of neural cells and dysfunction of normal cellular processes.[9,10,15] Cerebellar neurons treated with MG exhibited impaired neurotransmitter function, VGLUT1 and GAD65 – markers of excitatory and inhibitory neurotransmission, respectively – were reduced upon treatment with MG. In MG-insulted cells treated with morin, DBM, and MNP, this loss of neurotransmitter activity was attenuated. VGLUT functions to shuttle glutamate out of cell, and a reduction in VGLUT results in cellular glutamate accumulation, causing excessive Ca^{+2} influx and apoptosis.[5,23,55] GAD65 decarboxylates glutamic acid into GABA, and reduced GAD65 causes a shift in the balance of excitatory and inhibitory neurotransmission, which can lead to inflammation and apoptosis.[4,53,54] Morin derivatives were able to effectively preserve both excitatory and inhibitory neurotransmitter function. MAP2 is a structural membrane protein involved in outgrowth of neurites and dendritic branching, and it is essential for growth and connectivity of the neural network.[4,53,54] MG treatment significantly reduced expression of MAP2 in cerebellar neurons, but this reduction was attenuated by treatment with morin, DBM, and MNP. Morin derivatives exhibited the ability to retain neural growth and protect mechanisms of neural signaling even under states of extreme OS. Nrf2 is the primary transcription factor that binds and activates ARE, leading to the expression of antioxidant proteins including HO-1, GSH, Glo-1, and

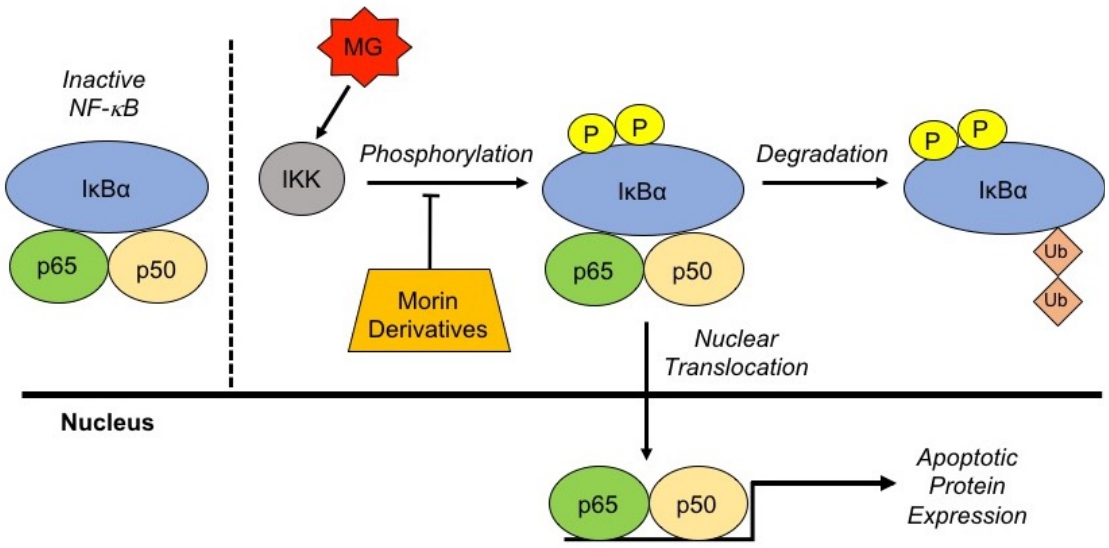

FIGURE 3-10: ACTIVATION OF NF-κB PATHWAY.

The NF-κB protein complex is bound in the cytoplasm by IκB. MG/ROS bind and activate IKK, which phosphorylates IκB and targets it for degradation. NF-κB proteins are translocated to the nucleus and induce gene transcription and protein expression.

Glo-2. [129,144,146] Under a state of MG-mediated OS, morin derivatives induced the expression of cytoprotective proteins, significantly elevating expression of Nrf2 and HO-1 compared to cells only treated with MG. Treatment with morin, DBM, and MNP significantly elevated the expression of Nrf2 and HO-1 compared to cells treated with MG alone. The NF-κB pathway is activated in response to cellular stress, and induces apoptosis (**FIG 3-10**). [123,152,161] Inactive NF-κB proteins are located in the cytoplasm, where they are bound by IκB of the IKK complex. [123,152,161] High levels of inflammation and OS trigger ROS and cytokines to bind and activate the IKK complex. [123,152,161] After activation, IKK phosphorylates IKB and it is ubiquitinated and degraded, allowing NF-κB to undergo nuclear translocation. [123,152,161] NF-κB interacts with transcription factors and induces the expression of apoptotic proteins. [123,152,161] Treatment with MG increased the production of ROS in cerebellar neurons, and significantly elevated the levels of p-IKKα. These neurons also had a significant reduction in IκBα, indicating it was degraded, and

subsequently unbound from NF-κB. The MG treated neurons exhibited significantly elevated cleaved caspase-3 through immunocytometric analysis. Conversely, the neurons treated with MG and morin derivatives had significantly lower levels of ROS, and P-IKKα levels similar to the non-MG treated controls. Morin derivative treated neurons also expressed IκBα levels significantly higher than the MG treated control. This is evidence that the IκB complex is still bound to cytoplasmic NF-κB. The inactivation of NF-κB is evidenced by the significantly lower cleaved caspase-3 found in the morin derivative treated cells. These results indicate that morin's anti-inflammatory and anti-apoptotic mechanisms are mediated by activation of the cellular protective Nrf2, and inhibiting the activation of the NF-κB signaling pathways.

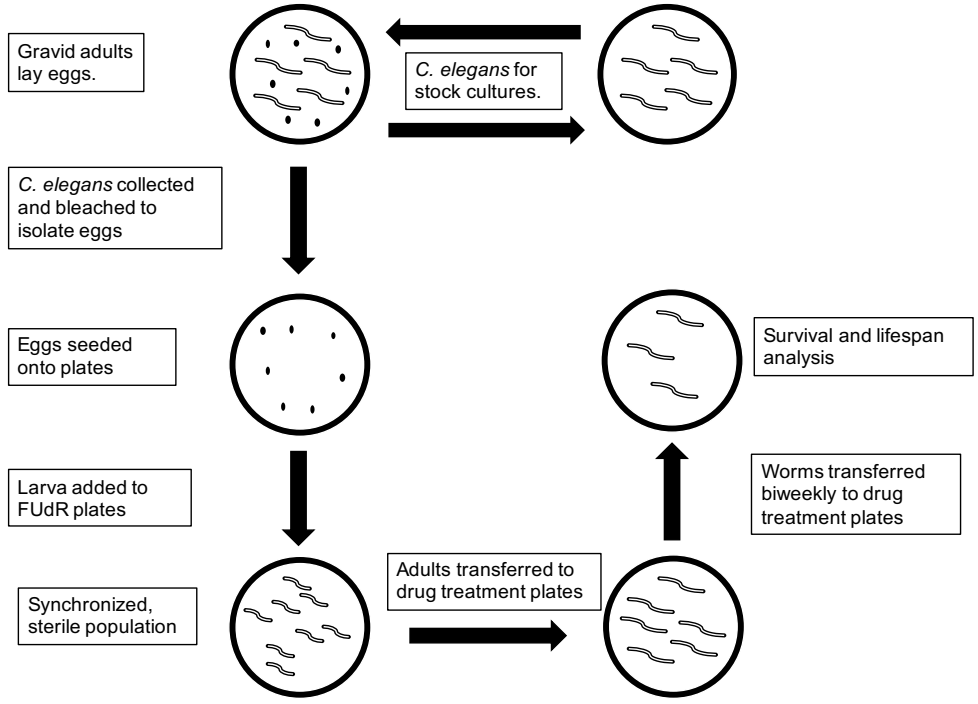

FIGURE 3-11: C. ELEGANS LIFECYCLE.

C. elegans must be separated from progeny for accurate measurement of lifespan. Adult *C. elegans* plated on NGM lay eggs, and are collected. Washing in a bleach solution kills the gravid adults. Eggs are added to NGM plates with FUdR to prevent progeny, resulting in a population of age-synchronized worms, which are used for lifespan analysis.

The use of model organisms for determining effect of anti-aging agents is an important tool for elucidating the function of antioxidant ability.[186] The use of an *in vivo* model system of *C. elegans* is useful for aging studies because of their relative ease of ability to culture, rapid propagation, and genetic homology to humans, and their short lifespan makes them an ideal species to study diseases associated with aging (**FIG 3-11**). [186,187] Isolating adult worms from their progeny is important to properly evaluate changes in lifespan and aging. We found that treatment of CL2006 with all three flavonoid compounds lead to a significant increase in their lifespan. While we did not investigate

the mechanism of lifespan extension, it is known that flavonoid treatments induce the expression of antioxidant and cytoprotective proteins.[30,186-188]

To improve on the inherent antioxidant capacity of flavonoids, we utilized the versatility of halogen bonding to synthesize a brominated morin derivative, and formulated a morin encapsulated nanoparticle. Our hypothesis was confirmed, evidenced by the increased ability of DBM and MNP - compared to the parent compound - to prevent MG-mediated OS through enhancement of the neural GP. DBM and MNP treatment increased detoxification of MG, evidenced through elevated expression of Glo-1 and Glo-2, and increased Glo-1 protein activity. These results were in parallel to increased D-lactate and reduced production of ROS. Neurons exhibited a reduction in caspase-mediated apoptosis, retained both excitatory and inhibitory neurotransmitter function, and exhibited increased dendritic branching. This elevated antioxidant activity was mediated by activation of the Nrf2 pathway and suppression of apoptotic NFκB signaling. The lifespan of DBM or MNP treated *C. elegans* (CL2006) was significantly higher than the untreated control confirming their potential as CNS drug.

CHAPTER 4: CANNABIDIOL ENHANCES NEURAL GLYOXALASE PATHWAY AND IMPROVES LIFESPAN IN C. ELEGANS

INTRODUCTION

The effect of secondary plant metabolites and naturally-produced compounds have been targeted for AD therapeutics, with recent focus on phytocannabinoids as anti-AD agents.[51,92,116,138,189-191]

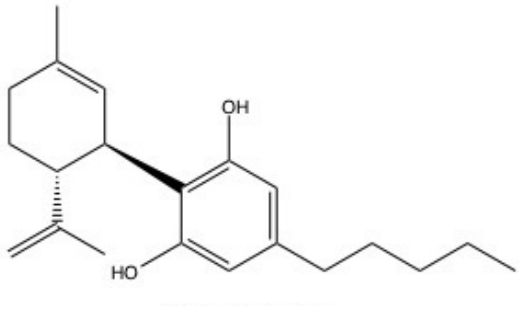

FIGURE 4-1: CBD STRUCTURE.

