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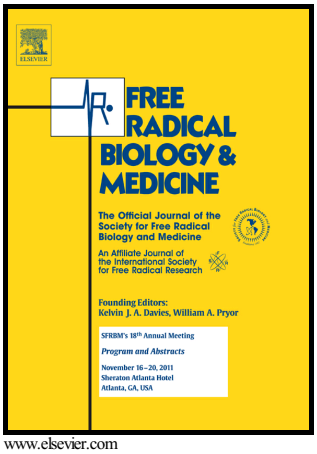

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Chemical Basis for the Disparate Neuroprotective Effects of the Anthocyanins, Callistephin and Kuromanin, Against Nitrosative Stress

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Abstract

Oxidative and nitrosative stress are major factors in neuronal cell death underlying neurodegenerative disease. Thus, supplementation of antioxidant defenses may be an effective therapeutic strategy for diseases such as amyotrophic lateral sclerosis, Parkinson's disease, and Alzheimer's disease. In this regard, treatment with nutraceutical antioxidants has garnered increasing attention; however, the differential neuroprotective effects of structurally similar nutraceuticals, which may affect their suitability as therapeutic agents, has not been directly examined. In this study we compare the ability of two anthocyanins, callistephin (pelargonidin-3-*O*-glucoside) and kuromanin (cyanidin-3-*O*-glucoside) to protect cerebellar granule neurons from damage induced by either oxidative or nitrosative stress. These anthocyanins differ by the presence of a single hydroxyl group on the B-ring of kuromanin, forming a catechol moiety.

While both compounds protected neurons from oxidative stress induced by glutamate excitotoxicity, a stark contrast was observed under conditions of nitrosative stress. Only kuromanin displayed the capacity to defend neurons from nitric oxide (NO)-induced apoptosis. This protective effect was blocked by addition of Cu, Zn-superoxide dismutase, indicating that the neuroprotective mechanism is superoxide dependent. Based on these observations, we suggest a unique mechanism by which slight structural variances, specifically the absence or presence of a catechol moiety, lend kuromanin the unique ability to generate superoxide, which acts as a scavenger of NO. These findings indicate that kuromanin and compounds that share similar chemical characteristics may be more effective therapeutic agents for treating neurodegenerative diseases than callistephin and related (non-catechol) compounds.

Keywords: Anthocyanins; Neuroprotection; Neurodegeneration; Nitrosative Stress; Nitric Oxide; Excitotoxicity

1. Introduction

Conditions of oxidative and nitrosative stress are well documented factors contributing to the pathophysiology of a number of neurodegenerative diseases [1-4]. In recent years, it has become apparent that these conditions arise largely as a consequence of diverse initiating mechanisms, including excitotoxicity, mitochondrial dysfunction, and neuroinflammation, which produce large quantities of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Accumulation of ROS, such as superoxide, hydrogen peroxide, and hydroxyl radicals, or RNS, such as nitric oxide and peroxynitrite, results in significant damage to vital cellular components, culminating in neuronal cell death. It is therefore unsurprising that strategies aimed at mitigating the damaging effects of ROS and RNS are being actively pursued as potential therapeutic treatments for a number of neurodegenerative disorders including Alzheimer's disease,

Parkinson's disease, and amyotrophic lateral sclerosis (ALS). In this respect, flavonoids, naturally occurring compounds found in a number of highly consumed fruits and vegetables, are promising therapeutic candidates due to their impressive antioxidant, anti-inflammatory and anti-apoptotic properties, which make them well suited to combat multiple facets of the neurodegenerative process [5-12].

In particular, anthocyanins, a class of flavonoid compounds responsible for the brilliant red, blue, and purple coloration of many fruits, vegetables and flowers, have garnered significant attention as potent antioxidants and anti-inflammatory agents. Their positive effects on a myriad of health conditions, such as heart disease and cancer, have been extensively documented [reviewed by 13-15]. However, while promising, the use of these compounds in the context of neurodegenerative disease is novel and warrants further exploration.

While several studies have explored the benefits of anthocyanins in both *in vitro* and *in vivo* models of neurodegeneration, neuroinflammation, and aging [13, 16-19], interpreting the results of these studies is often complicated by the use of anthocyanin-enriched extracts, which contain multiple anthocyanins and small amounts of other (poly)phenolic compounds. While such studies have been invaluable as a starting point for identifying anthocyanins as new drug candidates, the presence of multiple compounds within a single extract severely limits the ability of investigators to discern which compounds are ultimately responsible for any observed health benefits, and eliminates the possibility of dissecting the mechanism of action of any particular compound. Interpretation of results is further confounded by the possibility that any positive effects observed after treating with an anthocyanin-enriched extract may actually be due to synergism between several unique compounds, rather than the activity of a single agent. Indeed, a recent study by Carey et al. [17] compared the anti-neuroinflammatory effects of two pure

anthocyanin species to an extract derived from blueberries using the BV2 microglial cell line. Their data revealed that a much higher concentration of pure anthocyanins was required to achieve significant reductions in inflammatory markers when compared to the blueberry extract, suggesting a high degree of synergism between its constituents.

In this regard, conclusions drawn using anthocyanin-enriched extracts alone are restricted at best to a particular family of compounds which contains several members, or at worst, to the use of a particular extract itself. However, despite these limitations, studies using pure anthocyanin species in models of neurodegeneration and neuroprotection are few in number, and almost exclusively limited to *in vitro* assessment [20-25]. Indeed, to the best of our knowledge, only two studies have been conducted *in vivo*, one using the pure anthocyanidin, pelargonidin, in a 6-hydroxydopamine lesioned rat model of hemi-Parkinsonism, and one using cyanidin-3-*O*-glucoside to preserve spatial memory in rats treated with beta-amyloid peptide [26-27]. Furthermore only one *in vitro* study previously conducted by our lab with pure anthocyanins has compared the neuroprotective effects of distinct anthocyanin species [20]. However, this study was limited to the evaluation of anthocyanins only in the context of mitochondrial oxidative stress.

While our previous work indicated that the two pure anthocyanins, callistephin (pelargonidin-3-*O*-glucoside) and kuromanin (cyanidin-3-*O*-glucoside), protect primary neurons equally from the Bcl-2 inhibitor and mitochondrial glutathione depleting agent, HA14-1, reports comparing the properties of different anthocyanins and related flavonoids in non-neuronal systems have indicated that subtle differences in molecular structure, particularly the number of hydroxyl groups located on the B-ring, have measurable effects on the ability to interact with lipid membranes, absorption, and free radical scavenging activities [20, 28-29]. With this in

mind, we sought to determine if structurally similar, yet distinct anthocyanin species also differ in their ability to confer neuroprotection against a more diverse array of insults related to neurodegenerative processes, such as excitotoxicity and nitric oxide toxicity. Our results revealed a striking contrast in the neuroprotective abilities of the anthocyanins callistephin and kuromanin against nitric oxide-induced nitrosative stress, with only kuromanin significantly preserving cell viability. As these compounds are structurally similar, differing only by the presence of a single hydroxyl group on the B-ring of kuromanin (Fig. 1), we determined that slight alterations in molecular structure can have a profound effect on neuroprotective capacity under conditions of nitrosative stress. Here, we propose a novel neuroprotective mechanism by which anthocyanins containing a catechol moiety on their B-ring, such as kuromanin, are able to effectively scavenge and detoxify harmful nitric oxide radicals.

2. Materials and Methods

2.1 Reagents

Callistephin chloride (≥ 97% purity), kuromanin chloride (≥ 95% purity), glutamic acid, glycine, potassium chloride, Hoechst 33258, paraformaldehyde, polyethyleneglycol-conjugated superoxide dismutase 1 (PEG-SOD), Cu, Zn-superoxide dismutase (SOD1) from bovine erythrocytes, DT diaphorase (quinone oxidoreductase 1; QR1), quininone oxidoreductase 2 (QR2), tween 20, and monoclonal antibody against β-tubulin were purchased from Sigma-Aldrich (St. Louis, MO). Sodium nitroprusside (SNP) was obtained from Calbiochem (San Diego, CA). 2-Methyl-6-(4-Methoxyphenyl) -3,7-Dihydroimidazo[1, 2-A]pyrazin-O-3ne, Hydrochloride (MCLA) was purchased from Molecular Probes (Grand Island, NY). Basal Medium Eagle's solution, L-glutamine solution, penicillin/streptomycin solution, fetal bovine serum (FBS), and lipofectamine 2000 transfection reagent was purchased from Invitrogen

(Grand Island, NY). Anti-mouse FITC-conjugated secondary antibody was obtained from Jackson Immunoresearch Laboratories (West Grove, PA). Nitric Oxide Assay kit (EMSNOTOT) was obtained from Thermo Scientific (Rockford, IL). MTT assay kit was purchased from Bioassays Systems (Hayward, CA). Nutri-Fruit freeze-dried strawberry and blackberry powders were purchased from the Scenic Fruit Company (Gresham, OR).

