Neuroprotective mechanisms of red clover and soy isoflavones in Parkinson's disease models

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Abstract

Parkinson's disease (PD) is a neurodegenerative disorder characterized by nigrostriatal degeneration and the spreading of aggregated forms of the presynaptic protein α-synuclein (aSyn) throughout the brain. PD patients are currently only treated with symptomatic therapies, and strategies to slow or stop the progressive neurodegeneration underlying the disease's motor and cognitive symptoms are greatly needed. The time between the first neurobiochemical alterations and the initial presentation of symptoms is thought to span several years, and early neuroprotective dietary interventions could delay the disease onset or slow PD progression. This study aimed at characterizing the neuroprotective effects of isoflavones, a class of dietary polyphenols found in soy products and in the medicinal plant red clover (*Trifolium pratense*). We found that isoflavone-rich extracts and individual isoflavones rescued the loss of dopaminergic neurons and the shortening of neurites in primary mesencephalic cultures exposed to two PD-related insults, the environmental toxin rotenone and adenovirus encoding the A53T aSyn mutant. The extracts and individual isoflavones also activated the Nrf2-mediated antioxidant response in astrocytes via a mechanism involving inhibition of the ubiquitin-proteasome system, and they alleviated deficits in mitochondrial respiration. Furthermore, an isoflavone-enriched soy extract reduced motor dysfunction exhibited by rats lesioned with the PD-related neurotoxin 6-OHDA. These findings suggest that plant-derived isoflavones could serve as dietary supplements to delay PD onset in at-risk individuals and mitigate neurodegeneration in the brains of patients.

Keywords: alpha-synuclein, astrocytes, mitochondria, neurodegeneration, neuroprotection, Nrf2, 6-OHDA, rotenone, flavonoids, polyphenols.

1. Introduction

Parkinson's disease (PD) is a neurodegenerative disorder that affects 5% of the global population over the age of 85 [1-3]. The disease involves a loss of dopaminergic neurons from the *substantia nigra* in the midbrain, and this neuronal loss is largely responsible for motor symptoms such as the inability to initiate movement, resting tremor, and reduced balance [4]. Pathological phenomena underlying neurodegeneration in PD include oxidative stress [5, 6], loss of mitochondrial function [7-9], aggregation of the presynaptic protein α-synuclein (aSyn) [10-12], and microglial activation [13]. Familial PD mutations in the *SNCA* gene encoding aSyn are thought to promote the formation of toxic aSyn oligomers by increasing the protein's expression levels or intrinsic aggregation propensity in the case of duplication/triplication mutations or substitution mutations, respectively [12, 14-18]. Epidemiological evidence suggests that exposure to environmental toxins such as rotenone and paraquat leads to an increase in PD risk [19-22], potentially via a mechanism involving the production in neurons of reactive oxygen species (ROS) that in turn lead to a build-up of oxidatively modified forms of aSyn with a high propensity to form potentially toxic aggregates [12, 23, 24]. Current PD treatments consist primarily of dopamine replacement therapy (DRT), including administration of the dopamine metabolic precursor L-DOPA or synthetic dopamine receptor agonists, but these agents only alleviate symptoms without reversing the underlying neuronal death [25]. Patients on long-term DRT eventually experience pronounced side effects that include L-DOPA-induced dyskinesias, aggression, and insomnia [26]. Accordingly, there is a critical need to identify disease-modifying therapies that slow PD progression.

Multiple lines of evidence suggest that isoflavone-rich extracts such as soy-derived products and the medicinal plant red clover (*Trifolium pratense*) could have neuroprotective effects in the brains of PD patients. An ethnopharmacological survey of Pikuni-Blackfeet traditional medicine led to the identification of red clover as a traditional treatment for symptoms related to PD [27]. In addition, epidemiological data suggest a correlation between estrogen replacement therapy, amelioration of cognitive functions (e.g 60 mg total isoflavone equivalent/day, 12 weeks), and delayed onset of PD and Alzheimer's disease (AD) [28-31], suggesting that the consumption of phytoestrogens in isoflavone-rich foods and plant medicines could slow functional decline of the aging brain. In support of this idea, isoflavones and isoflavone-rich extracts have exhibit neuroprotective activity in preclinical models of AD and cognitive impairment [32, 33]. Individual isoflavones such as genistein, a major isoflavone found in soy, have also been reported to attenuate Parkinsonian symptoms in rodents exposed to MPTP, 6-OHDA, or LPS by restoring motor functions and preserving dopaminergic neurons [34-39]. Although these observations suggest that isoflavones can alleviate neurodegeneration in PD models, the effects of isoflavones or isoflavone-rich botanical extracts on toxicity elicited by insults linked epidemiologically or genetically to PD are poorly understood. Polyphenols (including isoflavones) are well known for their ability to scavenge ROS [40, 41].