The chemical structure of non-psychoactive phytocannabinoid CBD.

The *Cannabis Sativa* (*C. sativa*) plant has been used for its medicinal properties for thousands of years, and recent scientific advances have elucidated the mechanism of the plants' chemical compounds.[189-196] *C. sativa* produces over 100 phytocannabinoids in addition to terpenes, flavonoids, and other polyphenols, with these secondary plant metabolites possessing a range of biological activity, including antioxidant, anti-inflammatory, and anti-apoptosis.[189-191,194,195,197] Cannabidiol (CBD) is one of the primary phytocannabinoids produced by the plant, and it possesses a myriad of medical and therapeutic uses (**FIG 4-1**).[154,195,198-205] CBD possesses structural characteristics – similar to our previously investigated flavonoids - that impart it with antioxidant activity, most importantly the linked heterocyclic rings and number and position of hydroxyl groups.[154,199,204]

In this chapter, we are the first to investigate CBD's influence on the GP. This chapter also investigates the influence of CBD of glyoxalase 3 (Glo-3) – also known as DJ-1 – as

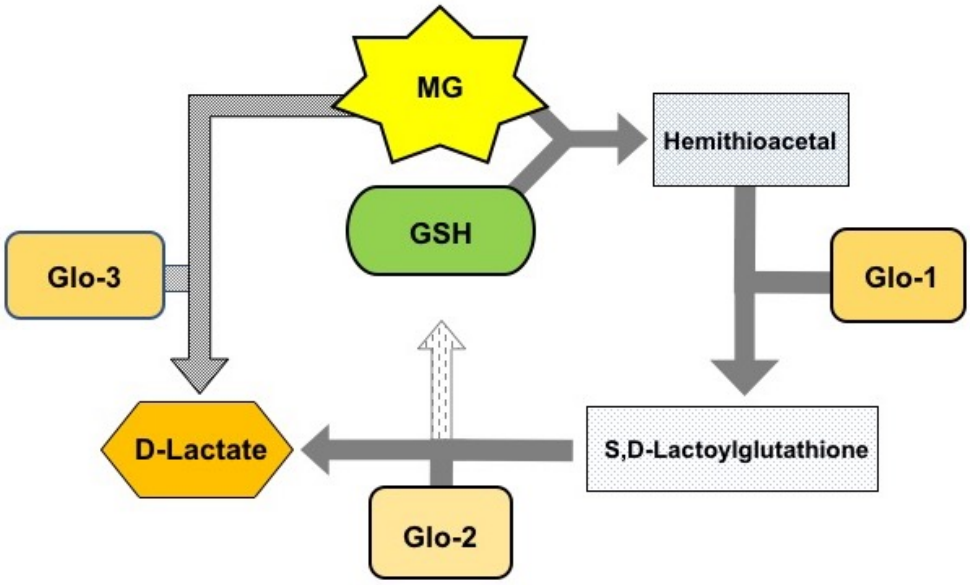

FIGURE 4-2: GLYOXALASE PATHWAY PROTEINS.

The GP consists of sequential reactions that results in the detoxification of carbonyl compounds primarily MG. Glo-1 and Glo-2 are involved in the GSH-mediated nonenzymatic neutralization of MG. Protein Glo-3 is involved in direct detoxification of MG in a GSH-independent manner.

an alternate mechanism of MG detoxification (**FIG 4-2**).[206,207] While the function of Glo-1 and Glo-2 have been elucidated through our previous works, the effect of Glo-3 has not been thoroughly investigated, however Glo-3 is thought to function during chronic states of OS during which diminished levels of GSH prevent efficient activity of Glo-1 and Glo-2.[206] We hypothesize that CBD exerts GP enhancing activity mediated by modulation of cellular signaling pathways, including Nrf2 and NF-κB. CBD is well documented as a potent antioxidant compound, and we aimed to elucidate its anti-inflammatory mechanism and function.[154,197,199,202-204] We utilized multiple models of AD including primary mouse cerebellar neurons and genetically modified *C. elegans* to investigate the effect of CBD on the GP. We determined CBD - like other plant produced compounds - is neuroprotective and induces an antioxidant response mediated by Nrf2, which causes the expression of cytoprotective proteins and inhibits the activation of apoptotic NF-κB

signaling. Through multiple models of MG-mediated OS in AD, utilizing *in vitro* cerebellar mouse neurons and *in vivo C. elegans*, we investigate the effect of CBD on the GP.

MATERIALS AND METHODS

Care and use of animals

Animal studies were approved and performed in accordance with the UNMC Institutional Animal Care and Utilization Committee (IACUC). C57BL/6 mice breeding pairs were obtained from The Jackson Laboratory (Bar Harbor, ME). *C. elegans* strains N2 (Bristol), VC343 (glod-4(gk189)), and GRU102 (dvls2), VH725, and *Escherichia coli* OP50 were purchased from the Caenorhabditis Genetics Center (CGC) at the University of Minnesota (MN, USA).

Chemicals and compounds

CBD was purchased from Cayman Chemical Company (Ann Arbor, MI). Sodium D-lactate was purchased from Santa Cruz Biotechnology (Dallas, TX). Lactate dehydrogenase was purchased from US Biological (Salem, MA). Poly-D-lysine hydrobromide, β-nicotinamide adenine dinucleotide hydrate, methylglyoxal, 5-fluorodeoxyuridine, and 2,4-Dinitrophenylhydrazine were purchased from Sigma Aldrich (St. Louis, MO). Antibodies used were B-Actin (sc-47778), MAP-2 (sc-32791), caspase-3 p17 (sc-373730), HO-1 (sc-390991), GAD-65 (sc-377145), Nrf2 (sc-81342), Glyoxalase I (sc-133214), Glyoxalase II (sc-166781), m-IgGκ BP-HRP (sc-516102), m-IgGκ BP-CFL 488 (sc-516176), m-IgGκ BP-CFL 555 (sc-516177) from Santa Cruz Biotechnology (Dallas, TX); DJ-1 (5933), P-Akt (4060), IKKα (11930S), IκBα (4812S), P- IKKα (2697S) from Cell Signaling Technology (Danvers, MA); VGLUT1 (AB5905), NeuN (MAB377) EMD Millipore (Temecula, CA). Unless otherwise noted, chemicals for this study were purchased from Thermo Fisher Scientific (Fair Lawn, NJ).

Primary Cell Culture

Cerebellar neurons were harvested from P3 C57/BL6 (Jackson Labs) as previously described. Corning plates were left under UV light for 30 mins, and poly-D-lysine HBr (MP Biomedicals) was added to wells (150 µg/mL) for 3 hours. Wells were washed three times with endonuclease-free water. The brains were removed via cervical dissection. The cerebellum was isolated, and the veins and meninges were removed. The tissue was treated with 2.5% trypsin for 15 minutes. The trypsin was removed, and 1% deoxyribonuclease was added and tissue was pipetted gently to form a homogenous mixture. Cells were centrifuged at 700 x g for 5 minutes. The supernatant was removed, and 1% DNase was added and resuspended. The solution was then filtered through a 40 µm nylon screen and centrifuged at 700 x g for 5 minutes. The supernatant was removed, and the pellet resuspended in BME serum media (Fetal Bovine serum, horse serum, glucose, glutamine). Cells were counted using a hemocytometer and seeded onto 6-well plates ($1.5x10^6$ cells/well), 12-well plates ($5×10^5$ cells/well), or 96-well plates ($5x10^4$ cells/well). Media were changed into serum-free DMEM (B27, N2, sucrose, glutamine, PS) after 4 hours. AraC was added (5 µM) after 24 hours to ensure a homogenous neural culture. Cells were incubated at 37˚C (5% CO_2) with half media changes every 2 days. Confluent cultures on day 5 were treated with MG (500 µM) and flavonoid (10 µM), or vehicle (0.1% DMSO) for 24 hours. Media and lysates were collected and stored at -80˚C.

MTT cellular toxicity

Cerebellar neurons were cultured in 96-well plates as previously described. At day 5, neurons were treated for 24 hours with varying concentrations of CBD (10 µM – 250 µM) to determine cellular toxicity of the compounds. After 24 hours, media was removed and wells were washed with PBS. MTT was added to each well (0.5 mg/mL) and incubated at 37˚C for one hour. DMSO was added to the wells, and plate was placed on a shaking

incubator for 30 minutes. Absorbance (570 nm) of MTT was measured using BioTek scanner (Winooski, VT).

SDS-PAGE Western Blot

Media was removed from 6-well plates and wells were washed with ice cold PBS. RIPA buffer (25 mM tris, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Na deoxycholate, 1% triton x-100, 3% glycerol) with protease and phosphatase inhibitors (Thermo-Fisher) were added to each well and rocked on ice for 15 minutes. Cells were scraped from the well, briefly sonicated, and centrifuged at 15,000 RPM for 10 minutes. The supernatant was removed. Protein concentration was quantified using a Pierce BCA assay (Thermo Fisher) to ensure equal loading. Cell lysate, β-Me, and lamelli dye were heated at 90 C for 5 minutes. Lysates were loaded into a 4-20% PAGE SDS gel (BioRad), and run at 100 V for 40 minutes. The gels were removed and washed, and transferred onto a PVDF membrane at 75 V for 2 hours. Blots were washed with TBST, and blocked for one hour at RT in TBST containing 5% bovine serum albumin. Blots were washed and conjugated with primary antibody (directed towards: Glo-1, Glo-2, Nrf2, HO-1, IκB-α, P-IKK, IKKα/ß, ß-actin) in 5% BSA, rocking overnight at 4˚C. Blots were washed with TBST, and conjugated with appropriate secondary antibodies in TBST containing 5% BSA for one hour at room temperature. Blots were washed and imaged using Western Dura Super Signal (Thermo Fisher) on an Azure C600 imager (Azure Biosystems, Dublin, CA). Densiometric analysis was performed with ImageJ (Madison, WI).

ROS detection

Cerebellar primary neurons were cultured in 96-well plates as previously described. Cells were treated with CBD [10 μM] for 24 hours, and then additionally treated with MG [500 μM] for 24 hours. Media was removed, cells were washed with PBS, and incubated with 1 μM CM-H_2DCF-DA (Life Technology) in EBSS in dark at 37 ˚C for 30 minutes.

Fluorescence was recorded at 485 nm excitation and 520 nm emission on a BioTek scanner.

