ACS Chemical Neuroscience

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ACS Chem. Neurosci., **Just Accepted Manuscript** • DOI: 10.1021/acschemneuro.9b00566 • Publication Date (Web): 07 Jan 2020

Just Accepted

is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

Neural Glyoxalase Pathway Enhancement by Morin Derivatives in an Alzheimer's Disease Model

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ABSTRACT

The gyloxalase pathway (GP) is an antioxidant defense system that detoxifies metabolic byproduct methylglyoxal (MG). Through sequential reactions, reduced glutathione (GSH), glyoxalase I (glo-1), and glyoxalase II (glo-2) convert MG into D-lactate. Spontaneous reactions involving MG alter the structure and function of cellular macromolecules through the formation of inflammatory advanced glycation endproducts (AGEs). Accumulation of MG and AGEs in neural cells contributes to oxidative stress (OS), a state of elevated inflammation commonly found in neurodegenerative diseases including Alzheimer's Disease (AD). Morin is a common plant-produced flavonoid polyphenol that exhibits the ability to enhance the GP-mediated detoxification of MG. We hypothesize that structural modifications to morin will improve its inherent GP enhancing ability. Here we synthesized a morin derivative, dibromo-morin (DBM) and formulated a morin encapsulated nanoparticle (MNP) – and examined their efficacy in enhancing neural GP activity. Cultured mouse primary cerebellar neurons and *Caenorhabditis elegans* were induced to a state of OS with MG, and treated with morin, DBM, and MNP. Results indicated the morin derivatives were more effective compared to the parent compound in neural GP enhancement and preventing MG-mediated OS in an AD model.

Key words: Glyoxalase, flavonoid, aging, brain, Alzheimer's Disease, methylglyoxal

INTRODUCTION

The brain's high metabolic requirements combined with limited antioxidant capacity render the organ highly vulnerable to damage mediated by oxidative stress (OS) and inflammation.[1-5] Elevated states of OS activate cellular death pathways – including nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) – which contributes to the loss of neural cells exhibited in neurodegenerative disorders, including Alzheimer's Disease (AD).[1, 4, 6] The glyoxalase pathway (GP) is an endogenous antioxidant system that functions in the detoxification of glycolytic and metabolic byproducts, mainly methylglyoxal (MG).[7-9] Ubiquitously produced MG is highly reactive and alters the structure and function of proteins, lipids, and nucleic acids through formation of advanced glycation endproducts (AGEs).[5-17] Through sequential reactions involving reduced glutathione (GSH), glyoxalase I (glo-1), and glyoxalase 2 (glo-2), the GP detoxifies MG and other dicarbonyl compounds into D-lactate (**Figure 1**).[7, 8, 10]Accumulation of MG is highly damaging to cells, and the resulting immune response involves elevated production of inflammatory cytokines, reactive oxygen species (ROS), and activation of apoptotic signaling pathways.[9-14] Elevated levels of MG have been identified as a contributing factor to neural death and pathogenic hallmarks of AD, extracellular β-amyloid plaques (Aβ) and intracellular neurofibrillary tangles (NFT).[12, 14-20] The GP performs indispensable functions in the brain by neutralizing and preventing accumulation of MG and producing an alternate energy source for neurons.[8, 9, 13] Increasing the activity of the GP can prevent elevated levels of MG in neural cells.[7, 21-24] Thus, the GP is a valid target for pharmacological intervention in AD and other neurodegenerative disorders. Naturally occurring chemicals have widely been utilized for their biological activity.[2, 25] Flavonoids are a class of plant-produced polyphenols encompassing over 5000 identified compounds, with a wide range of biological activity including antioxidant, anti-inflammatory, and anti-apoptotic functions.[7, 24, 26, 27]

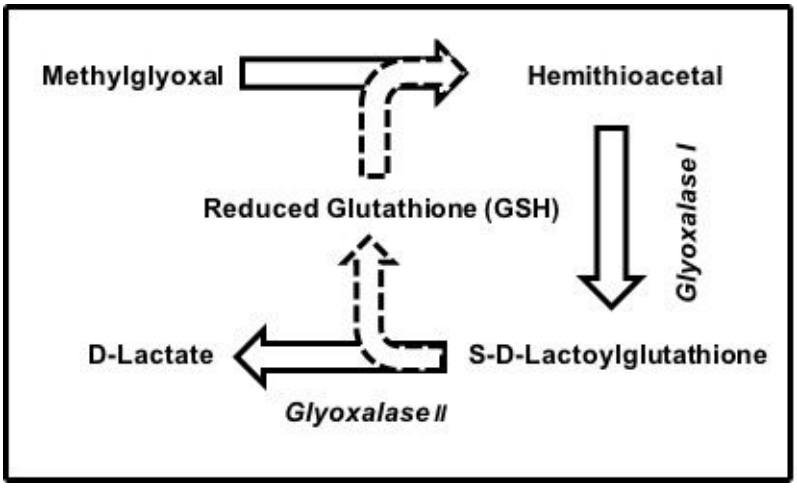

Figure 1. The glyoxalase pathway. MG reacts with GSH forming a hemithioacetal adduct, which is converted by glo-1 intoS-D-lactoylglutathione. Glo-2 converts the intermediate compound into D-lactate, while recycling GSH into the pathway.