2.2 Extraction and HPLC Analysis of Freeze-Dried Fruit Powders.

Anthocyanin enriched extracts from strawberry (SAE) and blackberry (BAE) were prepared using commercially available freeze-dried fruit powders. Extracts were prepared as described by Rodriguez-Saona and Wrolstad [30] using acidified methanol. Fifty grams of freeze-dried fruit powder was placed in a large flask and macerated with acidified methanol (0.01% HCl) for 1 hour at room temperature to create a crude extract of phenolic species. Methanol containing the extracted (poly)phenols was then collected by vacuum filtration using Whatman no. 1 filter paper. The remaining powder collected in a Buchner funnel, was then re-extracted with acidified methanol until a faint colored extract was obtained. The crude extract was pooled together and boiled under vacuum to remove the methanol until the extract had become viscous. This extract was then purified for anthocyanins using a C18 column prepared with acidified water (0.01% HCl). The extract was loaded onto the column then washed sequentially with one column volume each of acidified water and ethyl acetate, and then eluted into a collection flask using acidified methanol. This extract, containing purified anthocyanins, was then boiled once more under vacuum until a highly viscous and strongly pigmented solution was obtained.

HPLC analyses were conducted using HPLC-UV/Vis with an Agilent model HP1100 series II with programmable diode array detector (Avondale, PA). Separation of anthocyanins was carried out using a C18 bonded silica column (3µm, 3 x 150mm) from Dionex Inc. (Sunnyvale, CA). Mobile phases consisted of A, 0.1% trifluoroacetic acid (TFA) in water, and B, 70% acetonitrile in water, which were combined in a step-wise gradient over the course of the separation. Gradient conditions were as follows: initial conditions were composed of 90% mobile phase A and 10% mobile phase B; from time 5-10 minutes, the gradient changed to 30% mobile phase B; from 14.5-16 minutes, the gradient changed to 50% mobile phase B; from 20-21 minutes, the gradient was returned to 10% mobile phase B. Analysis was carried out on 10µL injections at 25˚C using a flow rate of 0.6 mL/min, and anthocyanins were detected at 520nm over the course of 25 minutes with a post run time of 5 minutes to clear and equilibrate the column for subsequent runs..

The concentration of the primary anthocyanin constituent in both SAE and BAE was determined by creating a standard curve from commercially available anthocyanin standards for both callistephin chloride and kuromanin chloride. For each anthocyanin, a 1mg/mL solution was prepared initially in 0.1% TFA in water, and diluted to 3/5, 2/5, 1/5, and 1/10 of the original concentration. Standards were injected individually and run under the HPLC conditions described above to create linear curves ($R^2 \geq 0.999$) for each anthocyanin species.

2.3 Cell Culture

Cerebellar granule neurons (CGNs) were isolated as previously described [31] from seven day-old Sprague-Dawley rat pups, including both sexes. Cells were plated on poly-L-lysine coated six-well plates (35mm-diameter), with a density of approximately $4x10^6$ cells/well

in Basal Medium Eagle's supplemented with 25mM potassium chloride, 10% fetal bovine serum (FBS), 2mM L-glutamine, and 2mM penicillin/streptomycin (100U/mL/100µg/mL). Twenty-four hours after CGNs were plated, 10µM cytosine arabinoside was added to the culture medium to inhibit the growth of non-neuronal cells. Granule neurons were subsequently incubated at 37˚C in 10% CO_2 for six to seven days in culture prior to experimentation. At this point, cultures were ~95% pure for CGNs.

Neuro2A cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS, 2mM L-glutamine, and penicillin/streptomycin (100U/mL/100µg/mL) at 37˚C in 10% CO_2. Cells were plated in 6-well plates (35mm-diameter), and allowed to reach approximately 80% confluency before transfection and treatment.

2.4 Transfection of Neuro2A cells

Neuro2A cells were plated at 50% confluency and transfected the following day when the cells had reached approximately 80% confluency using the Lipofectamine 2000 reagent according to the manufacturer's instructions. Briefly, culture medium was removed and replaced with OptiMEM transfection medium. Plasmids encoding either enhanced green fluorescent protein (EGFP) or an EGFP-Cu, Zn-superoxide dismutase (SOD1) fusion construct were incubated for 20 minutes in OptiMEM containing Lipofectamine 2000 at a dilution of 1:1000. This mixture was then added to culture dishes containing Neuro2A cells in OptiMEM transfection medium and cells were allowed to incubate for 6 hours at a temperature of 37˚C. Following the 6 hour incubation, the transfection medium was removed and replaced with culture medium at which time cells were treated as described below.

2.5 Treatment of cell cultures

Cell culture medium was removed and replaced with serum free medium in order to avoid any potential protective effects of the serum that might limit apoptosis. Cells were then treated with either 100µM glutamate (final solution contained 100µM glutamic acid and 10µM glycine co-factor) or 100µM SNP alone or in combination with 0.15% SAE, 0.25% BAE, 100µM callistephin, or 100µM kuromanin for 24 hours prior to quantification of apoptosis. For those experiments utilizing Cu, Zn-superoxide dismutase (SOD1), cells were treated as described above with the addition of either PEG-SOD or unconjugated SOD1 at a concentration of 30U/mL unless otherwise indicated. For all experiments, an untreated control was used for comparison in assaying cell death.

2.6 MTT Assay

An MTT assay kit was used to determine the viability of cells treated with SAE and BAE per the manufacturer's instructions. Briefly, MTT reagent was added to each well of a 6-well culture dish following treatment with SAE and BAE at a final concentration of 2mM, and cells were then incubated at 37˚C for 4 hours. Following this incubation, the purple formazan precipitate was solubilized using 2mL of dimethyl sulfoxide (DMSO) by gently rocking the culture dishes for 1 hour at room temperature until all of the precipitate had dissolved. This solution was then transferred to a 96-well plate, and the absorbance of the sample was determined at 570nm, and expressed as a percentage of the untreated control.

2.7 Immunocytochemistry and Assay of Apoptosis

Following treatment, CGNs were washed with phosphate buffered saline (PBS; pH=7.4) and fixed for one hour in 4% paraformaldehyde. Cells were then washed with PBS and incubated in blocking solution containing 5% bovine serum albumin (BSA) in 0.2% triton-X in PBS for 1 hour at room temperature. This was followed by overnight incubation at 4˚C with primary antibody against β-tubulin, prepared in a 1:200 dilution in 2% BSA in 0.2% triton-X in PBS. The following day, CGNs were washed, and then incubated for 1 hour at room temperature with FITC-conjugated secondary antibody prepared as a 1:250 dilution in 2% BSA in 0.2% triton-X in PBS with Hoechst stain at 10mg/mL. Neuro2A cells were similarly washed with PBS following transfection and treatment, then fixed for one hour at room temperature. These cells were then stained with Hoechst to visualize nuclear morphology. All cells imaged using a Zeiss Axiovert-200M epi-fluorescence microscope. Five images per well were captured to assay apoptosis, with either duplicate or triplicate wells for each treatment comprising one experiment. Cells were counted and scored as either living or apoptotic based on nuclear morphology using images showing decolorized Hoechst fluorescence. Cells having condensed or fragmented nuclei were scored as apoptotic, and a minimum of 50 cells per treatment per experiment were scored overall.

2.8 Nitric Oxide Assay

Nitric oxide assay was performed using a Nitric Oxide Assay kit from Thermo Fisher Scientific (Rockford, IL) by the Griess method per the manufacturer's instructions. This assay was used to determine the total amount of both nitrate and nitrite ions in solution, which are generated as the major degradation products of nitric oxide. Samples were prepared in sealed 1.5-mL mirocentrifuge tubes in the absence of cells in serum free CGN culture medium

containing 25mM KCl, and either 100µM callistephin or kuromanin ± 100µM SNP. Samples containing only SNP only in medium were prepared in parallel for comparison. Each experiment was run in parallel with a sample containing only culture medium and no SNP. All samples were placed in an Eppendorf thermomixer, and allowed to incubate overnight at 37˚C and 300 rpm before assaying nitric oxide concentration. Since the culture medium does not contain nitrates or nitrites, but was calculated to have an average nitrite/nitrate concentration of 15.704µM due to absorbance by phenol red in the medium, readings from a sample containing culture medium only were subtracted as background from the total absorbance for all of the remaining samples before calculating the concentration of nitric oxide in solution. Absorbance for samples containing callistephin and kuromanin without SNP were similarly subtracted from samples containing both anthocyanins and SNP in order to correct for the absorbance of these compounds when assaying for nitric oxide production.