Immunocytochemistry

Cerebellar primary neurons were cultured in 96-well plates as previously described. Cells were treated with MG [500 μM] for 24 hours, and then additionally treated with CBD [10 μM] for 24 hours. Media was removed, wells were washed with PBS, cells were fixed for 30 minutes with a 30% sucrose solution containing 4% PFA, and washed. Cells were solubilized for 10 minutes with PBS containing 0.1% Triton X-100, and washed with PBS. Cells were blocked for 1 hour at room temperature with PBS containing 2% BSA. Blocking solution was removed, and cells incubated overnight at 4˚C with PBS containing 2% BSA and primary antibodies directed towards NeuN, cleaved caspase-3, VGLUT1, GAD65, and MAP2. Wells were washed with PBS and incubated with the appropriate fluorescent conjugated secondary antibodies at room temperature for 1 hour. Wells were washed, covered with DAPI stain [1 μg/mL] for 10 seconds, washed and aspirated. Prolong Gold Antifade (Thermo Fisher Scientific, MO) was added directly to each well, and allowed to cure in the dark overnight. Plates were imaged on CLS Operetta confocal microscope (PerkinElmer, Waltham MA). Statistical analysis was performed through quantification of fluorescence normalized to the number of DAPI positive cells.

Glyoxalase activity

Cerebellar primary neurons were cultured in 12-well plates as previously described. After MG and CBD treatment, media was removed, and cells rinsed with PBS. Cells were lysed with buffer (10 mM HEPES, 0.02% Triton X-100, and 100 μg/mL BSA), briefly sonicated, and centrifuged. Reaction solution (60 mM sodium phosphate, 4 mM GSH, and 4 mM MG) in a 96 well plate was briefly incubated, followed by addition of cell lysates. S-lactoylglutathione synthesis was determined by measuring absorbance (240 nm) on a

BioTek scanner. Protein concentration was determined using a BCA protein assay reagent kit.

D-Lactate concentration

D-Lactate released into the extracellular space was measured spectrophotometrically using collected cell media. Culture media samples were loaded on a 96-well plate with 0.2 M glycine and semicarbazide buffer containing 2 mg/mL NAD and 40 U/mL D-lactate dehydrogenase. Samples were incubated at room temperature for 2 hours. A spectrophotometer (340 nm excitation, 450 nm emission) was used to measure conversion of NAD to NADH. Absolute values were determined from a standard curve of D-lactate concentrations.

MG concentration

MG concentration in cerebellar neuron cultures was determined using dinitrophenylhydrazine (2,4-DNPH). The reaction consisted of 0.2 mM 2,4-DNPH with 1 mM MG and previously collected culture media. Samples were heated in a thermomixer at 42 °C for 45 mins and 600 rpm. Spectrophotometer measurements were performed at 432 nm, according to absorbance of MG-bis- 2,4-DNPH-hydrazone for calculating concentration of MG.

***C. elegans* strains and maintenance**

C. elegans were cultured as previously described. *C. elegans* were maintained on nematode growth medium (NGM) plates [Bacto Agar 1.7%, Bacto Tryptone 0.25%, NaCl 50 mM, KPO_4 25 mM, $CaCl_2$ 1 mM, $MgSO_4$ 1 mM, and cholesterol 5 µg/mL], or in liquid S media [5.85 g NaCl, 1 g K_2HPO_4, 6 g KH_2PO_4, 1 ml cholesterol (5 mg/ml), 10 ml 1 M potassium citrate pH 6, 10 ml trace metals solution, 3 ml 1 M $CaCl_2$, 3 ml 1 M $MgSO_4$] at 20 Cº. A single colony of *E. coli* OP50 was cultured in LB media to OD 0.1, and 100 µL of

was spread on NGM plates and incubated overnight at 37 ºC. Synchronous L1 nematodes were added to NGM plates for experiments. *C. elegans* cultures were washed and collected from starved NGM plates, and added biweekly to fresh NGM plates with OP50.

Culture synchronicity

Synchronous populations of L1 *C. elegans* were obtained by bleaching as previously described. NGM plates were washed with M9 media, and cultures collected for centrifugation (200 g, 2 min at 25ºC). The supernatant was removed, pellet washed with M9 media, and recentrifuged. After removing supernatant, 2 mL of a bleaching solution (2 mL of 8% bleach, 200 µL of 10M NaOH , and 8 mL H_2O) was added for 7 minutes, with gentle agitation every minute. Cultures were checked under a microscope to ensure all adult worms died. M9 media was added to cultures to stop the reaction. The solution was centrifuged (400 gx3 mins at 25º), supernatant removed, pellet washed, and recentrifuged. The pellet of eggs was resuspended in S media, and allowed to gently rock for 24 hours until eggs hatched. An equal amount of L1 nematodes were added to plates with M9 media for culturing. 5-Fluoro-2'-deoxyuridine (FUdR) was added to cultures on the first day of adulthood to sterilize and prevent egg laying of gravid adults.

***C. elegans* CBD Treatment**

CBD was dissolved in DMSO at a final concentration of 0.1%, and added to freshly poured NGM plates at a concentration of 100 µM. MG was added directly to freshly poured NGM plates at a concentration of 500 µM. All NGM plates contained FUdR [250 µM] to prevent egg laying. Age synchronized L4 *C. elegans* were added to NGM drug treatment plates that were changed biweekly.

C. elegans thermotolerance

Nematodes were cultured to adulthood as previously described. Age synchronized adult worms were transferred to fresh NGM plates and incubated at 35º C. Worms were monitored every 2 hours to determine survival.

C. elegans OS resistance

Nematodes were cultured to adulthood as previously described. Adult nematodes were transferred to 96 well plate with S-complete media, with varying concentrations of MG [100, 250, 500, 750, 1000 µM]. Survival was monitored every day until all worms were dead.

Statistics

All experiments were performed in triplicate, with values are presented as the mean +/- SEM. Significance was determined by Student t-test, with $p<0.05$ being statistically significant. # denotes statistical significance between the non MG-treated control and MG-treated control; * denotes statistical significance between the MG-treated and CBD treated and MG-treated control; + denotes statistical significance between non MG-treated control and non MG-treated CBD treatment. Data were evaluated using Excel and SPSS.

RESULTS

CBD on Glyoxalase Pathway Function.

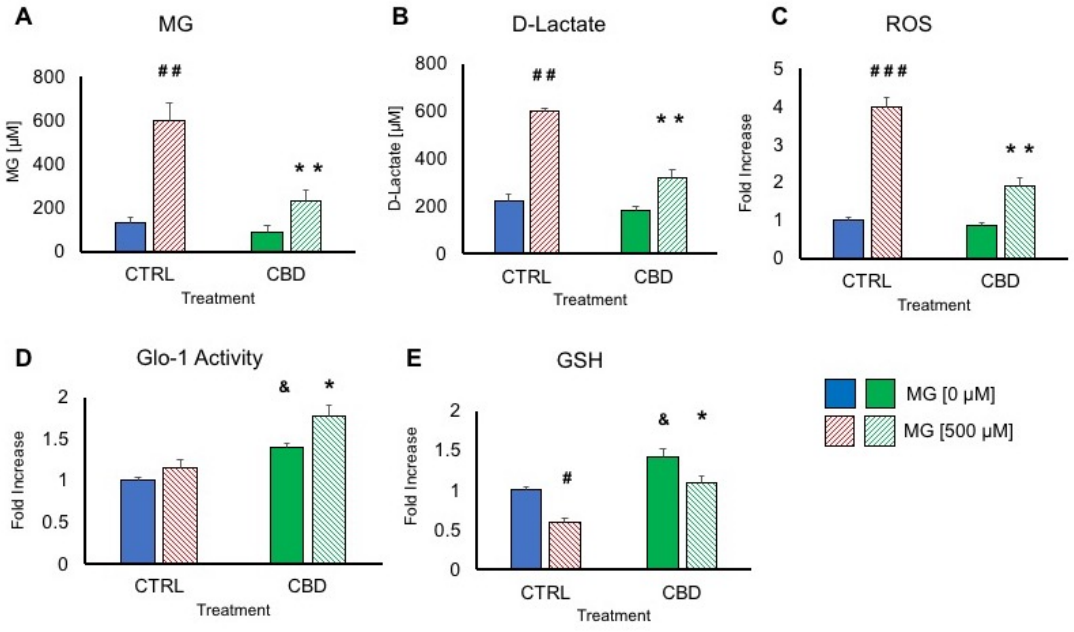

FIGURE 4-3: GP FUNCTION IN CEREBELLAR NEURONS.

CBD treatments on MG (**A**), D-lactate (**B**), ROS (**C**), Glo-1 protein activity (measured in mU/μg protein) (**D**), and GSH (**E**). Results are the mean +/- SEM of experiments performed in triplicate. #p<0.05, ##p<0.01, control compared to the MG-treated control; *p<0.05, **p<0.01 CBD and MG-treated compared to MG treated control; &p<0.05 CBD treated compared to control.

Cerebellar neurons treated insulted with MG and treated with CBD exhibited elevated function and activity of GP. Neurons insulted with MG [500 μM] exhibited elevated levels of MG compared to the non MG-treated control (# #p<0.01), which was significantly decreased (**p<0.01) upon treatment with CBD (**FIG 4-3A**). In accordance with this, MG treatment also resulted in elevated levels of D-lactate compared to the control (##p<0.01; however, in MG and CBD treated neurons we witnessed a concomitant elevation of D-lactate (**p<0.01), indicative of elevated MG detoxification (**FIG 4-3B**). We observed a drastic elevation in production of ROS upon MG treatment (###p< 0.005),

which CBD treatment significantly (**p<0.01) attenuated (**FIG 4-3C**). MG treatment is known to elevate Glo-1 activity, as cells respond to the oxidative insult. CBD treatment in non MG-treated cells significantly elevated Glo-1 activity compared to the control (&p<0.05), and also lead to an increase in Glo-1 activity in MG and CBD treated cells (*p<0.05) compared to the MG-treated control (**FIG 4-3D**). MG treatment depletes intracellular GSH levels, which was attenuated by CBD treatment (*p<0.05), and CBD treatment alone significantly elevated GSH levels (&p<0.05) compared to the non MG-treated controls (**FIG 4-3E**).