However, the fact that their brain levels are lower than endogenous glutathione levels suggests that they may mitigate neurotoxicity via additional protective mechanisms, including the activation of nuclear factor E2-related factor 2 (Nrf2), a transcription factor that regulates the expression of genes involved in the cellular antioxidant response, as well as modulation of the ubiquitin-proteasome system (UPS) and rescue of mitochondrial functional deficits [27, 42].

This research study is aimed at characterizing the neuroprotective effects of isoflavone-rich extracts and individual isoflavones and elucidating their mechanisms of action, with an emphasis on modulation of the Nrf2-mediated antioxidant pathway, the UPS, and mitochondrial function. Our findings suggest that medicinal plants enriched in isoflavones such as red clover and soy could form the basis of dietary interventions for individuals at risk for PD or therapeutic preparations to slow PD progression, and they yield insights into mechanisms by which isoflavone-rich extracts could mitigate neurodegeneration in the brains of patients.

2. Materials and methods

2.1. Materials

Chemicals were obtained from Sigma Chemical Co. (St. Louis, MO), except when noted. Dulbecco's Minimal Essential Media (DMEM), fetal bovine serum (FBS), penicillin streptomycin, and trypsin-EDTA were obtained from Invitrogen (Carlsbad, CA). Nuserum was purchased from Thermo Fisher Scientific (Waltham, MA). The SH-SY5Y cells were purchased from ATCC (Manassas, Virginia). Human iPSC-derived astrocytes (iCell astrocytes) were obtained from Cellular Dynamics International (CDI) (Madison, WI). The Novasoy 400 extract was obtained from Archer Daniels Midland (Chicago, IL). The vector pSX2_d44_luc [43] was provided by Dr. Ning Li (UCLA) with the permission of Dr. Jawed Alam (LSU Health Sciences Center), and the GFPu reporter adenovirus was provided by Dr. Xuejun Wang (University of South Dakota) [44].

2.2. Antibodies

The following antibodies were used in this study: chicken anti-microtubule-associated protein 2 (MAP2) (catalog number CPCA-MAP2, EnCor Biotechnology, Gainesville, FL); rabbit anti-tyrosine hydroxylase (TH) (catalog number 2025-THRAB, PhosphoSolutions, Aurora, CO); anti-rabbit IgG-Alexa Fluor 488 and anti-chicken IgG-Alexa Fluor 594 (Invitrogen, Carlsbad, CA).

2.3. Preparation and dissolution of botanical extracts

Red clover flowers were harvested, immediately dried at 37 °C with a food processor, and the water extract was prepared as described previously to reproduce the traditional methods of preparation of red clover-based herbal remedies [27, 42]. The soy extract was prepared as described from Tofu

soybeans [45, 46]. Prior to each experiment, extracts were dissolved in sterile deionized water (red clover) or ethanol (soy and Novasoy 400).

2.4. HPLC-TOF-MS profiling of a red clover extract

HPLC-TOF-MS analyses were conducted on an Agilent 6220a TOF-MS, equipped with a 1200 series HPLC (Agilent Technologies, Santa Clara, CA) with a Waters X-Bridge, 4.6 x 100 mm, 3.5 μm (Waters, Milford, MA) using a gradient method (0-5 min 2% CH3CN + 0.1 % formic acid/H_2O + 0.1% formic acid; 14 min 40%; 17-18 min 100%; 20-24 min 2%). TOF parameters included a drying gas of 10 L/min, nebulizer pressure of 45 psi, capillary voltage of 3.5 kV, fragment or voltage of 80 V, and a mass range of m/z 100-3000 in both positive and negative modes. The extract was run at 10 mg/mL with an injection volume of 10 μL.