2.9 Superoxide Assay

Superoxide production was assayed using MCLA luminescence as an indicator for the presence of superoxide radical. Samples were prepared in a total volume of 1mL in PBS containing 100µM MCLA and either 100µM kuromanin alone, or in combination with 5U/mL quinone reductase 1 (QR1) or quinone reductase 2 (QR2). Reactions containing QR1 also contained 200µM NADH to serve as a cofactor, while reactions containing QR2 also contained 200µM of 1-methyl-1, 4-dihydronicotinamide cofactor. A 30U/mL SOD1 aliquot was added to one sample for each experiment containing kuromanin and QR2 as a control to confirm that MCLA luminescence was due to superoxide, and not production of another reactive radical species. Additionally, samples containing only MCLA, MCLA with QR1 and NADH, or MCLA with QR2 and 1-methyl-1, 4-dihydronicotinamide in PBS were run in parallel for each

experiment as controls to ensure that neither QR1 nor QR2 generated a luminescence signal when incubated without a substrate. Luminescence was recorded every 15 seconds for a period of 8 minutes. A baseline was recorded for the first 4 minutes of each experiment after which time kuromanin, or kuromanin in combination with SOD1 was added to the reaction mixture. This procedure was repeated for control samples containing only MCLA with QR1 and NADH, or MCLA with QR2 and 1-methyl-1, 4-dihydronicotinamide in PBS. For those experiments in which the effects of QR1 or QR2 on kuromanin were assessed, these enzymes were added 1 minute after the addition of kuromanin or kuromanin in combination with SOD1. Luminescence was measured using a TD-20/20 model luminometer from Turner Designs (Sunnyvale, CA).

2.10 Data Analysis

Each experiment was performed using either duplicate or triplicate wells for each treatment, with each experiment being performed at least three times. Data represent the means +/- SEM for the total number (n) of experiments carried out. All data, unless otherwise noted, were analyzed using a one-way ANOVA with a *post hoc* Tukey's test. Data collected for the MCLA superoxide assay were analyzed using two-way ANOVA for repeated measures with a *post hoc* Bonferroni's test to compare all groups. A p-value <0.05 was considered statistically significant.

3. Results

3.1 Anthocyanin enriched extracts from strawberries and blackberries display differential neuroprotective effects against oxidative and nitrosative stress

Anthocyanin-enriched extracts were prepared from freeze-dried strawberry and blackberry powder and characterized by HPLC. Anthocyanins were detected at a wavelength of 520 nm and the identity of the primary anthocyanin constituent of each extract was determined by comparison to pure anthocyanin standards (Fig. 2).

By this method, it was determined that the primary anthocyanin constituent of strawberry anthocyanin extract (SAE) was callistephin, which made up 91% of the enriched extract, while blackberry anthocyanin extract (BAE) was composed primarily of kuromanin, which made up approximately 87% of the enriched extract (Table 1). Once the identity of the primary anthocyanin constituents of each extract had been determined, the concentration of this component was determined for both SAE and BAE by comparison to a standard curve created using varying concentrations of either callistephin or kuromanin accordingly. For SAE, callistephin was determined to have a concentration of 5mM. For BAE, the concentration of kuromanin was calculated to be 3mM (Table 1). In experiments using these extracts, the concentrations of SAE and BAE were adjusted on a percentage basis to yield equivalent concentrations (7.5μM) of their respective primary anthocyanin constituents.

As anthocyanins are known to be potent antioxidants, we next characterized the neuroprotective profile of the two extracts against distinct neurotoxic insults. Excitotoxicity, during which neurons become over stimulated by glutamate signaling, leading to massive calcium influx, and subsequent activation of multiple cell death processes, was used as a model of oxidative stress as increased production of ROS is a hallmark of this process [32]. Moreover, neuronal cell death associated with excitotoxicity is thought to play a role in several

neurodegenerative diseases such as ALS, Parkinson's disease and Alzheimer's disease, highlighting the relevance of this insult for screening potential neuroprotective compounds [33-35]. Alone, exposure to glutamate diminished cellular viability in CGNs by almost 50%, as assessed by MTT assay. However, co-treatment of the cells with either 0.15% SAE or 0.25% BAE abrogated this effect, preserving cell viability at a level that was comparable to untreated control cells (Fig. 3A).

While excitotoxicity is known to contribute to neurodegenerative disease processes, and particularly to oxidative stress, other factors, such as neuroinflammation, also play major roles in promoting cell death through production of free radical species. Indeed, a key aspect of neuroinflammation is the release of toxic nitric oxide radicals by astrocytes and microglia, which are capable of promoting conditions of nitrosative stress in neighboring neurons that ultimately culminate in the activation of cell death signaling cascades [36]. Since these processes do not occur independently of other disease factors such as excitotoxicity, it is important that potential therapeutics for neurodegeneration are capable of mitigating both oxidative and nitrosative stress in order to target multiple facets of disease pathology. To this end, we next tested the ability of the two anthocyanin extracts to defend cells from sodium nitroprusside (SNP), a nitric oxide donor, meant in this context to simulate conditions of nitrosative stress. Cells treated with SNP alone showed a drastic reduction in viability of 95%, and treatment with 0.15% SAE demonstrated no ability to preserve cellular viability in the presence of this insult (Fig. 3B). In striking contrast, treatment with 0.25% BAE significantly preserved neuronal survival in the presence of SNP (Fig. 3B). Moreover, BAE protection was dose dependent in this context (Fig. 3B) while SAE was ineffective at preserving neuronal survival at all doses tested (Winter and Linseman, unpublished data).

3.2 Both callistephin and kuromanin, the primary anthocyanin constituents of SAE and BAE, protect CGNs from glutamate excitotoxicity

The differential protective effects of the two anthocyanin extracts were thought to be attributed to differences in the neuroprotective potential of their respective primary anthocyanin constituents. However, given that neither extract was composed purely of a single anthocyanin, the possibility that the differential neuroprotective abilities were due to other compounds contained in the extract, or synergism between several compounds, could not be excluded. Therefore, in order to confirm that the observed differences in neuroprotection corresponded to the differing properties of the two primary anthocyanins, callistephin and kuromanin, we next examined the neuroprotective capabilities of the two pure anthocyanins.

Pure preparations of both callistephin and kuromanin were obtained commercially and tested for their ability to protect CGNs from glutamate excitotoxicity. Because pure anthocyanins were used at a higher concentration than either of the extracts and produced deeply pigmented solutions with absorbance in the same range as MTT products, cells were fixed following treatment, and nuclear morphology was observed in order to quantify apoptosis. In general, cells displaying condensed and/or fragmented nuclei were scored as apoptotic. In a preliminary dose response, it was found that pure anthocyanins were less efficient neuroprotective agents than the extracts, requiring a dose of either 75μM or 100μM for kuromanin and callistephin respectively in order to significantly protect CGNs from excitotoxic insult (Winter and Linseman, unpublished data). Given this observation, a dose of 100μM was selected to complete all subsequent analyses.

Treatment with glutamate alone produced death in approximately 50% of CGNs, consistent with our previous observations using the MTT assay (compare Fig. 3A and 4B). These cultures displayed a much higher proportion of cells with condensed or fragmented nuclei in addition to a compromised tubulin network when compared to untreated controls (Fig. 4A). This result is consistent with induction of neuronal apoptosis. Intriguingly, treatment with either callistephin or kuromanin significantly reduced the number of cells displaying apoptotic nuclei and fragmented tubulin (Fig. 4A). Quantitatively, callistephin and kuromanin reduced CGN apoptosis to 20% and 15% respectively, which is in good agreement with our previous observations using SAE and BAE against glutamate excitotoxicity (compare Fig. 3A and 4B). Collectively, these results suggest that callistephin and kuromanin, the primary anthocyanin constituents of SAE and BAE respectively, are capable of mediating similar neuroprotective effects against induced glutamate excitotoxicity to those observed using enriched anthocyanin extracts.

3.3 Kuromanin, but not callistephin, protects CGNs from nitric oxide-induced toxicity

In order to further compare the neuroprotective capacity of pure anthocyanin compounds to neuroprotection mediated by SAE and BAE, callistephin and kuromanin were next assessed for their ability to defend CGNs from nitric oxide-induced toxicity. CGNs that were treated with SNP alone uniformly displayed condensed and fragmented nuclear morphology as well as extensive disintegration of tubulin networks in comparison to untreated controls (Fig. 5A). Quantification of condensed nuclei revealed that 95% of cells exposed to SNP alone underwent apoptosis in concurrence with our previous results using MTT (compare Fig. 3B and 5B). Interestingly, cells that were co-treated with both callistephin and SNP displayed the same

features as cells treated with SNP alone, and no significant reduction in the number of apoptotic nuclei was observed (Fig. 5A, B). In stark contrast to this observation, co-treatment with kuromanin dramatically mitigated these effects, preserving tubulin networks and maintaining healthy nuclear morphology (Fig. 5A). These cells showed a reduction in apoptosis of nearly 70% in comparison to cells treated with SNP alone, or SNP with callistephin, consistent with the protective effect if BAE against SNP demonstrated using MTT (compare Fig. 3B and 5B).

3.4 Kuromanin, but not callistephin, reduces nitric oxide concentration in a cell free system and produces superoxide

Given the stark contrast between the abilities of callistephin and kuromanin to prevent the harmful effects of nitrosative stress in neurons, a nitric oxide assay was used to determine the final concentration of nitric oxide in cell-free medium incubated with callistephin or kuromanin and SNP. Analysis using a Griess assay to measure total nitrite and nitrate, decomposition products of nitric oxide, demonstrated that culture medium incubated overnight with SNP produced a substantial amount of nitric oxide in solution (Fig. 6A). Consistent with its inability to defend CGNs from nitric oxide toxicity, co-incubation with callistephin did not reduce the concentration of nitric oxide in solution (Fig. 6A). Incubation with kuromanin, however, significantly decreased the amount of nitric oxide that remained in solution following generation by SNP, suggesting that this anthocyanin is able to scavenge nitric oxide in some capacity (Fig. 6A).