CBD Elevates Glyoxalase Protein Expression

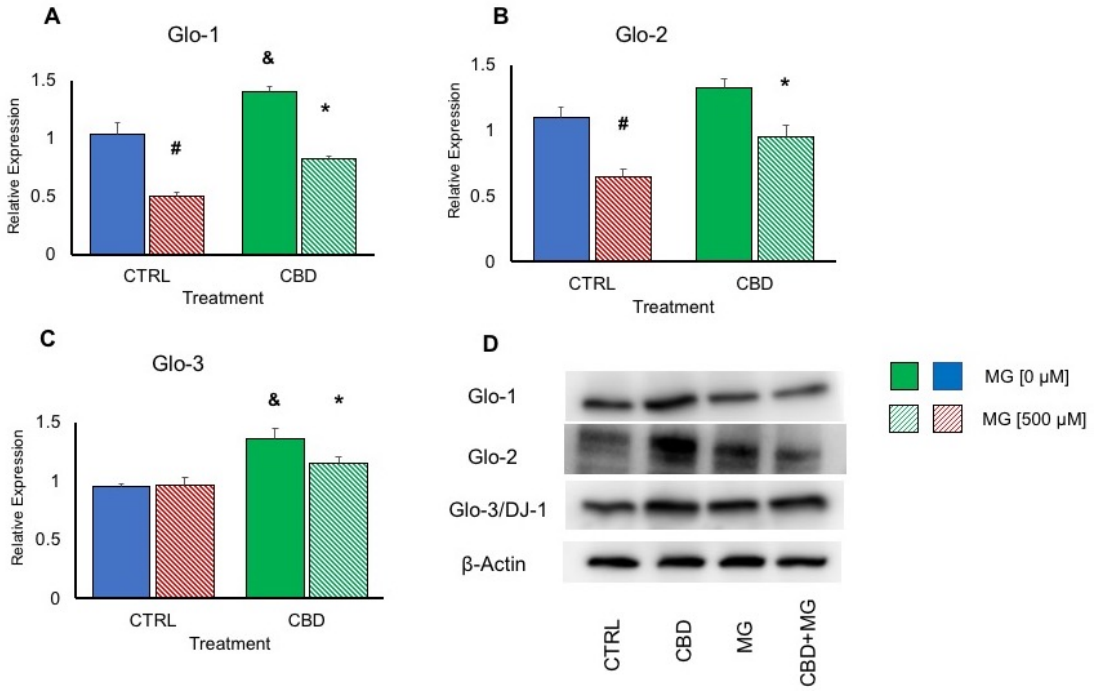

FIGURE 4-4: GLYOXALASE PROTEIN EXPRESSION.

Protein expression of Glo-1 (**A**), Glo-2 (**B**), and Glo-3 (**C**) in CBD and MG treated cerebellar neurons was determined through Western blotting (**D**). Results are the mean +/- SEM of experiments performed in triplicate. #p<0.05, ##p<0.01, control compared to the MG-treated control; *p<0.05, **p<0.01 CBD and MG-treated compared to MG treated control; &p<0.05 CBD treated compared to control.

Levels of glyoxalase protein expression in cerebellar neurons treated with MG and CBD was determined through Western blotting (**FIG 4-4D**). Glo-1 protein expression (**FIG 4-4A**) was significantly decreased with MG treatment (#p<0.05), and significantly increased with CBD treatment (&p<0.05) compared to the non-treated control. Treatment with both MG and CBD resulted in a significant increase (*p<0.05) compared to the MG treated cells. Glo-2 protein expression (**FIG 4-4B**) significantly decreased with MG treatment (#p<0.05) compared to the control, however treatment with both MG and CBD resulted in a significant increase (*p<0.05) in expression compared to the MG treated cells. CBD treatment alone increased protein expression, but not to a level of statistical

significance. DJ-1 – also known as glyoxalase 3 (Glo-3) – expression (**FIG 4-4C**) was significantly elevated with CBD treatment (&p<0.05) compared to the non-treated controls, and MG and CBD treatment resulted in a significant increase (*p<0.05) compared to the MG treated control. Here, CBD increased the expression of Glo-1,2 and 3 compared to control. We did not observe significant changes in protein expression upon MG treatment alone compared to the control.

CBD increases antioxidant protein expression

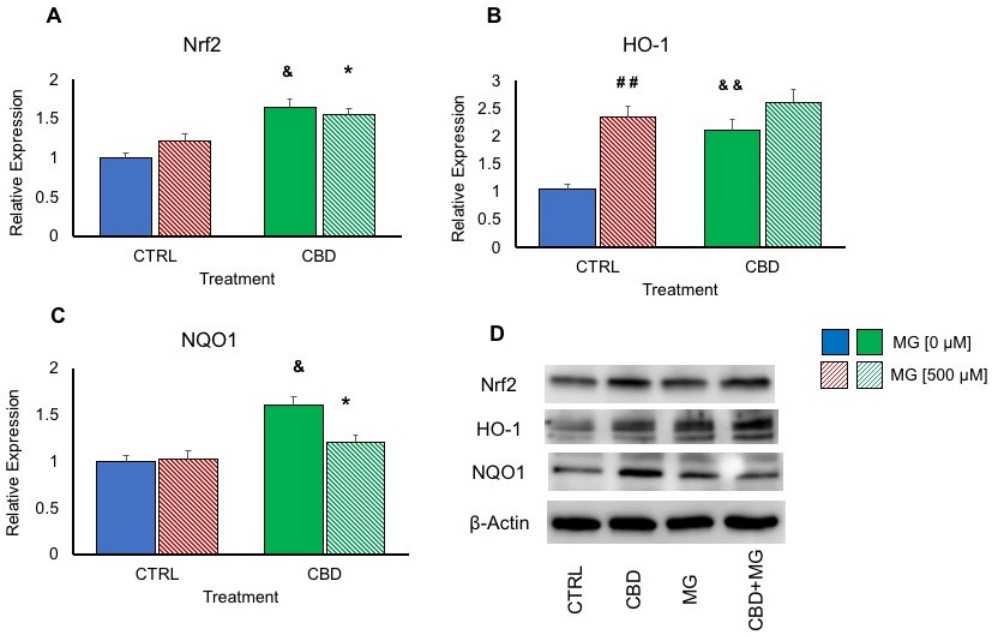

FIGURE 4-5: ANTIOXIDANT PROTEIN EXPRESSION.

Protein expression of Nrf2 (**A**), HO-1 (**B**), and NQ01 (**C**) in CBD and MG treated cerebellar neurons was determined through Western blotting (**D**). Results are the mean +/- SEM of experiments performed in triplicate. ##p<0.01, control compared to the MG-treated control; *p<0.05, CBD and MG-treated compared to MG treated control; &p<0.05, &&p<0.01 CBD treated compared to control.

Antioxidant protein expression levels in cerebellar neurons treated with MG and CBD was determined through Western blotting (**FIG 4-5D**). Nrf2 protein expression (**FIG 4-5A**) was significantly increased with CBD treatment (&p<0.05) compared to the non-treated control. Treatment with both MG and CBD resulted in a significant increase (*p<0.05) compared to the MG treated cells. HO-1 protein expression (**FIG 4-5B**) significantly increased with MG treatment (##p<0.01) and CBD treatment (&&p<0.01) compared to the non-treated control. NQO1 expression (**FIG 4-5C**) was significantly elevated with CBD treatment (&p<0.05) compared to the non-treated controls, and MG and CBD treatment resulted in a significant increase (*p<0.05) compared to the MG treated

control. Overall CBD increased the expression of HO-1, Nrf2, and NQO1. However, the effect is insensitive to MG treatment.

CBD modulates p38 cellular signaling

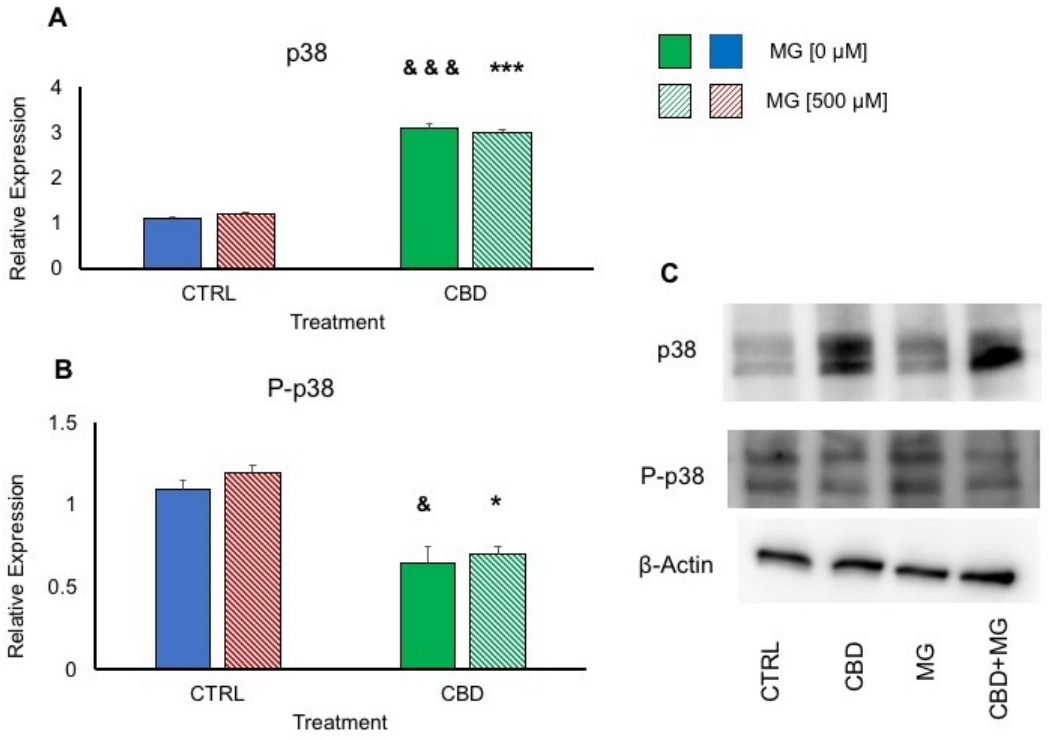

FIGURE 4-6: CELLULAR SIGNALING PATHWAYS.

Protein expression of p38 (**A**) and P-p38 (**B**) in CBD and MG treated cerebellar neurons was determined through Western blotting (**C**). Results are the mean +/- SEM of experiments performed in triplicate. ##p<0.01, control compared to the MG-treated control; *p<0.05, ***p<0.005 CBD and MG-treated compared to MG treated control; &p<0.05, &&&p<0.005 CBD treated compared to control.

Cellular signaling pathways in cerebellar neurons treated with MG and CBD was determined through Western blotting (**FIG 4-6C**). p38 protein expression (**FIG 4-6A**) was significantly increased with CBD treatment (&&&p<0.005) compared to the non-treated control. Treatment with both MG and CBD resulted in a significant increase (***p<0.005) compared to the MG treated cells. P-p38 protein expression (**FIG 4-6B**) was significantly decreased with CBD treatment (&p<0.05) compared to the non-treated control, and treatment with both MG and CBD resulted in a significant decrease (*p<0.05) compared to the MG treated cells.