2.5. Preparation of rat primary cultures

Primary, mixed midbrain cultures were prepared via dissection of E17 embryos obtained from pregnant Sprague-Dawley rats (Harlan, Indianapolis, IN) using methods approved by the Purdue Animal Care and Use Committee [15, 27, 42, 47, 48]. Briefly, the mesencephalic region containing the *substantia nigra* and ventral tegmental area was isolated stereoscopically, and the cells were dissociated with trypsin (final concentration, 26 μg/mL in 0.9% [w/v] NaCl). For experiments that involved analysis of neuroprotective activity (see Section 2.6), the dissociated cells were plated in the wells of a 48- or 96-well, plate (pretreated with poly-L-lysine, 5 μg/mL) at a density of 163,500 or 81,750 cells per well (respectively) in midbrain culture media consisting of DMEM, 10% (v/v) FBS, 10% (v/v) HS, penicillin (10 U/mL), and streptomycin (10 μg/mL). After 5 DIV, the cultures were treated with AraC (20 μM, 48 h) to slow the proliferation of glial cells. For experiments that involved monitoring activation of the Nrf2 pathway (see Section 2.7), the dissociated cells were plated in the wells of a 96-well, black clear-bottom plate (pretreated with poly-L-lysine, 10 μg/mL) at a density of 92,650 cells per well in midbrain culture media. After 5 DIV, the cultures were treated with AraC (20 μM, 72 h) before initiating experimental treatments.

Primary midbrain or cortical astrocytes were prepared via dissection of E17 embryos obtained from pregnant Sprague-Dawley rats (Harlan, Indianapolis, IN) using methods approved by the Purdue Animal Care and Use Committee, as described above. The dissociated cells were plated in a 6-well plate pre-treated with rat collagen (25 μg/mL) at a density of ~27x10^4 cells per well, while the cortical cells were plated in a 175 cm² flask pre-treated with rat collagen (25 μg/mL) at a density of ~14.5x10^6 cells per dish. The cultures were maintained in media consisting of DMEM, 10% (v/v) FBS, 10% (v/v) horse serum, penicillin (10 U/mL), and streptomycin (10 μg/mL). After 48 h, new media consisting of DMEM, 10% (v/v) Nuserum, 10% (v/v) horse serum, penicillin (20 U/mL), and streptomycin (20 μg/mL) was added to selectively propagate the astrocyte population (clusters of

cells that attached the dish) and remove unattached neurons. The media was replaced every two days until most of the astrocytes had spread out on the plate (generally by 7 DIV). The astrocyte-rich culture was passaged at least once (and no more than twice) before being used for the described experiments.

2.6. Analysis of neuroprotective activity of red clover or soy extracts in primary midbrain cultures

The analysis of neuroprotective activities of extracts and natural products was carried out as described previously [27, 42]. Briefly, primary midbrain cultures (7 DIV) were incubated in the absence or presence of botanical extract for 72 h, and then incubated for another 24 h in fresh media containing rotenone and extract or isoflavones. Alternatively, midbrain cultures were transduced with an adenovirus encoding A53T (A53T Ad) [15], at a multiplicity of infection (MOI) of 15 for 72 h in the presence of extract or isoflavones and then incubated in fresh media supplemented with extract for another 24 h. Control cultures were incubated in media without rotenone, A53T Ad, or extract. The cultures were then fixed, permeabilized, blocked [15, 27, 42, 47, 48], incubated with primary antibodies specific for MAP2 (chicken, 1:2000) and TH (rabbit, 1:1000) for 48 h, at 4 °C. The cells were then washed with PBS and incubated with a goat anti-chicken antibody conjugated to Alexa Fluor 594 (1:1000) and a goat anti-rabbit antibody conjugated to Alexa Fluor 488 (1:1000) for 1 h at 22 °C. The survival of dopaminergic neurons was determined by calculating the ratio of MAP2- and TH-immunoreactive neurons in a blinded manner, using a minimum of 12 images containing around 500–1000 MAP2$^+$ neurons. Each experiment was performed using at least 3 independent preparations of embryonic cultures, and presented the percentage of MAP2$^+$ neurons that were also TH$^+$ to normalize for variations in cell plating density. In addition, the lengths of MAP2$^+$ processes extending from TH$^+$/MAP2$^+$ neurons with an intact cell body (~90 neurons per sample) were measured in a blinded manner using the manual length measurement tool of the NIS Elements software (Nikon Instruments, Melville, NY).