We next aimed to determine the chemical mechanism by which kuromanin is able to scavenge nitric oxide, and prevent subsequent toxicity, in contrast to callistephin. These anthocyanins share a significant degree of structural homology at the molecular level, differing

only by the presence of a single hydroxyl group on the B-ring of kuromanin (Fig. 1). Given that these two compounds are otherwise identical, we postulated that the presence or absence of this catechol moiety on the B-ring is responsible for the differential neuroprotection of the anthocyanins against nitric oxide-induced cell death.

One unique aspect of catechol chemistry is the ability of catecholic compounds, such as kuromanin, to auto-oxidize and generate superoxide [37]. The superoxide produced from these reactions combines readily with other radical species, particularly nitric oxide. Therefore, we next assessed the ability of kuromanin to generate superoxide in a cell free system.

2-Methyl-6-(4-Methoxyphenyl) -3,7-Dihydroimidazo[1, 2-A]pyrazin-3-One, Hydrochloride (MCLA) luminescence was used to detect the presence of superoxide in solutions containing 100μM kuromanin alone or in combination with quinone oxidoreductase 1 (QR1) or quinone oxidoreductase 2 (QR2) in a cell free system. At this concentration, kuromanin alone did not produce detectable concentrations of superoxide in solution (Fig. 6B). Given that this assay was performed in the absence of cells and associated reducing factors that would allow kuromanin to undergo multiple auto-oxidation reactions, this result was not altogether unexpected. Without these reducing factors, each molecule has the potential to undergo only one auto-oxidation reaction, and detection would be limited by the rate at which this reaction occurred. QR1 and QR2 were added to the reaction to act as generic reducing factors for the quinone formed when kuromanin is fully oxidized, creating the potential for each molecule of kuromanin to undergo multiple oxidation reactions. Addition of QR1 to the system produced a modest luminescence signal while addition of QR2 significantly enhanced MCLA luminescence (Fig. 6B). Addition of SOD1 to solutions containing kuromanin and QR2 completely quenched MCLA luminescence, confirming that superoxide, and not another reactive radical species, was

responsible for the observed increase in the luminescence signal (Fig. 6B). Furthermore, neither QR1 nor QR2 produced an appreciable luminescence signal when incubated with only MCLA and their respective co-factors, NADH and 1-methyl-1, 4-dihydronicotinamide in comparison to a baseline set by incubating MCLA alone (Fig. 6C). It should be noted that baseline luminescence for all samples containing kuromanin was lower than that of all samples that did not contain kuromanin as the deeply pigmented solution produced by the addition of kuromanin partially quenched the baseline luminescence signal (Fig. 6B, C).

3.5 Kuromanin protects neurons from nitric oxide toxicity in a superoxide-dependent manner

To validate the data obtained using a cell free approach, we next examined the role of superoxide production in defending cultured neurons from nitric oxide toxicity. As before, CGNs were cultured with SNP alone, or in the presence of kuromanin, and evaluated for apoptosis. In addition to these treatment groups, cells were also treated with kuromanin and SNP in the presence of PEG-SOD, a cell permeable conjugate of SOD1, to scavenge any superoxide generated in culture. In agreement with our previous results, cells treated with SNP alone displayed fragmented tubulin networks and condensed nuclei indicative of widespread apoptosis, in comparison to untreated control cells (Fig. 7A). Kuromanin effectively mitigated these effects, preserving tubulin networks and healthy nuclear morphology. However, in the presence of PEG-SOD, the neuroprotective effects of kuromanin were completely ablated. Cells treated with PEG-SOD showed extensive disintegration of tubulin networks and condensed nuclei similar to cells that were treated with SNP alone (Fig. 7A). This effect was concentration dependent, as low concentrations of PEG-SOD had essentially no influence on the protective abilities of kuromanin, while concentrations of 30U/mL or more completely abolished kuromanin-mediated

neuroprotection (Fig. 7B). At these concentrations, up to 97% of cells possessed condensed or fragmented nuclei, and no significant difference was observed between the percentage of apoptotic cells under these conditions in comparison to cells treated with SNP alone (Fig. 7B). Moreover, treatment with PEG-SOD alone at the concentrations tested in these experiments had no effect on cellular viability (Winter and Linseman, unpublished data).

Following these experiments, we next sought to determine if superoxide generation by kuromanin occurred at the extracellular or intracellular level by comparing the effects of PEG-SOD and unconjugated SOD1, which is not cell permeable. As before, kuromanin protected CGNs from nitric oxide-induced toxicity, and these effects were blocked by concomitant treatment with PEG-SOD. However, treatment with unconjugated SOD1 had no significant effect on the neuroprotective capacity of kuromanin, suggesting that these effects occur at the intracellular level (Fig. 7C).

To confirm this observation using a molecular approach, we transfected Neuro2A mouse neuroblastoma cells with either a control vector expressing GFP or a vector expressing GFP-tagged, wild type SOD1, and treated them with SNP alone or in the presence of kuromanin. We then assessed the number of transfected cells displaying condensed or fragmented nuclei indicative of cell death. Cells transfected with the GFP vector and treated with SNP alone underwent significant cell death in comparison to untreated GFP-transfected controls, while cells that were co-treated with both SNP and kuromanin displayed a significant reduction in cell death in comparison to cells treated with SNP alone (Fig. 7D). In contrast, cells transfected with SOD1 and treated with SNP and kuromanin displayed the opposite result. Indeed, SOD1 overexpressing cells showed a marked increase in cell death in comparison to GFP-transfected cells that were

treated with SNP and kuromanin, and this level of death was comparable to that observed when cells were treated with SNP alone (Fig. 7D).

4. Discussion

Initially, two anthocyanin-enriched extracts were prepared from freeze-dried strawberry or blackberry powder in order to preliminarily characterize the neuroprotective effects of two distinct anthocyanin compositions. These fruits were chosen as their primary anthocyanin constituents, callistephin and kuromanin, have been previously described and are known to compose a majority of the anthocyanin profiles of strawberries and blackberries, respectively [38-39]. This increased the likelihood that any observed protective effects would be due to these two anthocyanins and not another phenolic species within the extracts. While it was found that treatment with either SAE or BAE was sufficient to defend primary neurons from excitotoxic insult, a stark contrast in neuroprotective ability between these two extracts was observed in cells treated with the nitric oxide donor, SNP. While BAE was able to significantly protect neurons from nitric oxide toxicity, SAE had no observable effect against this insult, suggesting that there is a distinct difference between the neuroprotective effects of the primary anthocyanin constituents of the two extracts. Similar effects were observed when pure preparations of both callistephin and kuromanin were assessed for their neuroprotective potential, confirming that these two compounds were likely responsible for the results observed when using enriched fruit extracts. However, it was notable that the protective concentration of kuromanin and callistephin in the extracts was much lower than that observed for the pure compounds. This may suggest synergism with other anthocyanin species or contaminating phenolics within the extract which was not observed when treating with the individual anthocyanins, and illustrates one of the

confounding factors of assessing the neuroprotective potential of particular species within enriched extracts. As anthocyanins are known to affect many pro-survival pathways (reviewed by Ross et al. [13 and 16], it is likely that the inclusion of multiple anthocyanin species or other contaminating phenolic compounds could lead to greater activation of neuroprotective signaling cascades. Similar results were observed by Carey et al. [17] in study of microglial inflammation where it was demonstrated that blueberry extract had greater anti-inflammatory capabilities than its pure anthocyanin constituents. Moreover, it should be noted that the actual concentration of free radical scavenging species within the extracts as a whole could not be determined due to lack of available standards to identify less concentrated anthocyanin species. Thus, the concentration and scavenging activity of the pure anthocyanin constituents of SAE and BAE, callistephin and kuromanin, is not reflective of the free radical scavenging activity of the anthocyanin extracts as a whole, which may account in part for the higher concentrations of pure anthocyanins required to achieve neuroprotection. Taken as a whole, these results indicate that subtle molecular differences between callistephin and kuromanin, specifically the absence or presence of a catechol moiety on the B-ring, was responsible for the differential neuroprotective effects observed under conditions of nitric oxide toxicity. This hypothesis was supported by the observation that kuromanin, which contains a catechol as part of its B-ring, was able to scavenge nitric oxide in solution to a significant degree, while callistephin, which lacks a catechol, was not.