CBD reduces SAPK/JNK activation

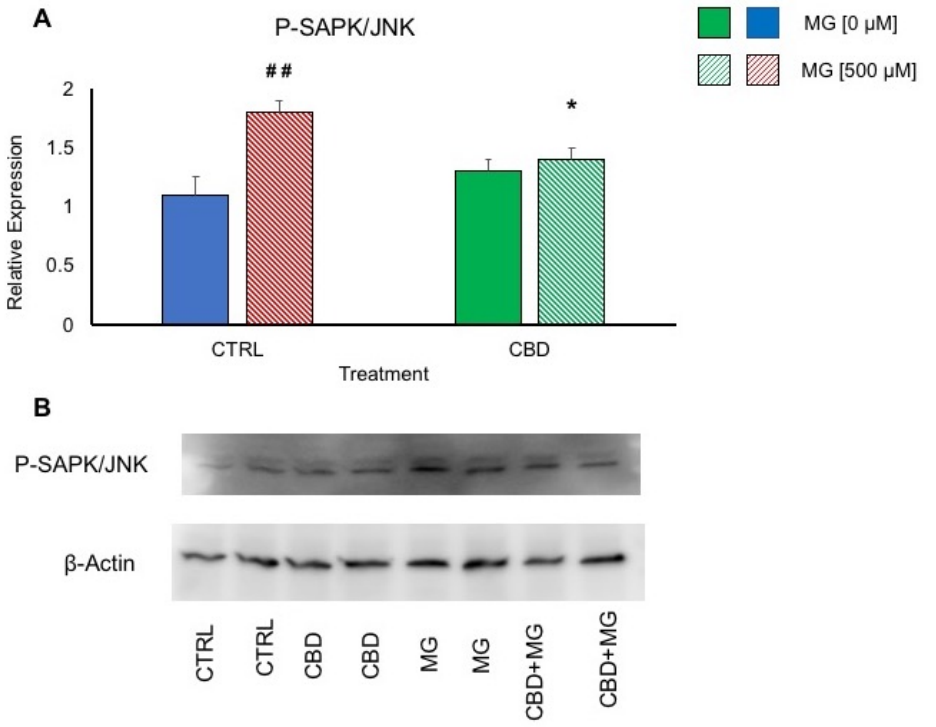

FIGURE 4-7: SAPK/JNK ACTIVATION.

Protein expression of P-SAPK/JNK (**A**) in CBD and MG treated cerebellar neurons was determined through Western blotting (**B**). Results are the mean +/- SEM of experiments performed in triplicate. ##p<0.01, control compared to the MG-treated control. *p<0.05 CBD and MG-treated compared to MG-treated control.

Activation of the SAPK/JNK cellular signaling pathway in cerebellar neurons treated with MG and CBD was determined through Western blotting (**FIG 4-7B**). Treatment with MG resulted in a significant decrease (##p<0.01) in P-SAPK/JNK (**FIG 4-7A**) compared to the nontreated control. Treatment with both MG and CBD resulted in a significant decrease (*p<0.05) of SAPK/JNK phosphorylation compared to the MG treated control. The CBD treatment also neutralized the effect of MG treatment on SAPK/JNK.

CBD increases excitatory and inhibitory neurotransmitter function

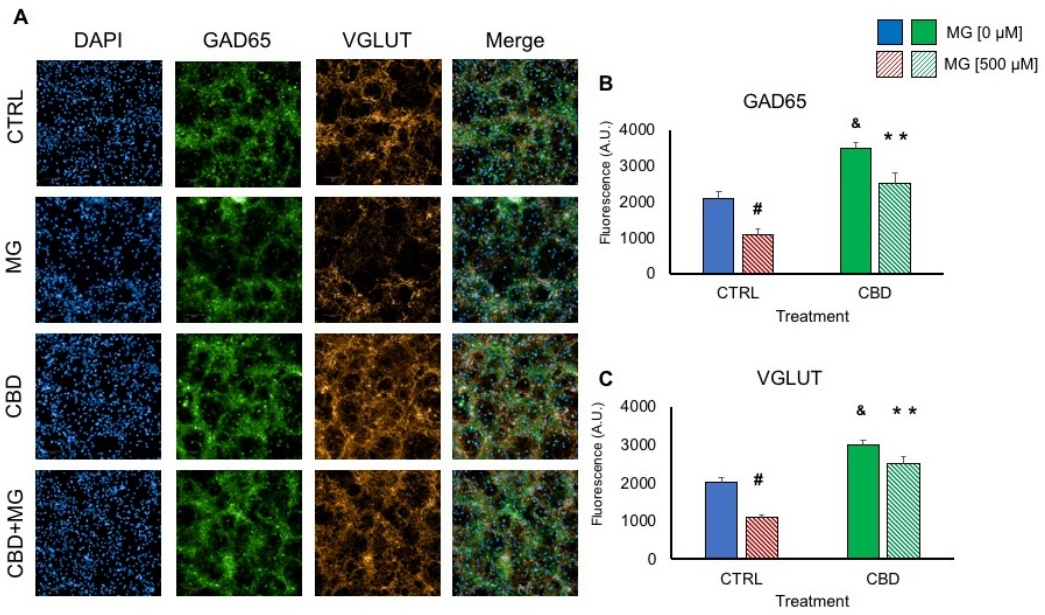

FIGURE 4-8: CEREBELLAR NEURON NEUROTRANSMITTER FUNCTION.

Representative confocal images (**A**) of GAD65 (**B**) and VGLUT (**C**) expression in CBD and MG treated cerebellar neurons. Results are the mean +/- SEM of experiments performed in triplicate. #p<0.05 control compared to the MG-treated control; **p<0.01 CBD and MG-treated compared to MG treated control; &p<0.05 CBD treated compared to control.

In cerebellar neurons insulted with MG [500 μM], treatment with CBD [10 μM] exhibited neuroprotective activity and resulted in retention of neurotransmitter function in cerebellar neurons (**FIG 4-8A**). Inhibitory neurotransmitter activity was determined through measuring fluorescence expression of GAD65 (**FIG 4-8B**). Treatment with MG resulted in a significant decrease (#p<0.05) of GAD65 expression compared to the control. Treatment with CBD resulted in a significant increase (&p<0.05) of GAD65 compared to the control. Neurons treated with MG and CBD resulted in a significant increase (**p<0.01) of GAD65 expression compared to the MG treated control. Excitatory neurotransmitter activity was determined through measuring fluorescence expression of VGLUT (**FIG 4-8C**). Treatment with MG resulted in a significant decrease

(#p<0.05) of VGLUT expression compared to the control. Treatment with CBD resulted in a significant increase (&p<0.05) of VGLUT expression compared to the control. Treatment with both MG and CBD resulted in a significant increase (**p<0.01) of VGLUT expression compared to the MG treated control.

CBD increases *C. elegans* Lifespan

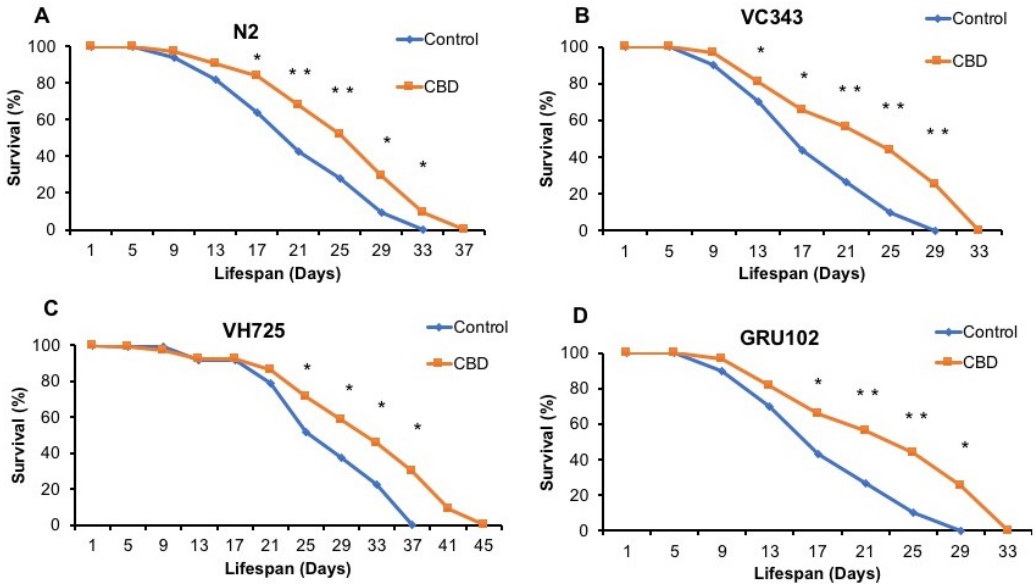

FIGURE 4-9: CBD INCREASES LIFESPAN IN C. ELEGANS.

CBD treatment increased lifespan and longevity in C. elegans strains N2 (**A**), VC343 (**B**), VH725 (**C**), and GRU102 (**D**). Results are the mean +/- SEM of experiments performed in triplicate. *p<0.05, **p<0.01 CBD treated compared to control.

C. elegans strains were treated with CBD [100 μM] and the effect on lifespan and longevity was determined. There was a significant increase in survival at various timepoints in different *C. elegans* strains: N2 (**FIG 4-9A**) exhibited elevated survival at days 17 (*p<0.05), 21(**p<0.01), 25(**p<0.01), 29 (*p<0.05), and 33 (*p<0.05); VC343 (**FIG 4-9B**) 13 (*p<0.05), 17 (*p<0.05), 21(**p<0.01), 25(**p<0.01), and 29(**p<0.01); VH725 (**FIG 4-9C**) 25 (*p<0.05), 29 (*p<0.05), 33 (*p<0.05), and 37 (*p<0.05); GRU102 (**FIG 4-9D**) 17 (*p<0.05), 21(**p<0.01), 25(**p<0.01), and 29 (**p<0.01).

MG Treatment Reduces *C. elegans* Lifespan.