Morin (2,3,4',5,7-pentahydroxyflavone) 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).[28-33] Morin exhibits antioxidant, anti-inflammatory, and anti-cancer activity.[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, multiple sclerosis, Parkinson's Disease, and AD.[7, 24, 28, 30, 32-40] Flavonoids protect against inflammation and OS through the modulation of cellular signaling pathways including nuclear factor erythroid 2-related factor 2 (Nrf2) and NF-κB.[28, 29, 32, 33, 36, 37] While flavonoid treatments have shown efficacy against AD *in vitro* and in animal models, no substantial benefit has been exhibited in clinical trials in AD patients.[25, 41, 42] 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).[41] Plant compounds are commonly utilized in drug design, as modification of chemical structural can influence and improve chemical activity.[43] Halogens are versatile atoms with a wide variety of chemical functions and commonly utilized in drug discovery.[44] 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.[44]

We have previously demonstrated the ability of flavonoids catechin, morin, and quercetin to enhance the GP to prevent MG mediated OS in neural cells.[7, 24] 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, and a morin encapsulated nanoparticle. We hypothesize structural modifications to morin will increase its ability to enhance the GP in a MG-mediated OS 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.

RESULTS AND DISCUSSION

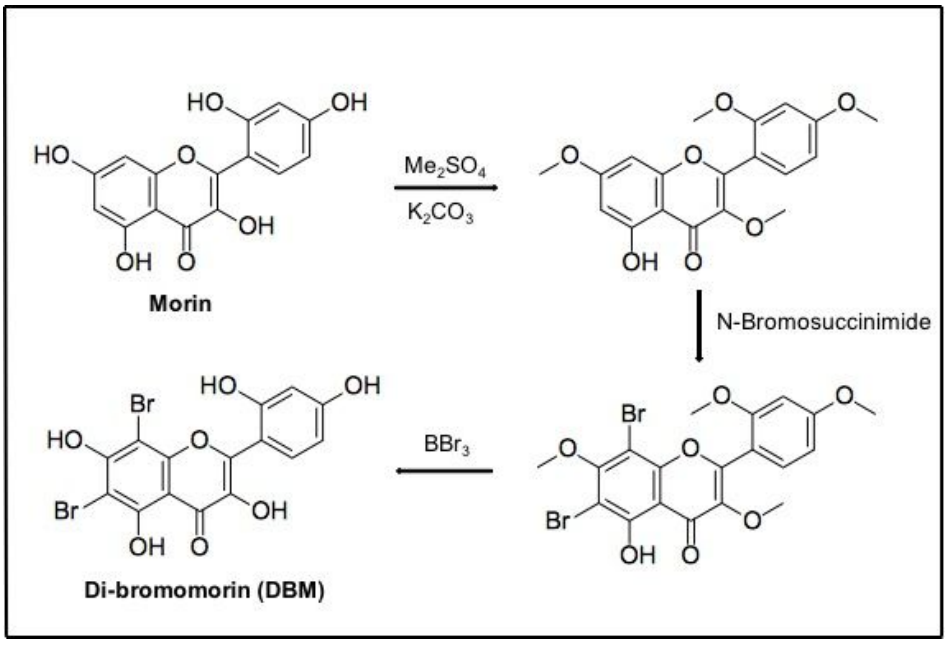

Figure 2. Synthesis of DBM. DBM was synthesized from morin, and identified through ^{1}H-NMR (Fig. **S1A**).

Synthesis of DBM and MNP

DBM was synthesized through a series of reactions involving protection, bromination and deprotection of morin (**Fig. 2**). Molecular structure was determined through ^{1}H-NMR and MS/MS analysis (Fig. **S1A**). Nanoparticles are small particles (less than 1000 nm) composed of an active chemical compound encapsulated in a polymer in a colloid solution.[45, 46] 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 (Fig. **S1B**). 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 (Fig. **S1C**). An MTT assay was performed to determine the toxicity of morin, DBM, MNP (Fig. **S2A**), and MG (Fig. **S2B**) in primary cerebellar neurons. All morin compounds exhibited low toxicity at concentrations up to 100 μM.