Interestingly, the chemistry of anthocyanins and many other natural products is such that these potent antioxidants can behave as pro-oxidants under certain conditions, such as at high concentrations [40-41]. In particular, catechol containing compounds such as kuromanin are thought to execute their pro-oxidant functions through the process of auto-oxidation. This

reaction proceeds using molecular oxygen as an electron acceptor to produce first a semiquinone and then a quinone molecule with a net production of one superoxide radical (Fig. 8; [37]). Within the cell where there are multiple reducing factors that may regenerate the original parent compound, this auto-oxidation cycle could proceed many times for a single molecule, increasing the abundance of superoxide that is ultimately produced.

This hypothesis is consistent with our observation that kuromanin produces superoxide, but this radical reached detectable concentrations only when kuromanin was incubated in the presence of a quinone reductase. Moreover, Chichirau *et al.* [37] propose that this reaction may only occur while the parent catechol is in its anionic form, which accounts for only a small proportion of aqueous anthocyanins at a given time, further hampering detection of superoxide. While both quinone reductases used in our experiments caused some increased superoxide production by kuromanin, the largest increase in superoxide production was observed in the presence of QR2. These results are consistent with the observation that QR1 works best on *meta*- and *para*-quinones, with a low affinity for *ortho*-quinones, while QR2 preferentially reduces *ortho*-quinones, as would be formed by kuromanin [42]. This also lends further support to the idea that catechol-containing species like kuromanin are not only capable of generating superoxide, but may do so with an enhanced capacity in the presence of appropriate reducing factors, as might occur within a cell.

Pro-oxidant processes are thought to be partially responsible for the anti-carcinogenic properties of anthocyanins and other nutraceuticals, and have spurred the development of the "quercetin paradox." This paradox suggests that by performing their antioxidant function, flavonoids and other polyphenols produce reactive species that are capable of depleting endogenous antioxidants like glutathione, ultimately causing cytotoxicity [43-46]. It is

noteworthy, then, that both callistephin and kuromanin were previously demonstrated to preserve neuronal viability and glutathione levels in the presence of the glutathione-depleting agent, HA14-1, which may suggest that anthocyanin chemistry is uniquely suited to avoid glutathione depletion, unlike quercetin [20, 43]. This is further supported by the observation that an anthocyanin-rich diet stimulates promoter activity for γ-glutamylcysteine ligase, the rate limiting enzyme in glutathione synthesis, and increases glutathione levels in kidney, liver, and brain tissue *in vivo* [47-48]. Most impressive is the observation that in the context of nitric oxide toxicity and subsequent nitrosative stress, superoxide generated through auto-oxidation of kuromanin appears to play the unexpected role of a neuroprotective nitric oxide scavenger rather than that of a toxic free radical, illustrating a role for the pro-oxidant activity of anthocyanins in mitigating this facet of neurodegenerative disease. Indeed, incorporation of excess SOD1 into the cell culture completely abolished the neuroprotective effect of kuromanin against nitric oxide, indicating that this process is superoxide-dependent. Moreover, this phenomenon was observed only when SOD1 was able to enter the cell, either as a cell-permeable form of the enzyme, PEG-SOD, or through transfection with wild type SOD1, demonstrating that nitric oxide scavenging by superoxide takes place at the intracellular level. This is significant in that damage from nitric oxide occurs largely at the intracellular level throughout many neurodegenerative processes, either by induction of nitric oxide synthase (NOS) through inflammatory cytokine signaling, or through diffusion into neuronal cells from neighboring glia [49-50].

Combination of superoxide and nitric oxide results in the formation of peroxynitrite. While high levels of peroxynitrite can also be toxic, this compound has a very short half-life and is readily scavenged by a number of endogenous antioxidants, such as glutathione, in addition to flavonoids such as kuromanin and quercetin, which possess a catechol as a part of their B-ring

[51]. Strikingly, there is also evidence to suggest that, in moderation, peroxynitrite is neuroprotective against nitric oxide toxicity in primary cortical neurons [52-53]. These effects are due to stimulation of the pentose phosphate pathway, which generates an abundance of NADPH that can be used in the synthesis of important antioxidants such as glutathione, and other cellular defenses [53]. Additionally, oxidative modification of PTEN by peroxynitrite has been shown to elicit a neuroprotective response by enhancing activation of the PI3K/Akt pro-survival pathway [52]. Taken together, these data suggest that any peroxynitrite generated through the proposed scavenging mechanism illustrated in Figure 8 is not only non-toxic, but may in fact contribute to the neuroprotective effect of kuromanin against nitric oxide, although further study is needed to confirm this hypothesis. Alternatively, it is also possible that peroxynitrite generated under these conditions is also a toxic species that may itself be scavenged by catechol-containing anthocyanins like kuromanin, as discussed above, resulting in an overall neuroprotective effect.

Our structure-activity analysis of callistephin and kuromanin indicates that although all anthocyanins bear a high degree of structural homology, the absence or presence of a catechol moiety on the B-ring of the compound is necessary for it to mitigate nitric oxide toxicity and subsequent cell death. This may also be true of delphinidin-based anthocyanins, and compounds such as EGCG, which possess a pyrogallol moiety as part of their structure. Such compounds are also capable of producing superoxide and reactive *ortho*-quinones through the process of autoxidation, similar to kuromanin [54]. However, it should be noted that while these compounds may undergo similar processes, and therefore protect similarly from nitric oxide toxicity, various anthocyanins also display differences in their abilities to interact with other oxidative species. For example, increasing hydrophilicity in anthocyanins through addition of hydroxyl groups

lowers the ability of these compounds to interact with lipid-rich environments, hampering their ability to prevent other prominent features of neurodegeneration such as lipid peroxidation [28]. It is also notable that while most common anthocyanins are known to cross the blood-brain barrier (BBB), highly hydrophilic anthocyanins interact less with lipid-rich environments, and cross the BBB at a lower rate than their more lipophilic flavonoid relatives in an *in vitro* model of the BBB [28, 55-56] This decreases the likelihood that anthocyanins that contain a high number of hydroxyl groups, particularly on the B-ring, can effectively mitigate oxidative damage in the brain. While further evidence is required to confirm this observation *in vivo*, it has been previously observed that the rate of transport across the basolateral membrane during digestion is directly correlated with the lipophilicity of the anthocyanin in question [29]. As a result, compounds such as delphinidin-based anthocyanins may prove to be less effective therapeutics than other more lipophilic anthocyanins like kuromanin.

This highlights the importance of choosing a drug candidate with versatile chemistry to target multiple aspects of neurodegenerative disease in the most effective way possible. For this reason, mechanistic studies examining individual compounds for their efficacy in various systems that mimic neurodegenerative conditions are advantageous. Moreover this approach also lends itself to the creation of a multi-agent treatment paradigm, which may ultimately be required to combat complex neurodegenerative diseases, as highly effective compounds with complimentary chemical properties can be readily identified and combined in high through-put screens to assess potential synergism. This allows for the rapid assessment and development of new drug combinations for further pre-clinical testing.

While it is true that some element of the multi-agent strategy is present in the use of enriched fruit extracts, it is often the case that a single extract may contain compounds that

directly hinder one another's performance, compromising its effectiveness. Additionally, such extracts are difficult to standardize and purify sufficiently to ensure safety during consumption for clinical development. Lastly, understanding the mechanistic aspects of neuroprotection for a single compound, such as kuromanin, may allow for the identification or design of similar substances that are able to perform the same functions with even greater efficiency, which cannot be accomplished using extracts. This is particularly true in cases of compounds like anthocyanins where concentrations used to achieve neuroprotection *in vitro* far exceed physiological concentrations due to issues of bioavailability and metabolism. Compounds that perform the same functions with greater efficiency or that achieve higher bioavailability may ultimately prove to be more efficacious therapeutic compounds. In the case of anthocyanins, this may be especially true of their phenolic acid metabolites, which are derived from the B-ring of the parent compounds, and appear at concentrations in the body that far exceed that of the parent anthocyanin following ingestion [57-58]. Moreover, physiological concentrations of neurotoxic species, such as nitric oxide, should also be taken into account, as concentrations needed to achieve relatively fast neuronal death *in vitro* are also likely to exceed physiologically relevant concentrations observed *in vivo*. Thus, lower concentrations of potential therapeutic candidates may be needed *in vivo* to observe similar neuroprotective effects as those achieved *in vitro*. With this in mind, parent anthocyanins may provide some therapeutic benefit; however it is likely that their metabolites could ultimately prove to possess greater therapeutic potential. Nevertheless, studies like these are important to gain mechanistic insights into neuroprotection by polyphenolic species in order to determine if related or derivative compounds behave in a similar beneficial manner. Indeed, it will be of great interest in the future to determine if anthocyanin metabolites behave in a manner similar to their parent compounds, as these derivatives may ultimately be

responsible for the observed benefits of anthocyanin consumption *in vivo* due to their higher bioavailability.

4.2 Conclusions

Collectively, our data demonstrate that some anthocyanins may be more uniquely suited than others to combat the multi-faceted nature of neurodegenerative disorders. Indeed, while anthocyanins appear to be universally capable of mitigating conditions of oxidative stress to some degree, our observations clearly show that this ability does not extend to all anthocyanin family members under conditions of nitrosative stress. This understanding is vital for determining which anthocyanin species have the highest potential as preventative and therapeutic agents in the context of neurodegeneration, and for the development of new, more effective therapeutics in the future.