2.7. Treatment with ARE-EGFP reporter adenovirus

An adenovirus encoding enhanced green fluorescent protein (EGFP) downstream of the minimal promoter of the mouse heme oxygenase-1 (HO-1) gene (pAd-ARE-EGFP-TKpolyA) was prepared [27, 43], and activation of the Nrf2 pathway was monitored in cortical astrocytic cultures and primary midbrain cultures as described [27, 42]. Briefly, primary cortical astrocytes and primary midbrain astrocytes were plated at a density of 5,000 cells per well on a 96-well black clear-bottom plate and transduced with the ARE-EGFP reporter virus at an MOI of 25. Additional experiments were carried out with human iCell astrocytes produced at CDI (Madison, WI) by differentiating an iPSC line that was reprogrammed from fibroblasts obtained from an apparently healthy female individual without

known PD-related mutations. Each lot provided by CDI consists of >95% astrocytes that express relevant markers (S100β and GFAP) and respond to pro-inflammatory stimulation by cytokines. iCell astrocytes were plated at a density of 10,000 cells per well on a 96-well, black clear-bottom plate (pretreated with laminin, 10 µg/mL) in DMEM/F12, HEPES media supplemented with 2% (v/v) FBS and 1% (v/v) N-2 supplement. After 24 h, the cells were transduced with the ARE-EGFP reporter virus at an MOI of 25. In other experiments, primary midbrain cultures (8 DIV) prepared as described above (see Section 2.5) were transduced with ARE-EGFP reporter virus at an MOI of 10.

After 48 h, the virus-containing media was removed, and the cells were treated with botanical extract for 24 h, incubated with 5 µM curcumin (positive control), or untreated (control). The cells were imaged in HBSS at 37 °C using a Cytation 3 Cell Imaging Reader equipped with a 4X objective (BioTek Instruments, Winooski, VT). Quantification of EGFP and Hoechst fluorescence was carried out with Gen 5 2.05 data analysis software (BioTek Instruments, Winooski, VT) as described previously [27, 42]. Briefly, ROIs were generated based on size (40 – 400 µm for astrocytes, 20 – 400 µm for mixed midbrain cultures) and a threshold for fluorescence intensity was calculated such that the fluorescence intensity in the positive control culture (5 µM curcumin) was 1.5- to 2.5-fold greater than in the negative-control. Finally, the number of ROIs for EGFP was divided by the total cell number for each condition and normalized to the control value.

2.8. RNA isolation and quantitative RT-PCR

Cortical astrocytes were harvested by centrifugation at 1,300 x g for 10 min at 4 ºC, and the mRNA was extracted using an E.Z.N.A Total RNA kit (Omega Bio-Tek, Norcross, GA). Total RNA (200 ng) was reverse-transcribed using an iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Quantitative PCR (qRT-PCR) was performed using an iQ SYBR Green PCR Kit (Bio-Rad) with forward and reverse primers specific for heme oxygenase 1 (HO1), glutamate-cysteine ligase, catalytic subunit (GCLC), Nrf2, or GAPDH. The primer sequences designed for the rat cDNA sequence were as follows (forward primer sequence relative to the coding sequence listed first for each primer pair): GCLC: $^{5'}$GTTCAACACAGTGGAGGACAA$^{3'}$ and $^{5'}$GGGACTTAGATGCACCTCCTT$^{5'}$; HO1: $^{5'}$ACAACCCCACCAAGTTCAAA$^{3'}$ and $^{5'}$CCTCTGGCGAAGAAACTCTG$^{3'}$; Nrf2: $^{5'}$GAGACGGCCATGACTGATT$^{3'}$ and $^{5'}$CAGTGAGGGGATCGATGAG$^{3'}$; GAPDH: $^{5'}$GAACATCATCCCTGCATCCA$^{3'}$ and $^{5'}$CCAGTGAGCTTCCCGTTCA$^{3'}$.