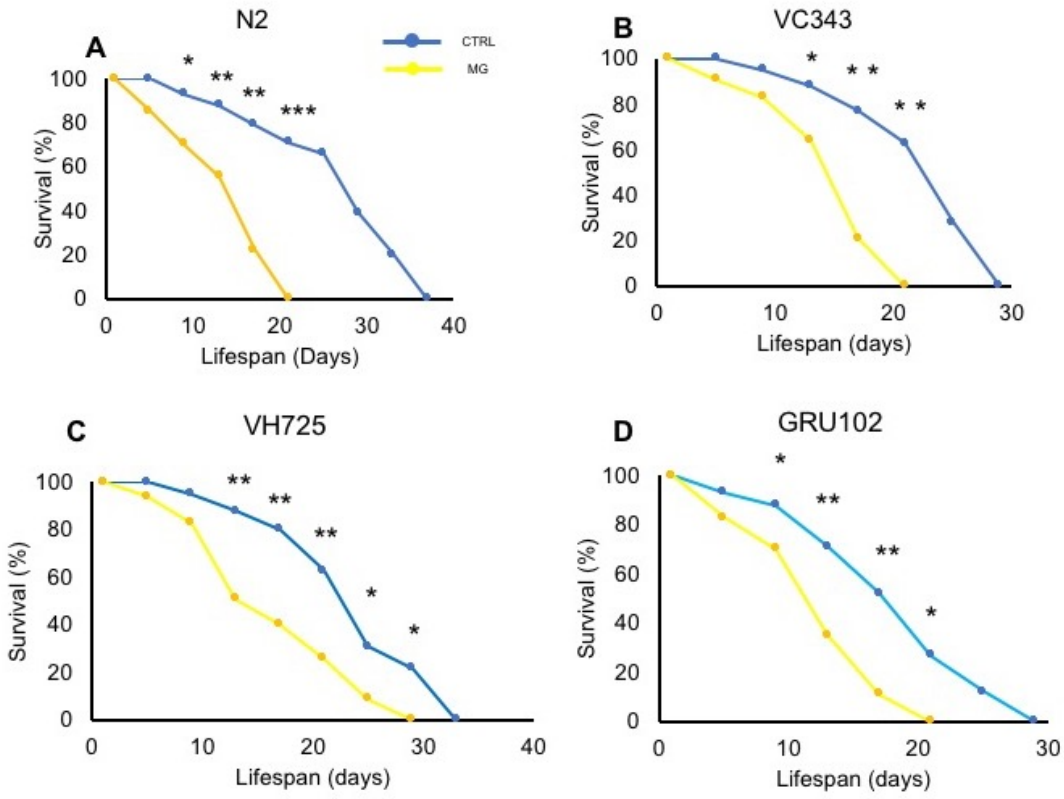

FIGURE 4-10: MG REDUCES C. ELEGANS LIFESPAN.

The effect of MG on lifespan of C. elegans strains N2 (**A**), VC343 (**B**), VH725 (**C**), GRU102 (**D**) was determined. Results are the mean +/- SEM of experiments performed in triplicate. *p<0.05, **p<0.01 CBD treated compared to control.

The effect of MG treatment [1 mM] on *C. elegans* lifespan was determined. There was a significant decrease in survival at various timepoints in different *C. elegans* strains: N2 (**FIG 4-10A**) 10 (*p<0.05), 15 (**p<0.01), 20 (**p<0.01), and 25 (***p<0.005), VC343 (**FIG 4-10B**) 12 (*p<0.05), 16 (**p<0.01), 20(**p<0.01), and 24(**p<0.01); VH725 (**FIG 4-10C**) 12 (**p<0.01), 16 (**p<0.01), 20 (**p<0.01), 24 (*p<0.05), and 28 (*p<0.05); GRU102 (**FIG 4-10D**) 8 (*p<0.05), 12(**p<0.01), 16(**p<0.01), and 20 (*p<0.05).

MG and CBD treatment on *C. elegans* Lifespan

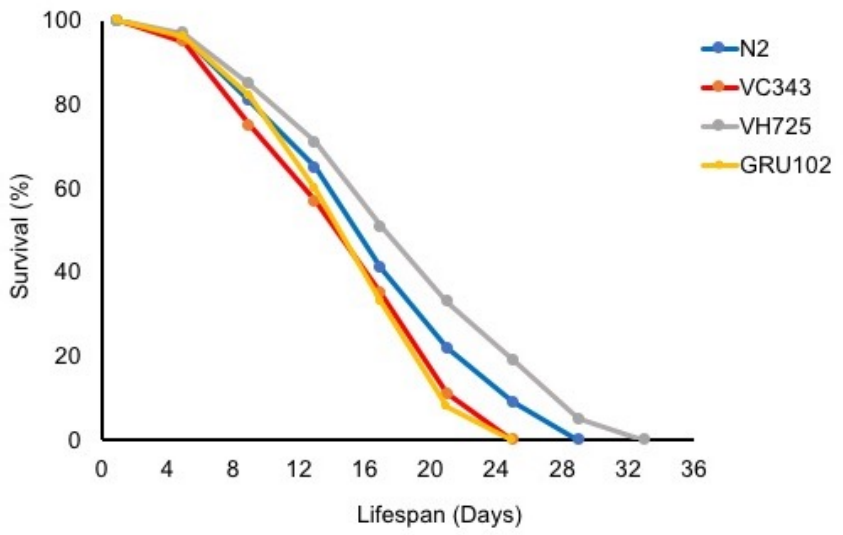

FIGURE 4-11: CBD AND MG TREATMENT ON C. ELEGANS LIFESPAN.

All *C. elegans* strains exhibited a significant (*p<0.05) increase in average lifespan compared to the MG treated *C. elegans*. Results are the mean +/- SEM of experiments performed in triplicate.

The effect of combination treatment of CBD [100 μM] and MG [1 mM] on lifespan was determined in *C. elegans* strains (**FIG 4-11**). While MG treatment alone significantly reduced *C. elegans* lifespan, the combination treatment with CBD exhibited a protective effect against MG toxicity. Compared to MG treatment alone, the combination treatment resulted in significant (*p<0.05) lifespan increases in N2 (7 days), VC343 (6 days), VH725 (6 days), and GRU102 (6 days).

Oxidative Stress and Thermotolerance Resistance in *C. elegans*

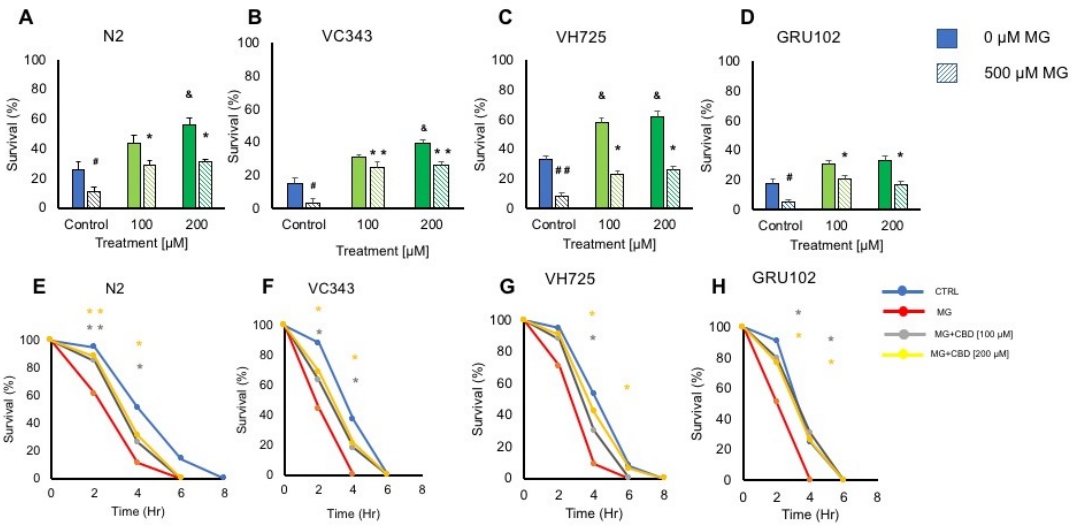

FIGURE 4-12: OXIDATIVE STRESS AND THERMOTOLERANCE RESISTANCE IN C. ELEGANS.

Resistance to OS induced by H2O2 was measured in N2 (**A**), VC343 (**B**), VH725 (**C**), and GRU102 (**D**). Thermotolerance was measured in N2 (**E**), VC343 (**F**), VH725 (**G**), and GRU102 (**H**). Results are the mean +/- SEM of experiments performed in triplicate. #p<0.05, ##p<0.01, control compared to the MG-treated control; *p<0.05, **p<0.01 CBD and MG-treated compared to MG treated control; &p<0.05 CBD treated compared to control.

C. elegans strains were treated with H_2O_2 to induce OS and treated with CBD [100 or 200 µM], MG [1 mM], or a combination. Results showed a significant decrease in survival in MG treated *C. elegans* strains N2 (#p<0.05) (**FIG 4-12A**), VC343 (#p<0.05) (**FIG 4-12B**), VH725 (##p<0.01) (**FIG 4-12C**), and GRU102 (#p<0.05) (**FIG 4-12D**). In all strains of *C. elegans*, treatment with CBD lead to a significant increase in survival. There was not significant survival determined between treatments of 100 or 200 µM CBD. Their survival in the MG treated condition was miniscule, and significantly increased upon treatment with CBD at 100 and 200 µM. The Glo-1 overexpressing strain VH725 exhibited significantly extended survival with CBD treatment, and significantly extended survival in the CBD and MG combination treatment. The survival of N2 and VH725 *C. elegans* was not significant.

CBD Reduces Aβ Expression in GRU102 *C. elegans*

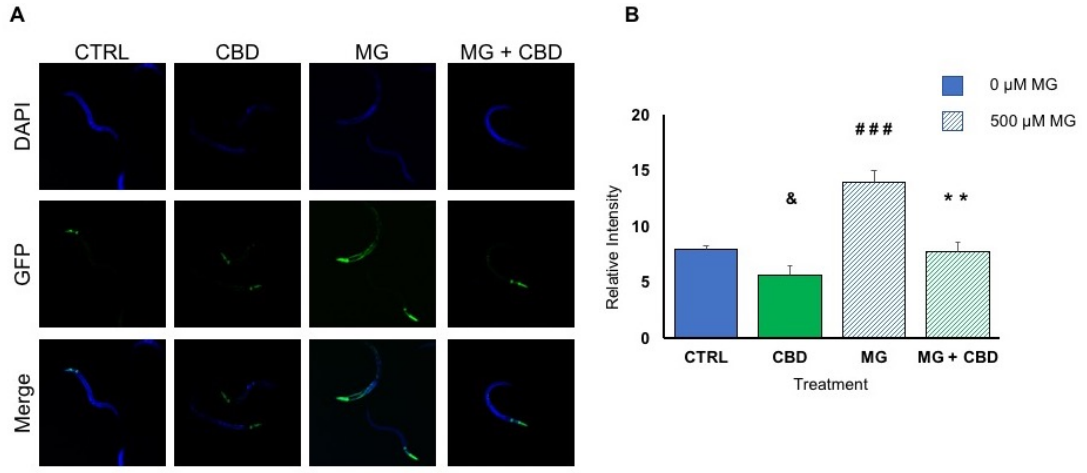

FIGURE 4-13: Aβ EXPRESSION IN GRU102 C. ELEGANS.

Representative confocal images (**A**) of GRU102 *C. elegans* treated with MG and CBD (**B**). Results are the mean +/- SEM of experiments performed in triplicate. ###p<0.005, control compared to the MG-treated control; **p<0.01 CBD and MG-treated compared to MG treated control; &p<0.05 CBD treated compared to control.