Conflict of Interest: None

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the preparation of the anthocyanin extracts used to conduct experiments and aided in performing the HPLC analysis of the extracts to characterize anthocyanin content and HPLC data analysis. SK conducted most of the experiments on neuroprotection utilizing the anthocyanin extracts. KM developed the HPLC protocol for characterization of anthocyanins in the two anthocyanin extracts and aided in conducting HPLC analysis and analyzing HPLC data. DAL conceived of the project and contributed most the experimental design in addition to aiding in the writing the manuscript with ANW.

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Figure 1. Molecular structures of callistephin (left) and kuromanin (right). Note the catechol moiety on the B-ring of kuromanin.

Figure 2. Representative HPLC chromatograms of anthocyanin-enriched extracts from strawberry (SAE) and blackberry (BAE). *A,* Purified anthocyanin extract from freeze-dried strawberry powder was analyzed by HPLC with UV/Vis detection at 520nm (top panel) and compared to a pure callistephin standard (bottom panel) for peak identification of the major anthocyanin constituent. Callistephin appears at rt=13.22 min for SAE and rt=13.22min for the pure standard; *B,* Purified anthocyanin extract from freeze-dried blackberry powder was analyzed by HPLC with UV/Vis detection at 520nm (top panel) and compared to a pure kuromanin standard (bottom panel) for peak identification of the major anthocyanin constituent. Kuromanin appears at rt=12.56 min for BAE and rt=12.55 min for the pure standard. rt: retention time.

Figure 3. Effects of SAE and BAE on glutamate excitotoxicity and nitric oxide toxicity induced by sodium nitroprusside (SNP). *A,* Primary rat cerebellar granule neurons (CGNs) were treated with 100μM glutamate alone or in combination with 0.15% SAE or 0.25% BAE for 24h. Cell viability was then assessed by MTT assay; *B,* CGNs were treated with 100μM SNP alone or in combination with SAE or varying concentrations of BAE for 24h. Cell viability was then assessed by MTT assay. All data are represented as mean ± SEM. *** indicates $p<0.001$ compared to untreated control cells, ††† represents $p<0.001$, †† represents $p<0.01$, and † represents $p<0.05$ in comparison to cells treated with the insult alone as determined by one-way ANOVA with a *post hoc* Tukey's test.

Figure 4. Both pure callistephin and kuromanin protect CGNs from glutamate-induced excitotoxicity. *A,* Representative fluorescent micrographs showing untreated control CGNs, or CGNs treated with 100μM glutamate alone or in combination with 100μM callistephin (Calli) or 100μM kuromanin (Kuro) for 24 hours. Top panels show immunocytochemistry for β-tubulin (green) and Hoechst stain (blue). Panels on the bottom show decolorized Hoechst fluorescence to visualize nuclear morphology. Scale bar=10μm; *B,* Quantitative assessment of cellular apoptosis CGNs treated as described in *(A)*. Cells were counted and scored as either living or apoptotic based on nuclear morphology, and the percent of cells showing apoptotic nuclei (indicated by the white arrows) was determined. Data are represented as the mean ± SEM. *** indicates $p<0.001$ compared to untreated control cells and ††† represents $p<0.001$ in comparison to cells treated with glutamate alone, as determined by one-way ANOVA with a *post hoc* Tukey's test.

Figure 5. Pure kuromanin, but not callistephin, protects CGNs from nitric oxide-induced toxicity. *A,* Representative fluorescent micrographs showing untreated control CGNs, or CGNs treated with 100μM SNP alone or in combination with 100μM callistephin (Calli) or 100μM kuromanin (Kuro) for 24 hours. Top panels show immunocytochemistry for β-tubulin (green) and Hoechst stain (blue). Panels on the bottom show decolorized Hoechst fluorescence to visualize nuclear morphology. Scale bar=10μm; *B,* Quantitative assessment of cellular apoptosis CGNs treated as described in *(A)*. Cells were counted and scored as either living or apoptotic based on nuclear morphology, and the percent of cells showing apoptotic nuclei (indicated by the white arrows) was determined. Data are represented as the mean ± SEM. *** indicates $p<0.001$ compared to untreated control cells and ††† represents $p<0.001$ in comparison to cells treated with SNP alone, as determined by one-way ANOVA with a *post hoc* Tukey's test.

Figure 6. Kuromanin, but not callistephin scavenges nitric oxide present in culture medium following incubation with SNP through a superoxide dependent mechanism. *A,* 100μM SNP at 37°C alone or in combination with 100μM callistephin (Calli) or 100μM kuromanin (Kuro) for 24 hours. The amount of total nitrate and nitrite was then assessed using the Griess assay to quantify the amount of nitric oxide remaining after anthocyanin addition. Data are represented as the mean ± SEM. * indicates $p<0.05$ compared to culture medium containing SNP alone by one-way ANOVA with *post hoc* Tukey's test; *B,* Superoxide production was monitored using MCLA luminescence assay. A baseline with just MCLA in PBS was recorded for the first 4 minutes of the assay (data not shown) before addition of 100μM kuromanin alone or in combination with 30U/mL Cu, Zn-superoxide dismutase (SOD1). MCLA luminsecence was then assessed for 1 minute before the addition of 5U/mL of either quinone reductase 1 or 2 (QR 1/2)

and either NADH or methyl-1, 4-dihydronicotinamide (MDHNA) cofactor, respectively. *C,* Samples containing MCLA alone, MCLA with QR1 and NADH co-factor, or MCLA with QR2 and MDHNA co-factor were monitored to measure baseline luminescence in the absence of kuromanin substrate. Time points at which additions were completed for *(B)* and *(C)* are indicated below the x-axis of the graphs. *** indicates $p<0.001$, ** indicates $p<0.01$, and * indicates $p<0.05$ compared to MCLA luminescence with kuromanin alone, ††† indicates $p<0.001$ compared to MCLA luminescence in sample containing kuromanin, QR2, and SOD1, and ‡‡‡ indicates $p<0.001$ compared to samples containing kuromanin and QR2. All statistics were conducted using two-way ANOVA for repeated measures with a *post hoc* Bonferroni test to determine differences between groups.

Figure 7. Delivery of SOD1 intracellularly blocks the neuroprotective effects of kuromanin against SNP. *A,* Representative fluorescent micrographs showing untreated control CGNs, CGNs treated with 100μM SNP alone or in combination with 100μM kuromanin (Kuro), and CGNs treated with SNP, kuromanin, and PEG-SOD at the indicated concentrations (U/mL) for 24h. Top panels show immunocytochemistry for β-tubulin (green) and Hoechst stain (blue). Panels on the bottom show decolorized Hoechst fluorescence to visualize nuclear morphology. Scale bar=10μm; *B,* Quantitative assessment of cellular apoptosis for CGNs treated as in *(A).* Cells were counted and scored as either living or apoptotic based on nuclear morphology, and the percent of cells showing apoptotic nuclei (indicated by the white arrows) was determined; *C,* Quantitative assessment of cellular apoptosis for untreated control CGNs, CGNs treated with SNP alone or in combination with kuromanin, and CGNs treated with SNP, kuromanin, and either unconjugated SOD1 or PEG-SOD for 24 hours; *D,* Quantitative assessment of apoptosis in

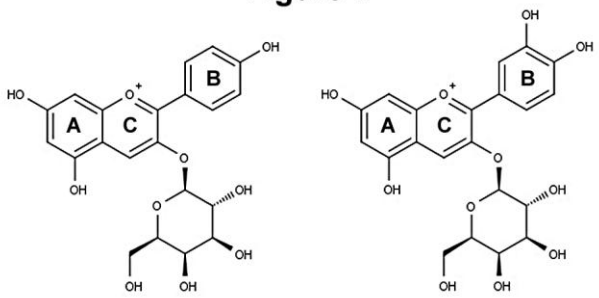
Neuro2A cells transfected with either GFP or GFP-tagged SOD1 and treated with 200μM SNP alone or in combination with 100μM kuromanin. Transfected (GFP-positive) cells were counted and scored as either living or apoptotic based on nuclear morphology, and the percent of cells showing apoptotic nuclei (either condensed or fragmented morphology) was determined. Data are represented as the mean ± SEM. *** indicates $p<0.001$ compared to untreated control cells, ††† represents $p<0.001$ in comparison to cells treated with SNP alone, and ‡‡‡ indicates $p<0.001$ in comparison to cells treated with kuromanin and SNP in combination as determined by one-way ANOVA with a *post hoc* Tukey's test.

Figure 8. Proposed auto-oxidation reaction of kuromanin to produce superoxide. Kuromanin reacts with molecular oxygen to produce a semiquinone and one superoxide radical ($HO_2^{\bullet}$). Superoxide produced from this reaction is consumed to produce hydrogen peroxide (H_2O_2), while molecular oxygen interacts with the semiquinone radical to form more superoxide. This results in the net production of one molecule of H_2O_2, one superoxide radical, and the oxidized quinone form of kuromanin. The superoxide radical produced from this reaction is then able to scavenge nitric oxide ($NO^{\bullet}$) radicals to form peroxynitrite ($ONOO^-$). The oxidized quinone form of kuromanin is then recycled to the reduced catechol by a quinone reductase or other reducing factor (QR). This mechanism is adapted from a mechanism proposed by Chichirau et al. [37].