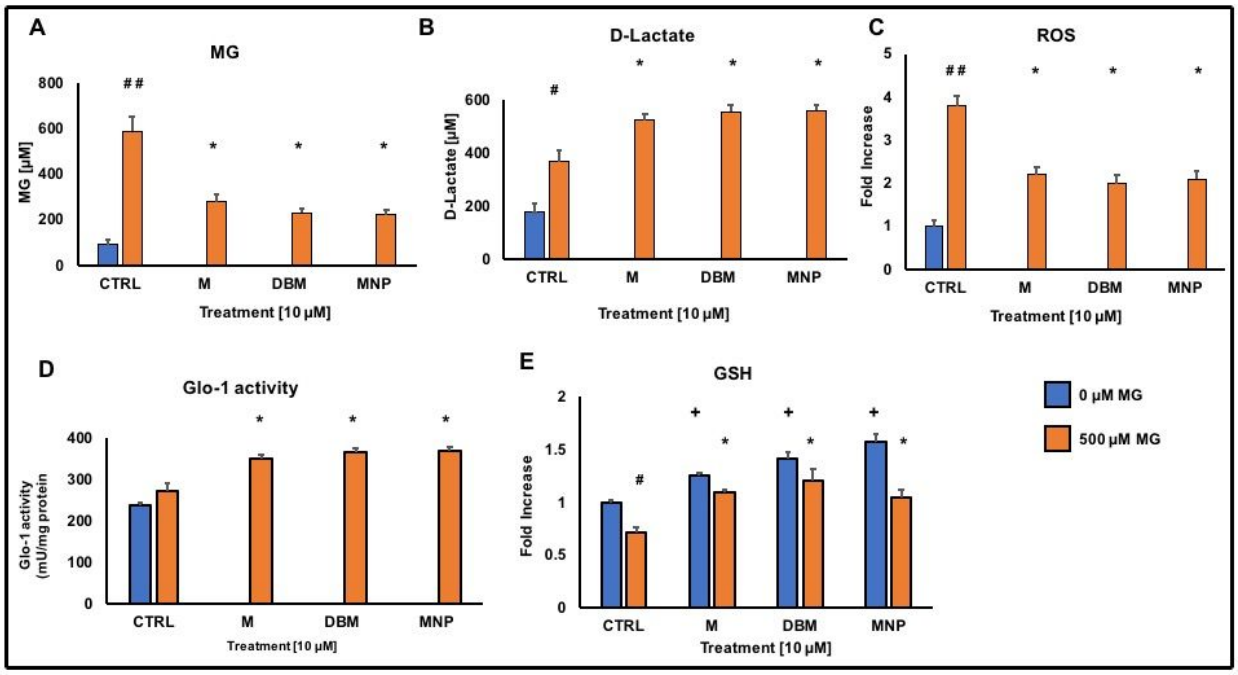

Figure 3. GP and antioxidant function in cerebellar neurons. Morin derivative treatments on MG (**A**), D-lactate (**B**), glo-1 protein activity (measured in mU/μg protein) (**C**), ROS production (**D**), and GSH (**E**). Results are the mean +/- SEM of experiments performed in triplicate. #p<0.05, ##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.

Morin derivatives increase detoxification of MG to D-lactate, and reduce production of ROS.
Addition of MG to cerebellar neuron cultures resulted in significant elevation of MG (#p<0.05) compared to the control, and morin, DBM, and MNP significantly (*p<0.05) decreased the concentration of MG compared to the MG treated control (**Figure 3A**). Treatment with MG significantly (#<0.05) elevated D-lactate concentration compared to the control, and morin, DBM, and MNP significantly (*p<0.05) elevated the concentration of D-lactate compared to the MG treated control **(Figure 3B**). The production of ROS was significantly (##p<0.01) increased by treatment with MG compared to the control, and morin, DBM, and MNP significantly (**p<0.01) reduced the production of ROS compared to the MG treated control (**Figure 3C**).

Morin derivatives increase Glo-1 activity and GSH concentration.
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 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 3E**). In the non-MG treated conditions, morin, DBM, and MNP significantly (*p<0.05) elevated GSH compared to the control.