Aβ expressing GRU102 *C. elegans* were treated with CBD [100 µM], MG [1 mM], or a combination, and visualized through confocal microscopy (**FIG 4-13A**) to determine GFP expression of Aβ. CBD treatment reduced the fluorescence expression of Aβ in mutated *C. elegans* (**FIG 4-13B**). Insulting *C. elegans* with MG resulted in significantly elevated GFP expression of Aβ (###p<0.005), which was drastically reduced upon treatment with CBD (**p<0.01). CBD treatment alone also lead to a significant reduction (&p<0.05) in fluorescence expression in the control. MG treatment significantly increased expression of Aβ in GRU102 C. elegans. Treatment with CBD significantly lowered Aβ expression, and the combination treatment lowered Aβ expression to levels indistinguishable from the control group.

DISCUSSION

While phytocannabinoids have been long utilized for their medicinal properties, their exact mechanisms of pharmacological activity have only recently been elucidated.[190,191,208] Cannabinoids are separated into three groups: phytocannabinoids produced by the C. sativa plant, endogenously produced endocannabinoids, and synthetic cannabinoids.[190] In addition to the over 100 identified phytocannabinoids, C. sativa produces a host of other secondary metabolites including terpenes, sterols, polyphenols, and flavonoids.[189,193,209] These chemical compounds are synthesized primarily in the flowers of the female plants, and secreted through the trichrome glands; the leaves, stalks, and root matter also contain secondary metabolites, although in significantly smaller concentrations.[191,208,209] The most abundantly produced phytocannabinoids are ∆9-tetrahydrocannabinol (THC) and CBD, with cannabigerol (CBG), cannabinol (CBN), and cannabichromene (CBC) present in lower concentrations.[210,211] The phytocannabinoids are synthesized in the carboxylic acid form in the plant, and must be decarboxylated and converted to the neutral form for bioactivity.[189,191,210] A large part of CBD's utility stems from its lack of psychoactivity - mediated through its low affinity for CB1 – unlike THC.[190,212] CBD is a very durable and pharmacologically valuable compound with antibacterial, antiviral, and anti-cancer properties, in addition to its well documented antioxidant and anti-inflammatory activity.[190,213,214] It is neuroprotective in a variety of illnesses including epilepsy, ischemia, MS, HD, PD, and AD.[171,196,200,205,215-220] The mechanism of CBD's neuroprotective activity involves modulation of signaling molecules, suppressing inflammatory cytokines while increasing production of anti-inflammatory cytokines and compounds.[196,198,203,205,221] In an experimental autoimmune encephalitis model of multiple sclerosis, CBD attenuated the neuroinflammation mediated by autoreactive T cells.[218] CBD is also able to modulate

neuroinflammatory immune response by preventing overactivation of glial cells, a common hallmark in neurodegenerative diseases.[215,218,222-224] CBD treatment downregulated gene transcription of pro-inflammatory cytokines (IL-17, IL-6, IL-1β, TNF-α), while simultaneously upregulating gene transcription of anti-inflammatory cytokine IL-10.[218] In AD, CBD has shown evidence of multiple mechanisms of neuroprotection.[154,171,217] CBD was shown to attenuate Aβ toxicity by reducing the production of TNF-α, IL-1β, and NO through activation of PPAR-γ and inactivation of NF-κB. CBD is able to directly modulate the expression of AD pathology, through ubiquitination of APP and reducing reactive Aβ production.[194]

The activity and molecular effects of CBD are determined and mediated through the structure activity relationship (SAR) of the chemical compound.[189,190,212] Thus, the specific composition and arrangement of CBD is inexorably linked to its bioactivity.[189,190,212] CBD is conferred its antioxidant activity through several structural characteristics: hydroxyl groups of the B phenolic ring, the methyl group of the A cyclohexene ring, and the pentyl side chain.[190,194,212] The key structural features of CBD's antioxidant effects are the aromatic B ring hydroxyl groups.[199,203,204] CBD exists in several resonance structures, where unpaired electrons are located on the benzene ring and alkyl side chains.[225] This mediates direct antioxidant activity through proton transfer and electron donation with ROS and other free radical molecules.[192] The aromatic hydroxyl groups also afford amino acid and nucleic acid binding ability.[204] Both the number and location of hydroxyl groups are essential for antioxidant activity.[184,185,204] Although CBD and THC are structurally very similar, the CBD contains two hydroxyl groups on the B ring compared to a single hydroxyl group of THC. [204,226] Consequently, CBD was found to be a better free radical scavenger and have higher antioxidant activity than THC. [226] The same structural characteristics that impart antioxidant activity in CBD

are shared in flavonoid compounds (**FIG 4-14**). CBD and our previously utilized flavonoids – catechin, morin, and quercetin - possess similar carbon backbone.[19,184,185] The di-hydroxylation of the B ring is an essential component of antioxidant activity, which is shared by CBD, morin, quercetin, and catechin.[118,184,185] However, the arrangement of hydroxyl groups is an important factor in activity. The meta configuration of hydroxyls at 2' and 6' is shared by both morin and CBD, while quercetin and catechin possess an ortho configuration of hydroxyls at 4' and 5'.[184,185,199] Flavonoids with a resorcinol structure we found to have higher antioxidant activity than either catechol or hydroquinone structures.[51,119,120,163]

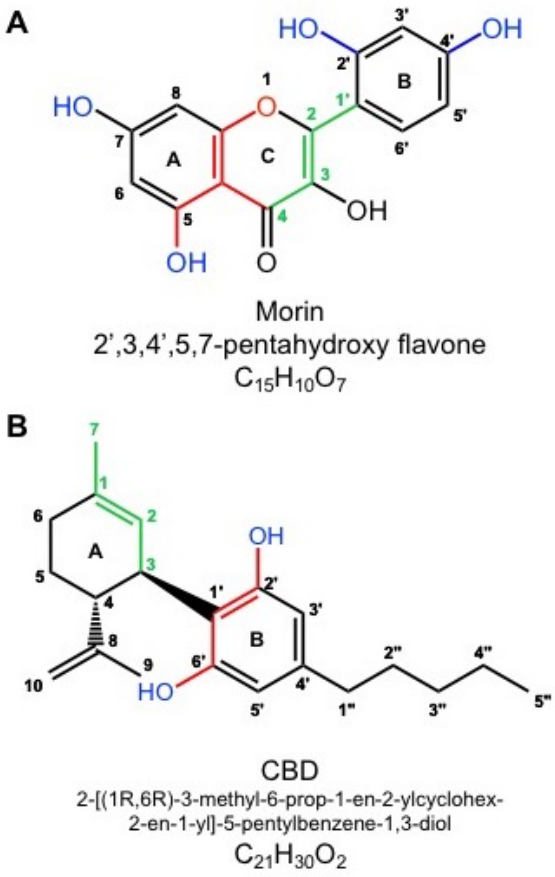

FIGURE 4-14: STRUCTURAL SIMILARITIES OF CBD AND MORIN.

The presence of multiple hydroxyl groups (blue), heterocyclic rings with C=C (green), and 3-carbon alcohol moieties (red) confer antioxidant activity in both morin and CBD.

Linked heterocyclic rings - including a phenol ring and C=C bond – confers chemical activity and are present in CBD and flavonoids.[118-120] Another consideration of activity is the spatial arrangement of ring structures. Both CBD and morin possess rings (A and B, respectively) that are in almost perpendicular planes, which influences binding activity.[19,116,120] For example, THC and flavonoid apigenin exist in a rigid, planar configuration, and were determined to have decreased antioxidant activity compared to CBD and non-planar flavonoids. [19,75,76,116,226] Direct redox regulation by CBD occurs

through modulation of both pro and anti-oxidant molecules.[199,203] CBD can bind ROS, metal ions, and other reactive metabolic byproducts, preventing their interaction with cellular macromolecules and avoiding excessive lipid and protein oxidation.[197,199,202-204,227] *In vitro* assays determined CBD exhibits higher activity than α-tocopherol, the standard chemical used to determine antioxidant activity.[197,199] However, the indirect modulation of apoptotic and anti-inflammatory signaling pathways is the primary mechanism of CBD's antioxidant activity; mediating a response through induction or suppression of antioxidant and apoptotic signaling pathways, respectively.[199,203,204,218,221] CBD can act on transient receptor potential vanilloid receptors (TRPV), peroxisome proliferator-activated receptors (PPAR-γ), 5-hydroxy tryptophan receptors (serotonin), and adenosine receptors, to modulate inflammatory activity at both the protein expression and transcription levels.[199] In our research, limited CBD treatment activated Nrf2 signaling as evidenced by the elevated transcription and expression of antioxidant proteins including Nrf2, HO-1, GSH, SOD.[218] We also witnessed elevated protein expression of Glo-1, Glo-2, and Glo-3 in CBD treated neurons insulted with MG, in a Nrf2 dependent manner. Our measures of GP activity were enhanced and improved with CBD treatment. Cerebellar neurons insulted with MG and treated with CBD exhibited significantly lower levels of MG and ROS, with concomitant elevation in D-lactate, GSH, and Glo-1 activity.

It was previously believed that flavonoids – and other antioxidant compounds – exhibited a protective effect through direct neutralization with ROS and inflammatory molecules; however, the physiological concentration of dietary flavonoids is a far smaller molar equivalency than ROS produced in the body.[18,51,55,76,117,122,136] More recent research suggests the protective function of flavonoids is indirect, through modulation of endogenous cellular signaling pathways.[1,19,51,75,116,132] Antioxidant compounds – like

flavonoids and phytocannabinoids – can activate or inhibit cytoprotective and apoptotic pathways, respectively.[11,58,125,132,135,148,150,151,198,218] Upon stimulation via ROS, UV irradiation, and inflammatory compounds, SAPK/JNK proteins enter the nucleus and act as transcription factors to induce protein expression. MAPK activation is associated with caspase activation and cell death, mediated in part through the NF-κB pathway.[60,124,225,228] In a mouse model of aging, hippocampal astrocytes exhibited elevated ROS production and a concomitant activation of SAPK/JNK.[80] MAPK protein p38 is also involved in Ca^{2+} mediated apoptosis. Elevated production of ROS causes phosphorylation of p38, inducing C-AMP response element binding protein (CREB) inactivation.[80] Hypertension and cardiovascular disease also exhibit elevated inflammatory cytokine signaling and ROS production.[84] In a rat model of neural hypertension, chronic elevated angiotensin elevated NADPH oxidase-produced ROS, and phosphorylation and activation of SAPK/JNK and p38, resulting in glutaminergic neuron dysfunction.[84] Activation of SAPK/JNK and other MAPKs is exhibited in AD, and is exacerbated by the presence of Aβ.[225] Insoluble Aβ peptides induce the production of ROS, and can directly stimulate the activation of SAPK/JNK.[225] In a high-glucose model of neural apoptosis, treatment of cells with p38 and SAPK inhibitors attenuated apoptosis, and reduced phosphorylation levels of the proteins.[225] In our research, we observed a significant increase in SAPK/JNK phosphorylation upon treatment with MG, however CBD treatment attenuated SAPK/JNK activation. CBD treatment – with and without MG – resulted in elevated expression of p38 compared to the controls. In accordance with this, phosphorylated p38 was significantly decreased in CBD treated cells, while the MG-treated condition exhibited elevated P-p38. A model of diabetes-associated cognitive decline utilized high glucose conditions to induce markers of OS in SH-SY5Y cells, which was attenuated with quercetin pretreatment, and Nrf2

activators.[165] It was determined that inhibition of p38 had an influence on Nrf2 phosphorylation and nuclear translocation; preventing activation of p38 through pretreatment with a p38 inhibitor resulted in decreased presence of AGEs, elevated Nrf2 nuclear translocation and activation.[165] In our research we found that MG treatment elevated expression of P-p38, while treatment with CBD prevented phosphorylation of p38, even in the presence of MG. In accordance with the previous report, antioxidant treatment of neurons with CBD elevated Nrf2 levels, while preventing activation of inflammatory cell signaling pathways.