Highlights

- Anthocyanins are polyphenolic compounds with neuroprotective properties
- Distinct anthocyanins display opposing neuroprotective effects against nitric oxide
- Protection from nitric oxide relies on catechol-dependent superoxide generation
- Catecholic anthocyanins may be effective therapeutic agents for neurodegeneration

Figure 1



Pelargonidin-*O*-3-Glucoside (Callistephin)

Cyanidin-*O*-3-Glucoside (Kuromanin)

Figure 2

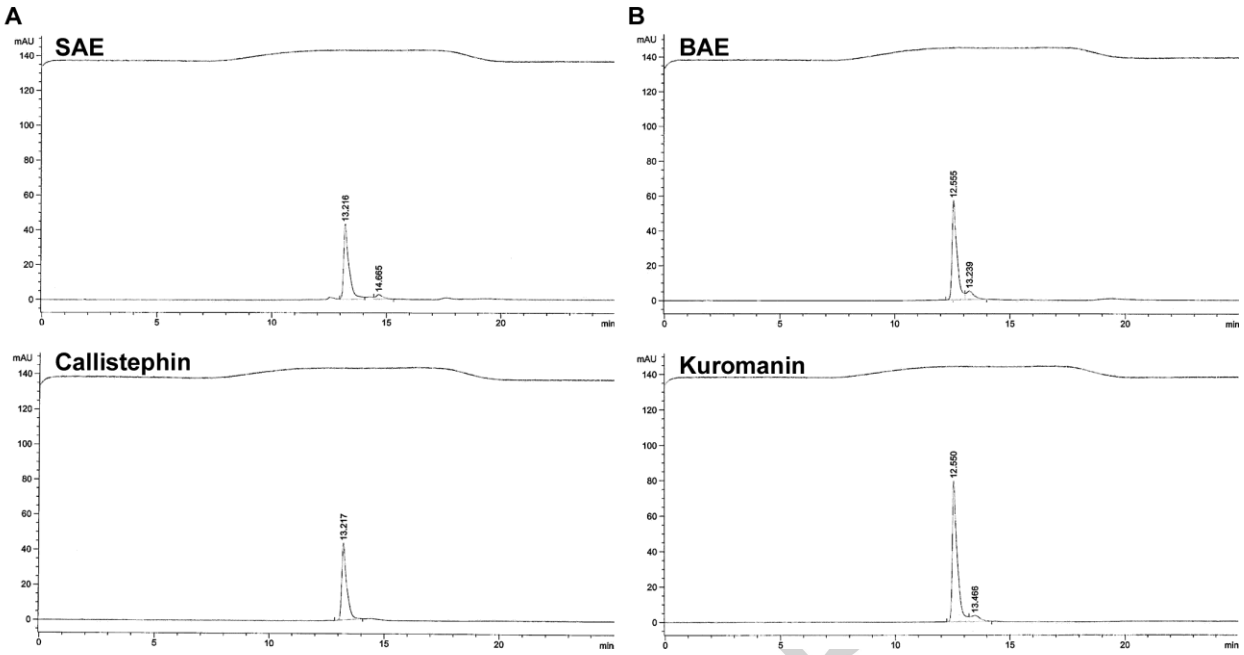


Figure 3

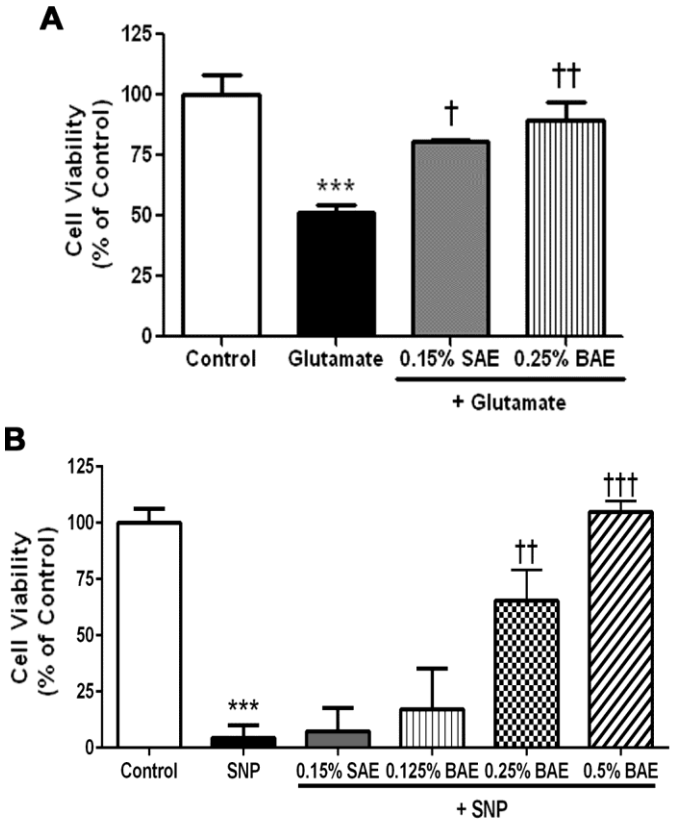


Figure 4

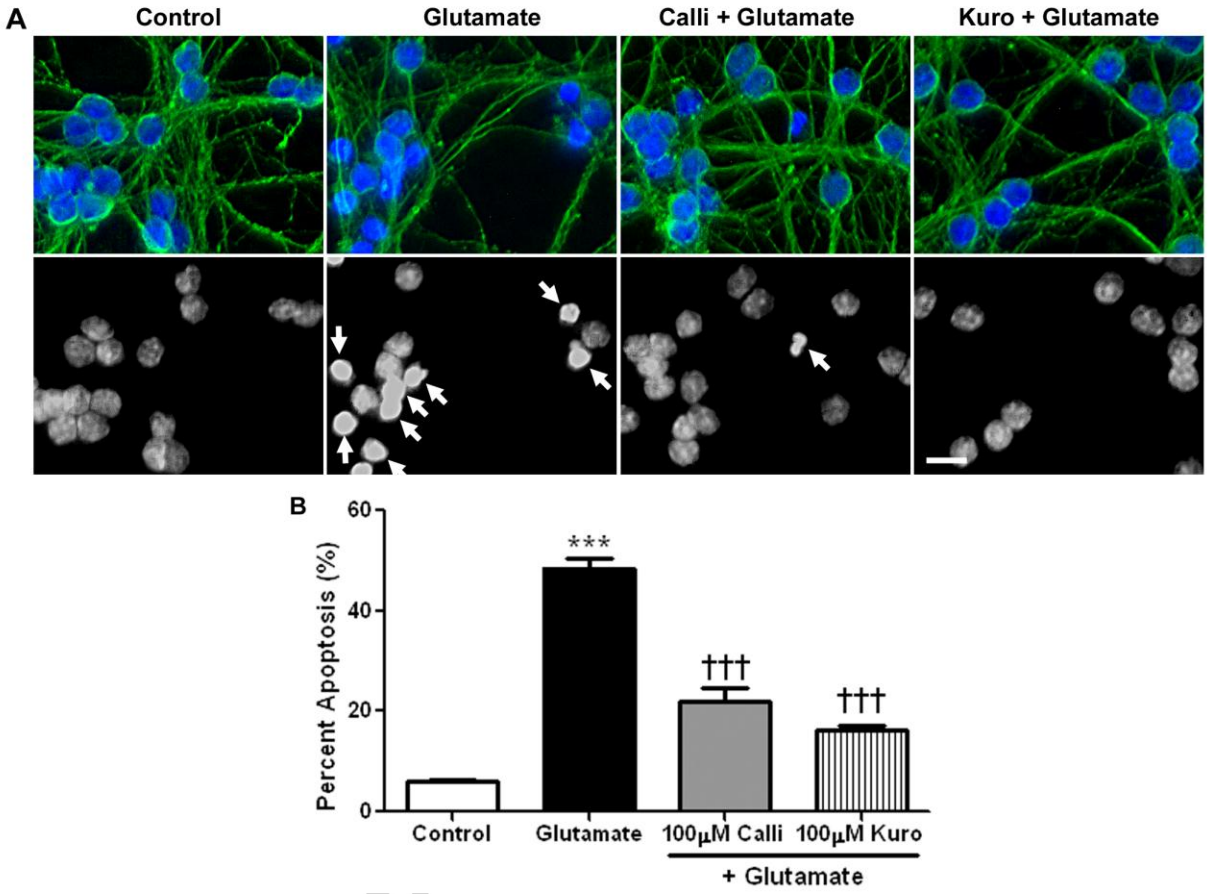


Figure 5

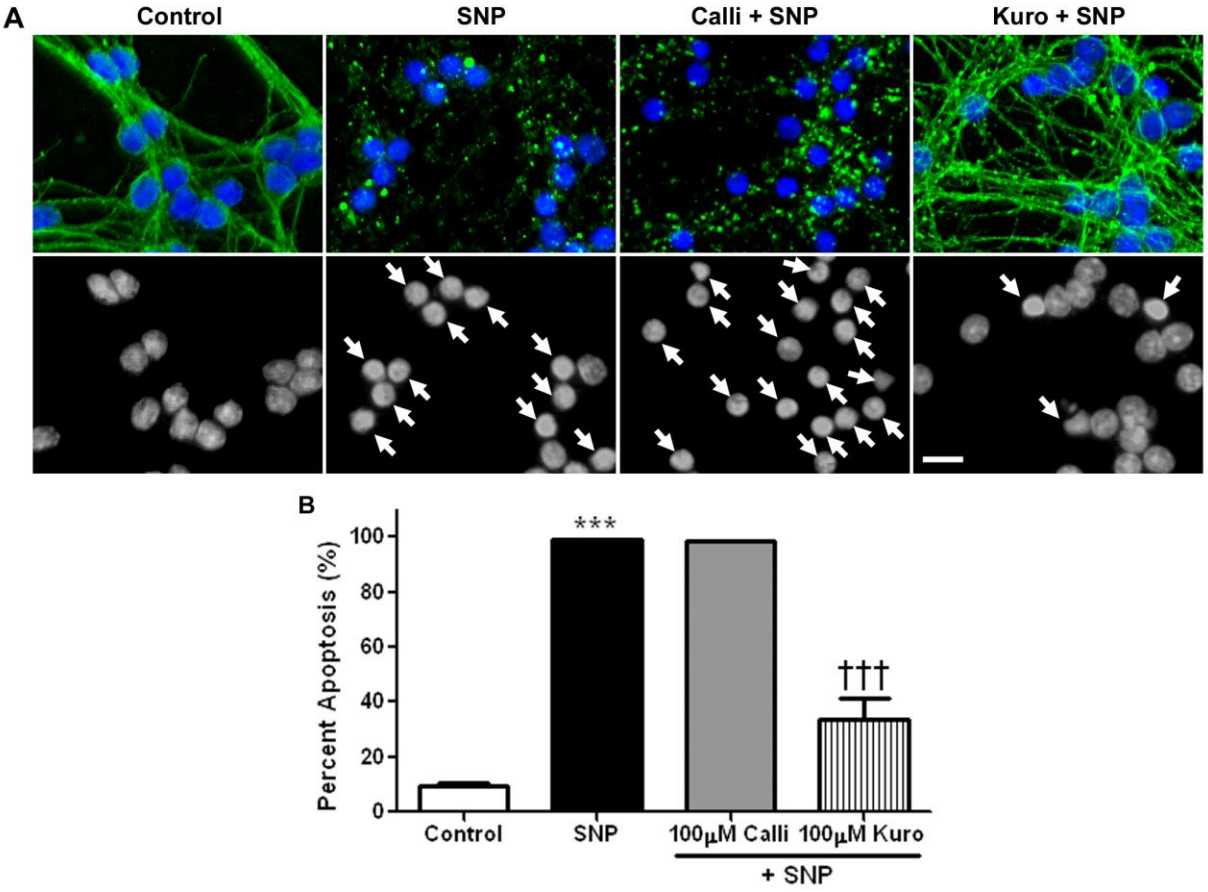


Figure 6

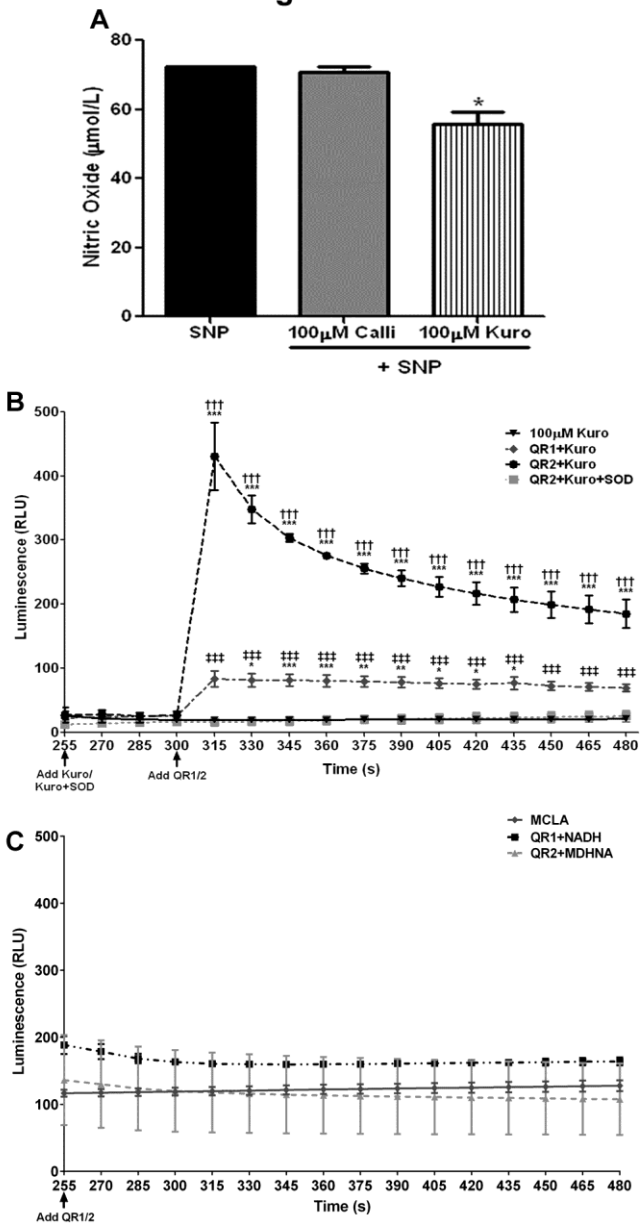


Figure 7

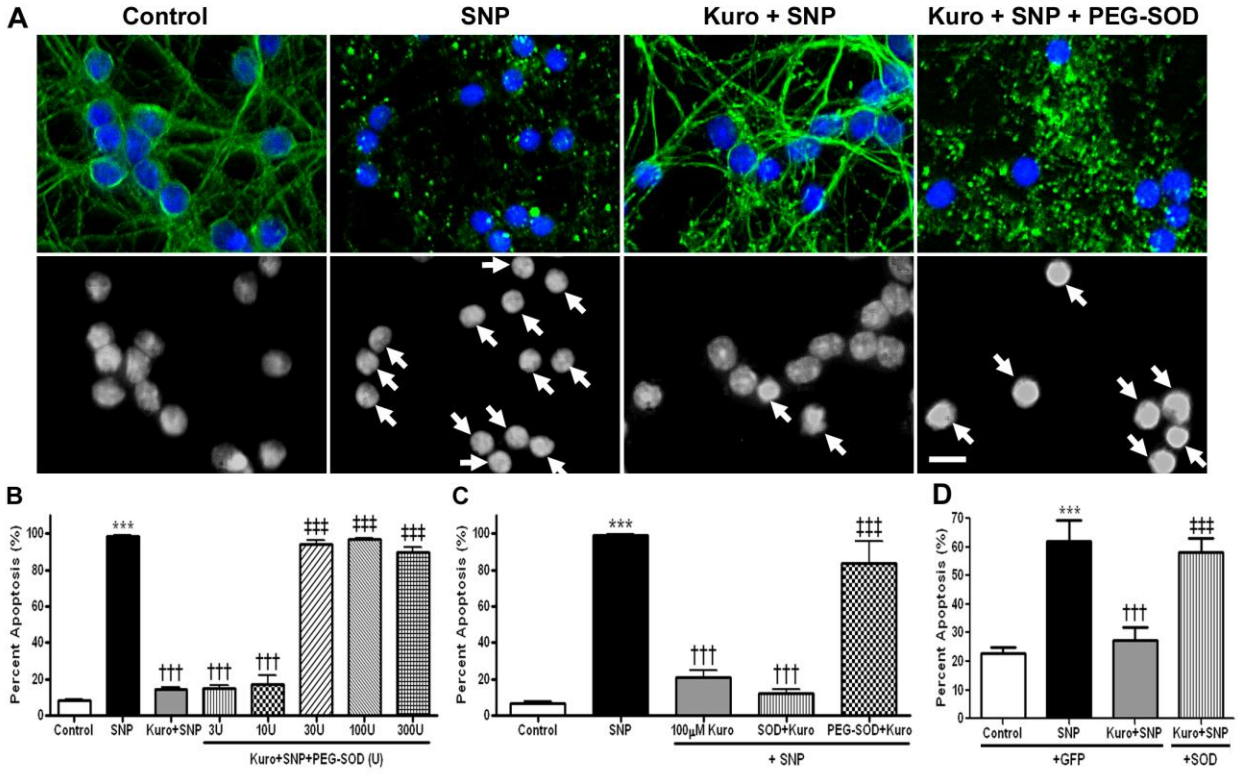


Figure 8