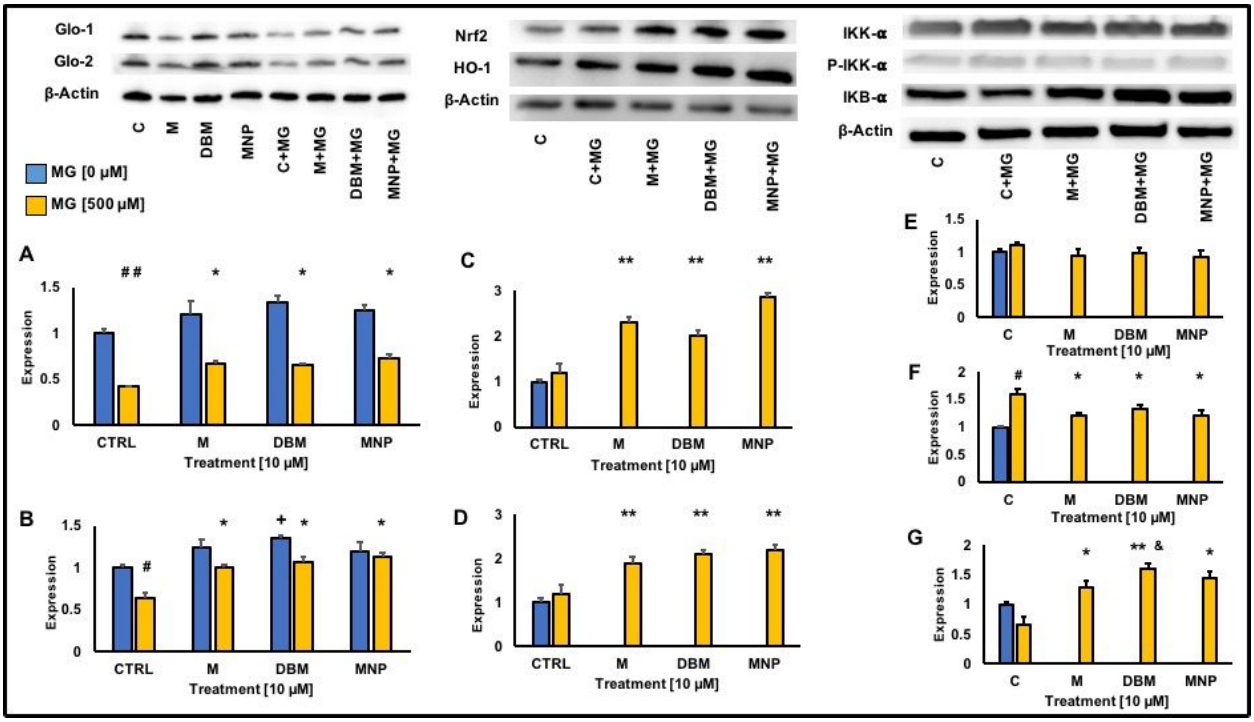

Figure 4. Protein expression in cerebellar neurons. Morin derivative treatments in expression of glo-1 (**A**) and glo-2 (**B**); Nrf2 (**C**) and HO-1 (**D**); IKKα (**E**), p-IKKα (**F**), and IκBα (**G**). Images are from representative Western blots. Results are the mean +/- SEM of experiments performed 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.

Morin derivatives increase expression of glyoxalase pathway proteins

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 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 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. 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 4C**) and HO-1 (**Figure 4D**). Cells insulted with MG exhibit activation of antioxidant response, including expression of Nrf2 (**Figure 4C**). and 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.[28, 32, 33] In its inactive form, it is bound in the cytoplasm to the IκBα complex. [28, 32, 33] Continued interaction with IκBα protein IKKα causes NF-κB to be retained in the cytoplasm and degraded. [28, 32, 33] MG treatment significantly elevated p-IKKα (*p<0.05) compared to the control (**Figure 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 4G**).

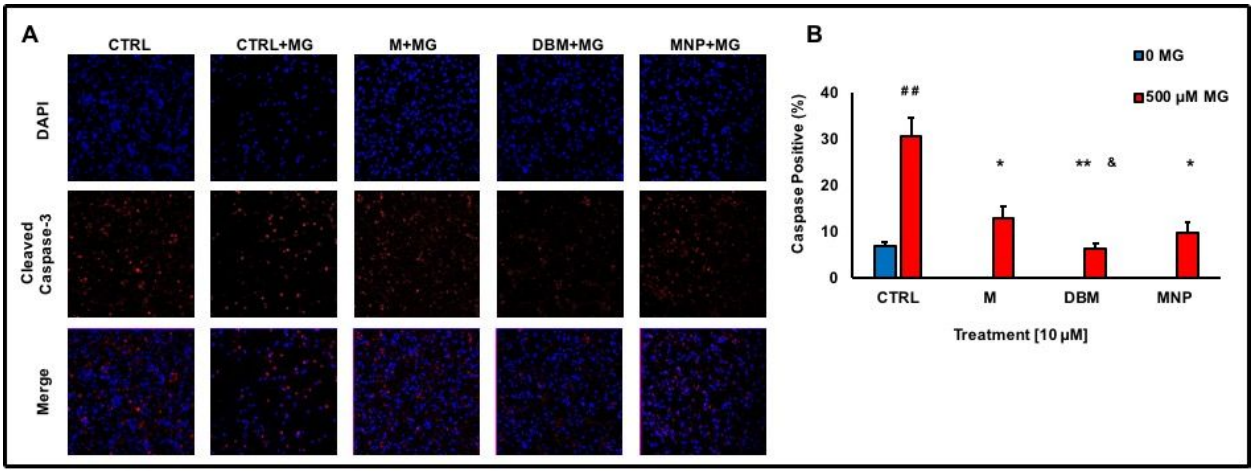

Figure 5. Apoptosis in cerebellar neurons. Morin derivative treatments in MG insulted cells measured by the percentage of cleaved caspase-3 positive cells (**B**). Representative images are shown (**A**). Results are the mean +/- SEM of experiments performed in triplicate. #p<0.05, ##p<0.01, control 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.

Morin derivatives prevent MG-mediated apoptosis in neurons.

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 (**Figure 5**). In MG insulted cells, we witnessed significant decreases 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.