Imbalanced excitatory and inhibitory neurotransmission is common in neurological disorders, and contributes to the elevated excitotoxicity and apoptosis present in neurodegenerative conditions.[214] Our research determined limited CBD as possessing neuroprotective activity in both inhibitory and excitatory neurotransmitter function, as measured through fluorescence expression of GAD65 and VGLUT. Cerebellar neurons incubated with CBD exhibited significantly elevated fluorescence expression of GAD65 and VGLUT compared to MG treated controls. CBD also protected neural morphology, as evidenced by the elevated expression of structural protein MAP2 compared to MG treated cells.

CBD exhibited remarkable protective effects *in vitro*, and we desired to replicate this success *in vivo* through utilizing model organism *C. elegans*. For our experiments, we used several strains of *C. elegans* possessing genetic mutations in Glo-1 (VC343 and VH725) and Aβ (GRU102) in addition to the WT (N2). The lifespan of *C. elegans* is known to be decreased in the presence of oxidative and inflammatory environments.[186,188,229,230] We determined MG treatment to significantly decrease the lifespan of all strains, compared to untreated controls. Strains VC343 and GRU102 featured increased susceptibility to MG-mediated OS, due to their deficits in endogenous

antioxidant activity. The Glo-1 overexpressing strain VH725 had a less significant decrease in lifespan, a consequence of its elevated Glo-1 protein expression. In all strains, CBD treatment exhibited an extension of lifespan compared to non-treated controls. CBD and MG cotreatment resulted in a decrease of lifespan compared to the CBD only and control conditions, however there was a significance in lifespan length compared to the MG treatment condition. *C. elegans* are sensitive to oxidative changes in environments, including elevated H_2O_2, UV, and temperature.[186,188,229,230] To determine the effect of CBD and MG on environmental stressors in *C. elegans*, we performed OS and thermotolerance experiments to investigate survival under extreme conditions. *C. elegans* were pretreated with CBD, MG, or a combination and exposed to either H_2O_2 or increased temperatures. In the OS tolerance assay, CBD exhibited a protective effect in all strains exposed to H_2O_2. As expected, MG pre-treated *C. elegans* had significantly decreased survival under oxidative conditions. All combination treatments exhibited in elevated survival compared to the MG treated condition. The antioxidant compromised VC343 and GRU102 *C. elegans* had lower survival percentages compared to the N2 and VH725 strains. CBD treatment also conferred elevated thermotolerance to all strains, whereas MG treatment reduced survival. All CBD and MG combination treatments exhibited elevated thermotolerance compared to the MG only condition. CBD pretreatment conferred protection against elevated levels of OS and temperature. Although we did not investigate the exact mechanism, CBD is known to activate antioxidant signaling pathways and induce the expression of cytoprotective proteins against ROS, and heat-shock proteins to prevent instability and denaturing of proteins.[199-202] To investigate the effect of CBD and MG on production of AD pathology, we utilized GRU102 *C. elegans*, which harbor a mutation causing them to overexpress Aβ protein. Because the mutation is tagged with green fluorescent protein (GFP), we were able to observe expression changes through confocal microscopy. *C. elegans*

treated with MG exhibited elevated GFP expression, indicative of increased Aβ burden. CBD treatment significantly decreased Aβ expression compared to the non/MG-treated controls. *C. elegans* treated with both CBD and MG exhibited a blunting of GFP expression compared to the MG treated control. In this experiment, results indicated that CBD is protective against Aβ *in vivo*, possibly through decreased expression or disrupted fibrillation of Aβ. CBD was determined to have a protective effect against MG in *C. elegans* strains, regardless of the specific mutations they harbored.

Through measurement of GP function, constituent proteins, and markers of antioxidant function, we determined CBD to be an effective enhancer of the GP. Under states of MG-mediated OS, CBD treatment induced an antioxidant response that effectively attenuated the insult from MG treatment. Our hypothesis that limited CBD enhanced GP function through modulation of cell signaling pathways was confirmed, and we are the first to report CBD's effect on the GP. Our use of both primary neurons and model organisms provides evidence that CBD is effective in GP enhancement in both *in vitro* and *in vivo* models of MG-mediated OS. We determined CBD's neuroprotective effect is mediated through modulation of cellular signaling pathways, and not necessarily directly involved in MG and ROS neutralization.

CHAPTER 5: CONCLUSION

Neurodegenerative conditions exhibit progressive severity in disease pathology, of which overproduction of ROS and reactive metabolic byproducts is prominently featured.[5,10,12,19] AD and aging features deficits in endogenous responses contribute to the imbalance of antioxidant and pro-oxidant molecules, resulting in chronic activation of apoptotic and inflammatory cellular signaling pathways.[3,7,8,93] The pathological hallmarks of AD - Aβ, NFT, and loss of brain tissue – can be exacerbated by deficiencies in endogenous antioxidant systems, in which MG accumulation and elevated ROS production plays a prominent role.[10,13,15,31,92,148] GP dysfunction has a direct role in the development and progression of AD pathology, and contributes to the cognitive decline present in the disease.[1,3,21,22,26,37,49,64,65,87,128] However, pharmacological modulation of the GP can attenuate oxidative damage, reduce the pathological burden, and lessen cognitive deficits associated with aging and AD.[1,20,34,37,38,48,49,54,57,58,87,128] Humans have long utilized naturally-produced plant compounds for therapeutic uses, and the specific mechanisms of their functions have only recently been elucidated.[51,116] The Free Radical Theory of Aging posits that elevated production of ROS and free radicals is the contributing factor in human diseases, however the primary mechanism of damage from free radical production occurs through modulation of cellular signaling pathways.[2,18,75,76] It was previously believed that the benefits of flavonoids functioned through direct binding with ROS, however the concentration of dietary antioxidant molecules would not be sufficient enough to offset the elevated concentration of ROS found in aging and AD.[9,18,37,51,55,75,76,92,116,121,122,136] Thus the protective effect of flavonoids was determined to be modulation of antioxidant and apoptotic signaling pathways. [123,127,147,151,152,156,158,161] Our first research project focused on three flavonoid compounds – catechin, morin, and quercetin – and their effect on the GP.[37] We investigated the mechanism of flavonoid enhancement of GP function through evaluating glyoxalase proteins, markers of neurotransmitter function, and antioxidant signaling activation.[37] We determined the GP-

enhancing ability of the aforementioned compounds was mediated through activation of the antioxidant Nrf2 signaling pathway.[37] The flavonoid compounds possessed the ability to attenuate oxidative damage from MG, which is commonly elevated in diseases of aging and neurodegeneration.[37] While all compounds exhibited GP enhancing ability, our subsequent research project focused on improving morin's inherent antioxidant ability through chemical structural additions to the parent compound.[128]

Metabolic degradation and poor BBB permeability are likely factors in the lack of success flavonoids have exhibited in *in vivo* replicates.[131] We modified the morin parent structure - through addition of two bromine molecules, and encapsulation in a polymer nanoparticle – to produce DBM and MNP and tested efficacy in GP enhancement.[128] We repeated previous experimental techniques utilizing DBM and MNP in primary cerebellar neurons and *C. elegans*.[128] *C. elegans* are useful organisms in determining effectiveness of chemical compounds on aging and longevity, due to their genetic homology with humans and relatively short lifespan.[186-188,229-231] We determined the structural additions to be beneficial in elevating the capacity of the GP compared to the unmodified morin compound itself.[128] DBM and MNP exhibited potent antioxidant and neuroprotective properties, and attenuated OS-mediated damage through mechanisms involving induction of Nrf2 signaling and inhibiting NF-κB-mediated apoptosis.[128] While results were successful, the synthesized compounds most promising activity needs to be tested in higher level organisms, to determine efficacy in preventing premature metabolic breakdown and BBB permeability. Our final chapter of original research utilized limited phytocannabinoid CBD to investigate its effect on the GP. While CBD has well-established antioxidant and neuroprotective activity in neurodegenerative disorders, we aimed to elucidate its effect in an MG-mediated model of aging found in AD.[154,197,199,201-204] We are the first to report results on the effect of CBD in the GP in a neural model of

MG-mediated OS. Our results showed CBD had a protective effect against MG-mediated toxicity in both primary cerebellar neurons and *C. elegans* strains featuring overexpression and knockdown of Glo-1 (VH725 and VC343, respectively), and a mutation in Aβ (GRU102). As with our previous research, we intended to elucidate the cellular signaling pathways involved in CBD's protective effect. While we found similarities in the effect of CBD and flavonoids – no doubt due to the structural similarities shared between the classes of chemical compounds – we also observed differing modulation of apoptotic and antioxidant signaling pathways. We found that the antiapoptotic effect of both flavonoids and CBD was mediated through inhibition of NF-κB pathway signaling, albeit through different pathway mechanisms. Flavonoids were found to inhibit activation through modulation of direct NF-κB complex proteins, and CBD through modulation of stress-activated signaling pathways – SAPK/JNK and p38. Both classes of compounds induced expression of antioxidant proteins through Nrf2, the primary transcription factor and activator of the ARE, and thus exhibited similar protein expression patterns in glyoxalase proteins, HO-1, and GSH. CBD and flavonoids were found to attenuate the damaging effects of MG accumulation, which occurs in a host of inflammatory diseases within and outside the CNS, through enhancement of the GP.[25,86] Our results show that secondary plant metabolites of different structural classifications can increase the capacity of the GP and allow for the retention of neurotransmitter activity and neuron morphology even in the presence of a cytotoxic and highly inflammatory cellular environment. These findings necessitate further research in higher organisms to fully elucidate the mechanisms of protection the GP, and how it can be best manipulated for therapeutic effects in aging and AD.

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