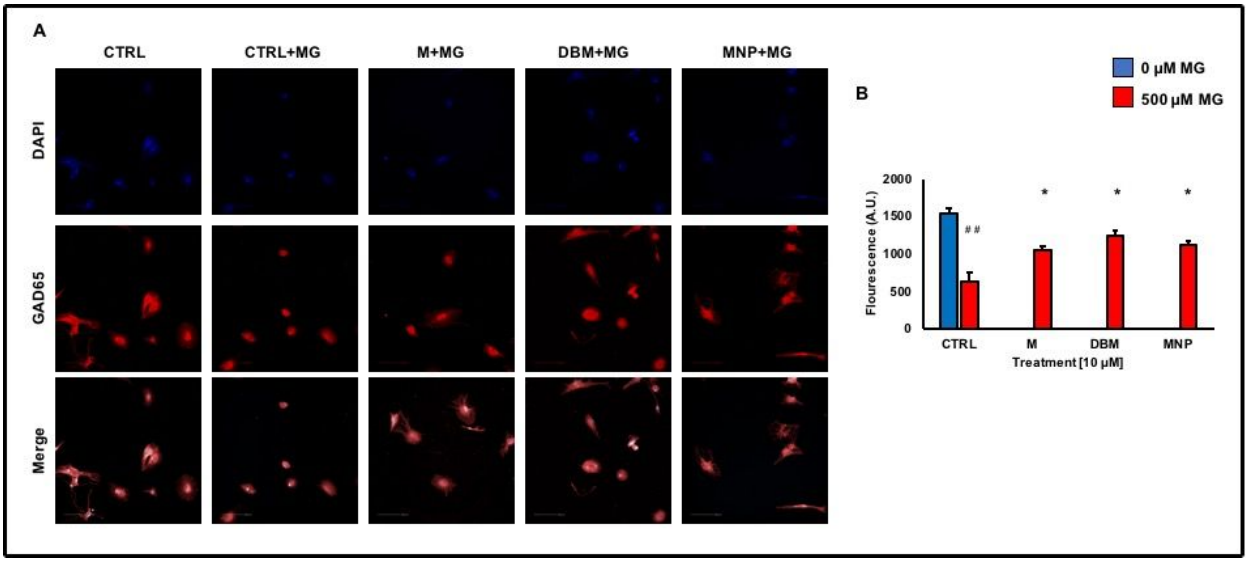

Figure 6A. Inhibitory neurotransmitter function.
Representative confocal images and graphs of morin derivative treatments in MG insulted neurons of GAD65. Results are the mean +/- SEM of experiments performed in triplicate. Fluorescence was normalized to amount of DAPI positive cells, with representative 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.

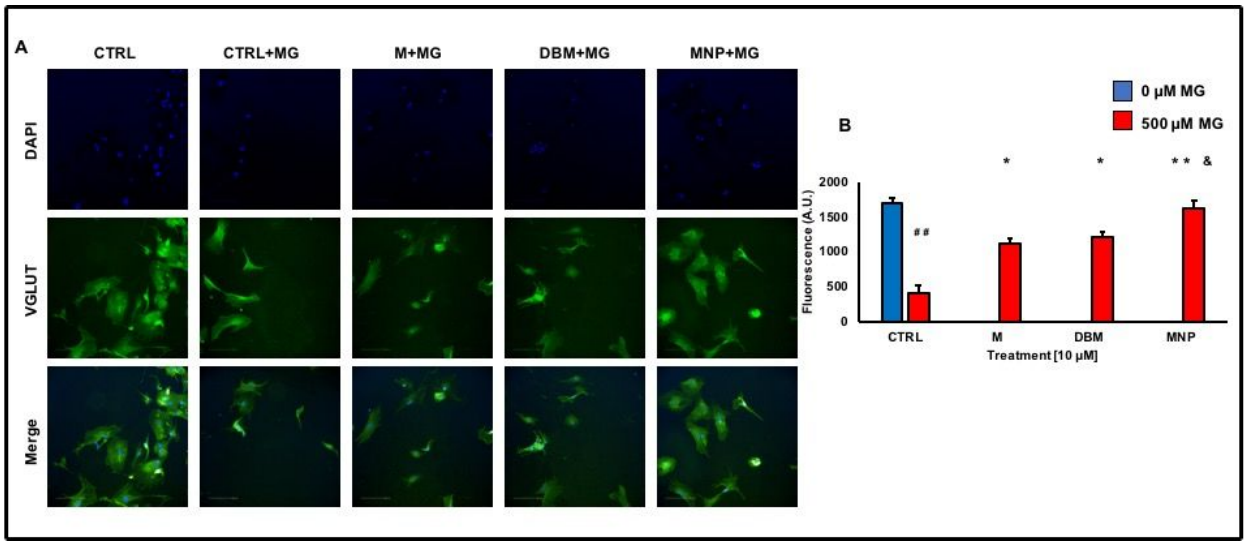

Figure 6B. Excitatory neurotransmitter function.
Representative confocal images and graphs of morin derivative treatments in MG insulted neurons of VGLUT. Results are the mean +/- SEM of experiments performed in triplicate. Fluorescence was normalized to amount of DAPI positive cells, with representative 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.

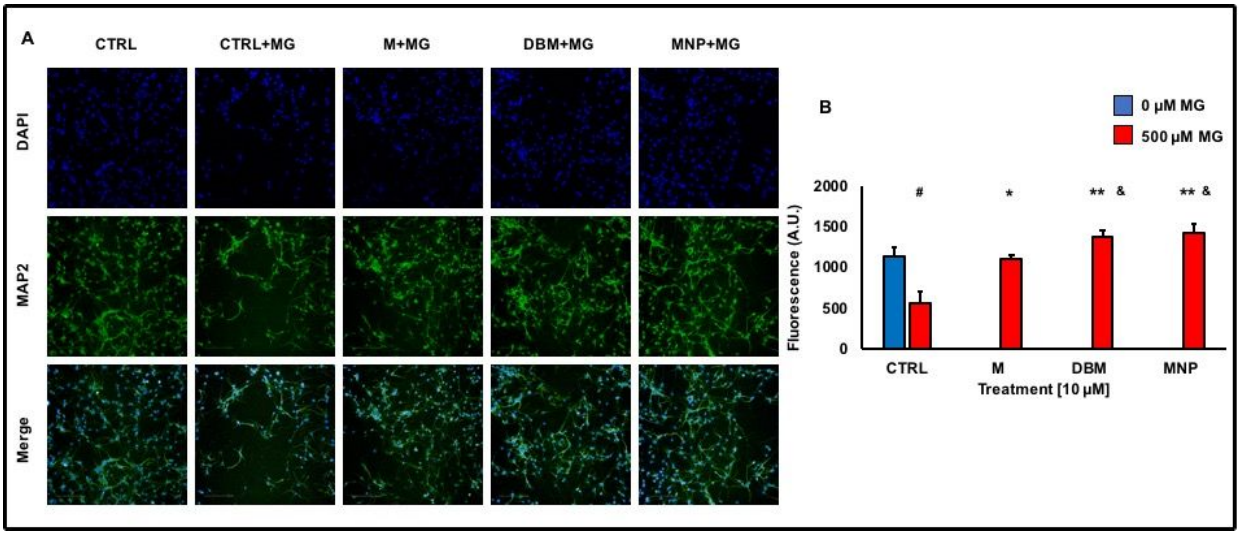

Figure 6C. Synaptic structural proteins.
Representative confocal images and graphs of morin derivative treatments in MG insulted neurons of MAP2. Results are the mean +/- SEM of experiments performed in triplicate. Fluorescence was normalized to amount of DAPI positive cells, with representative images 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.

Neurons treated with morin derivatives retained excitatory and inhibitory neurotransmitter function and elevated expression of synaptic structural proteins.
Glutamic acid decarboxylase (GAD65) is the protein responsible for synthesis of γ-aminobutyric acid (GABA) - the primary inhibitory neurotransmitter- from glutamic acid or glutamate. GAD65 decarboxylates glutamate into GABA, reducing the concentration of excitatory glutamate into GABA. Vesicular glutamate transporter (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. 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 decreases in GAD65 (**6A**), VGLUT (**6B**), and MAP2 (**6C**) compared to the control. MG insulted cells treated with morin exhibited significant elevation in GAD65 (*p<0.05), VGLUT (*p<0.05), and MAP2 (*p<0.05); DBM significantly elevated GAD65 (*p<0.05), VGLUT (*p<0.05), and MAP2 (*p<0.05); MNP significantly elevated GAD65 (*p<0.05), VGLUT (*p<0.01), and MAP2 (*p<0.05). There was a significant increase in VGLUT expression in MNP (&p<0.05) treated cells compared to morin. DBM (&p<0.05) and MNP (&p<0.05) significantly increased MAP2 expression compared to morin.

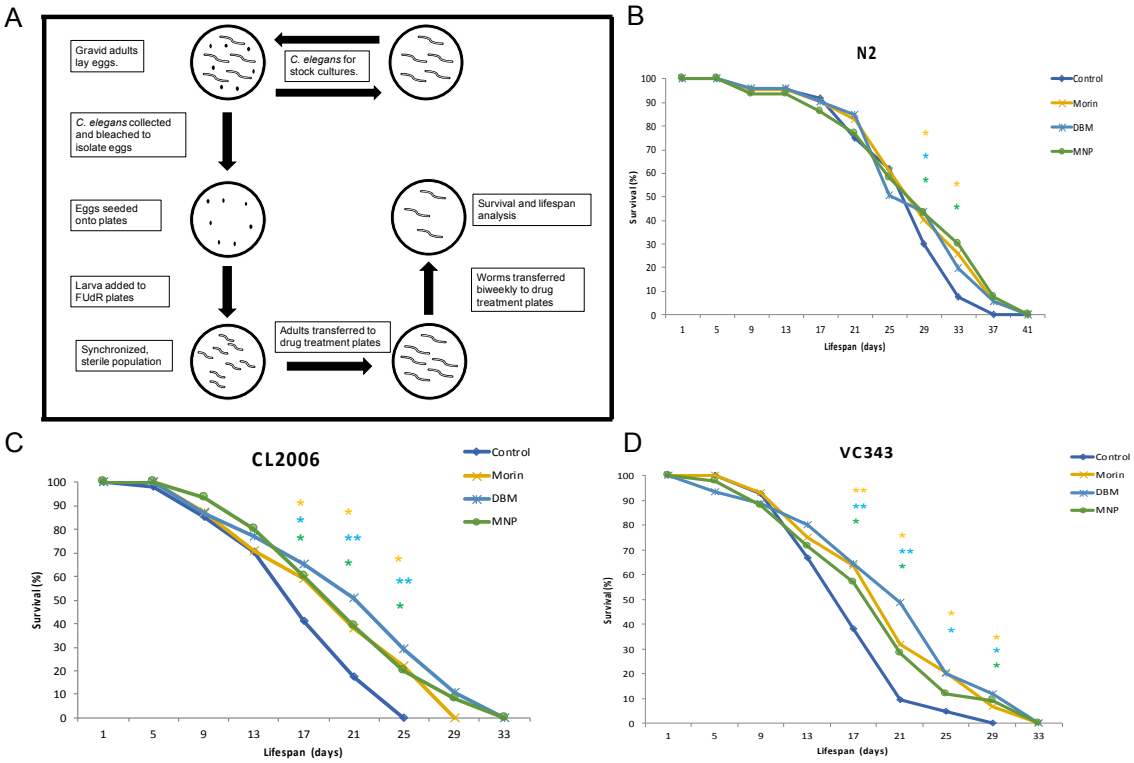

Figure 7. *C. elegans* lifecycle and lifespan extension. *C. elegans* must be separated from progeny for accurate measurement of lifespan.(A) Adult *C. elegans* plated on NGM lay eggs, and are collected. Washing in a bleach solution kills the gravid adults. Eggs are added to NGM plates with FUdR to prevent progeny, resulting in a population of age-synchronized worms, which are used for lifespan analysis. 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 derivatives extend lifespan of C. elegans

Morin, DBM, and MNP were utilized to treat *C. elegans* strains N2 (**Figure 7B**), CL2006 (AD model overexpressing Aβ, **Figure 7C**), and VC343 (glo-1 knockdown) (**Figure 7D**). 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).

Proper functions of neural cells are heavily reliant upon GP detoxification of MG. GP dysfunction can result accumulation of MG, with concomitant elevation of inflammation mediated by AGEs, ROS, and pro-inflammatory signaling molecules.[3, 8, 24, 47, 48] MG is ubiquitously produced in all cells through both enzymatic and non-enzymatic processes, however the rate of formation depends on the cell and tissue type, metabolism, and physiological environment.[12, 47, 48] Cell permeable MG is formed from the breakdown of glycolysis intermediates and metabolism of proteins and lipids.[12, 47, 48] MG glycation occurs through a chemical mechanism called the Maillard reaction, where through sequential reactions, carbonyls and aldehydes spontaneously modify lysine and arginine residues of cellular macromolecules to form AGEs.[11, 14, 48-50] The resulting process produces irreversible, covalent modifications to molecular structures preventing proper function, with modified molecules often recognized as misfolded and targeted for proteolytic degradation.[11, 48, 49]

Conversion of hemithioacetal to S,D-lactoylglutathione by Glo-1 is the rate limiting step in the GP.[12, 47, 48] The function of glo-1 is influenced by the expression levels of the protein and its enzymatic activity.[8, 9, 13] Therefore, modulating glo-1 expression influences the activity of the GP and impacts the levels of cellular OS and inflammation. 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.[51] Glo-1 KO mice showed significantly elevated concentrations of MG and AGEs.[51] Glo-I KO in mammalian Schwann cells exhibited increased toxicity to MG, and elevated levels of oxidized glutathione (GSSG).[52] The decrease in the GSH/GSSG ratio corresponded to a decrease in glo-1 activity, and an increase in MG and AGEs.[52] However, treatment with antioxidant compounds prevented OS and ROS production.[53] 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.[54, 55]

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.[8, 9] 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. Levels of VGLUT 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. The VGLUT protein functions to shuttle glutamate out of the cell.[38, 56, 57] A reduction in VGLUT causes glutamate – the primary excitatory neurotransmitter- to accumulate in the cell, causing excessive Ca^{+2} influx and apoptosis. GAD65 decarboxylates glutamic acid into GABA, the primary inhibitory neurotransmitter.[4,53,54] A reduction in GABA 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. Elevated apoptosis and reduced neurotransmitter function damages neural cells and prevents interneuron signaling. 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 glo-2. [58-60] Cellular antioxidant response occurs with the activation of Nrf2, which is sequestered in a cytoplasmic complex with Keap1.[58-60] Prior to undergoing nuclear translocation, Nrf2 must be released from Keap1. [58-60] Strong electrophilic molecules – like MG and ROS – target cysteine residues on Keap1 and cause a conformational change releasing Nrf2 for nuclear translocation. [58-60] Upon entering the nucleus, Nrf2 interacts with the ARE, a DNA regulatory sequence commonly found in the promoter regions of genes encoding antioxidant and detoxifying enzymes.[23, 29, 58] The human GLO1 gene promoter contains multiple ARE sequences, evidence that the glyoxalase pathway is involved in antioxidant response.[61] Under a state of MG-mediated OS, morin derivatives induced the expression of cytoprotective proteins. Treatment with morin, DBM, and MNP significantly elevated the expression of Nrf2 and HO-1 compared to cells treated with MG alone.

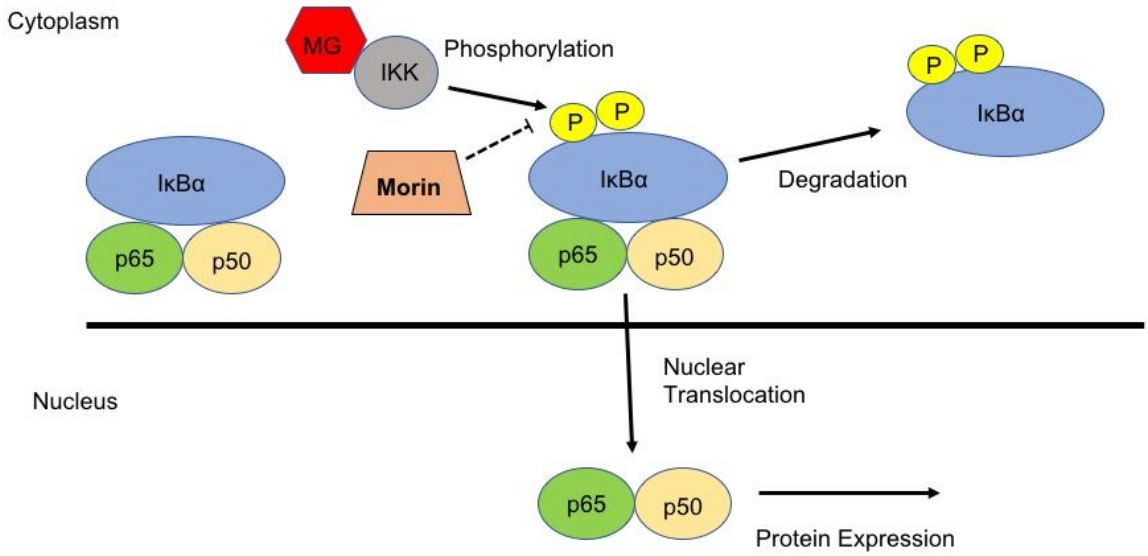

Figure 8. Activation of NF-κB pathway. The NF-κB protein complex is bound in the cytoplasm by IκB. MG/ROS bind and activate IKK, which phosphorylates IκB and targets it for degradation. NF-κB proteins are translocated to the nucleus and induce gene transcription and protein expression.

The NF-κB pathway is activated in response to cellular stress, and induces apoptosis (**Figure 8**).[28, 32, 33] Inactive NF-κB proteins are located in the cytoplasm, where they are bound by IκB of the IKK complex . [28, 32, 33] High levels of inflammation and OS trigger ROS and cytokines to bind and activate the IKK complex. [28, 32, 33] After activation, IKK phosphorylates IKB and it is ubiquitinated and degraded, allowing NF-κB to undergo nuclear translocation. [28, 32, 33] NF-κB interacts with transcription factors and induces the expression of apoptotic proteins. [28, 32, 33] 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. The use of model organisms for determining effect of anti-aging agents is an important tool for elucidating the function of antioxidant ability.[62] 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. [62, 63] Their relatively short lifespan makes them an ideal species to study diseases of aging. [62, 64] Isolating adult worms from their progeny is important to properly evaluate changes in lifespan and aging. We found that treatment of CL2006 *C. elegans* with all three flavonoid compounds lead to a significant increase in their lifespan. We believe the flavonoid treatments induced the expression of antioxidant and pro-cytoprotective proteins that lead to the significant results we presented.[11, 62-64]

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. Future directions will involve testing morin derivatives *in vivo* in a murine model, as it is challenging to fully recreate the cellular milieu to determine the potential ability for a CNS drug.

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.[13, 24] 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.

Synthesis of Dibromo-morin

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.

MNP 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.

MTT cellular toxicity

Cerebellar neurons were cultured in 96-well plates as previously described.[13, 24] 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).

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, Dubin, CA). Densimetric analysis was performed with ImageJ (Madison, WI).

ROS detection

Cerebellar primary neurons were cultured in 96-well plates as previously described.[65] 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.

Immunocytochemistry

Cerebellar primary neurons were cultured in 96-well plates as previously described,[13, 24] 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.

Glyoxalase activity

Cerebellar primary neurons were cultured in 12-well plates as previously described, [13, 24]
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.

D-Lactate concentration

D-Lactate released into the extracellular space was measured spectrophotometrically using collected cell media.[13, 24] 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-DMNPH).[13, 66] 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.

Caenorhabditis elegans strains and maintenance

C. elegans were cultured as previously described.[67] *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.[67] 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.

Flavonoid 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 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.

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.

Associated Content:

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at:
It contains characterization of the compounds and Toxicity study.

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Author contributions:

J.R.F designed experiment, performed all experiments, analyzed results and wrote the manuscript. S.C Synthesized DBM and Characterized DBM. P.N. conceived the idea for the project, designed the experiment, coordinated the project, analyzed the results and edited the manuscript. All authors have given approval to the final version of the manuscript.

Funding Sources:

This work was partially supported by Nebraska Research Initiative funding -2014

Notes

The authors declare no competing financial interest.

Abbreviations:

Dibromo-morin (DBM), morin nanoparticle (MNP), Alzheimer's Disease (AD), Methylglyoxal (MG), Advanced Glycation Endproducts (AGEs), Reactive Oxygen Species (ROS), Oxidative Stress (OS), Reduced glutathione (GSH), nuclear factor erythroid 2-related factor 2 (Nrf2), Kelch-like ECH-associated protein 1 (Keap1), Glyoxalase 1 (glo-1), Glyoxalase 2 (glo-2), Antioxidant Response Element (ARE), Blood Brain Barrier (BBB), Heme Oxygenase-1 (HO-1).

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