The red clover cDNA levels were normalized to GAPDH cDNA levels, and the fold change for samples treated with a red clover extract relative to control samples was calculated using the following formula:

$$2^{-\Delta\Delta Ct} = 2^{-\{(Ct,TG-Ct,CG)RC-\{(Ct,TG-Ct,CG)control\}}$$

Where Ct,TG represents the crossing threshold for the target gene, Ct,CG represents the crossing threshold for the control gene, 'RC' refers to mRNA obtained from cells incubated in the presence of red clover extract, and 'control' refers to mRNA obtained from cells incubated in the absence of red clover extract.

2.9. UPS reporter assay

UPS function was monitored as described [42] using an adenoviral reporter construct encoding GFPu, a form of GFP with a C-terminal fusion of the degron CL1 [44, 49, 50]. Cortical astrocytes were plated on a 96-well black clear-bottom and were transduced with the GFPu reporter adenovirus at an MOI of 2. After 48 h, the cells were incubated in fresh media containing red clover extract, MG132 (2 µM, positive control), or untreated (control) for an additional 24 h. Quantification of GFP fluorescence was carried out as described previously [42]. Briefly, ROIs were generated by the Cytation software GEN5 2.05 based on size (20 – 400µm) and the threshold for fluorescence intensity was calculated such that a 6-7 fold increase in the number of ROIs above the threshold was observed for the MG132-treated culture compared to the negative-control culture. The number of ROIs for GFP was then divided by the total cell number for each condition and normalized to the control value.

2.10. Oxygen consumption assay

Oxygen consumption was monitored as described previously [42, 51]. Briefly, neuroblastoma SH-SY5Y cells were grown in glucose-free media supplemented with galactose. The cells were incubated in the absence or presence of botanical extract for 19 h and then treated with 30 nM rotenone for 5 h, as opposed to competition assays for which both rotenone and extract were added at the time of the oxygen consumption measurement. In this case, the cells were exposed to a higher concentration of rotenone (50 nM instead of 30 nM) to ensure a rapid and robust inhibition of cellular respiration. The cells were harvested, suspended in O_2 consumption buffer (10 mM $MgCl_2$, 20 mM HEPES pH 7.2, 8.6 % sucrose (w/v), 0.026 % KH_2PO_4 (w/v)), and cellular respiration was measured using a Clark-type oxygen electrode attached to a voltmeter (Digital Model 10 Controller, Rank Brothers, Ltd, Cambridge, UK). An aliquot of 4.5 x 10^6 cells was loaded into the respiration chamber, and the sample was constantly stirred at 840 rpm. Using the Pico Technology software program (PicoTechnology, Ltd., Cambridgeshire, UK), the O_2 level remaining in the chamber at any time during respiration was automatically logged (with 10 sec intervals) as a voltage, VO_2, which corresponded to the voltage generated by the reaction of O_2 with the electrode and steadily decreased as O_2 was consumed. The rate of O_2 consumption was calculated as previously described using the following formula [52]:

$$Rate\ of\ O_2\ consumption\ (pmol\ s^{-1}10^6cells^{-1}) = \frac{\left(-slope\ [\frac{V}{seconds}]\right)\ \times\ 200\ \times 10^3\ (pmol/mL)}{number\ of\ cells/mL\ (\times\ 10^6)}$$

Mean O_2 consumption rates were obtained from 3 independent experiments and normalized to control values.

2.11. *In vivo* experiments

A soy extract was examined for protective activity in adult male Sprague-Dawley rats (Harlan, Indianapolis, IN) using methods approved by the Purdue Animal Care and Use Committee. The rats (250–300 g) were divided into four groups: control (n = 3), sham (n = 7), 6-OHDA/DMSO (n = 7), and 6-OHDA/soy (n = 7). The control group received no injections. The sham, 6-OHDA/DMSO, and 6-OHDA/soy groups received a stereotaxic injection of saline or 6-OHDA in saline (4 µg/µL). The 6-OHDA/DMSO and 6-OHDA/soy groups received a daily ip injection of 30% (v/v) DMSO vehicle (1 mL/kg) or soy extract (5-20 mg/kg), respectively, over a period of 39 days. Stereotaxic injections were performed unilaterally in the right medial forebrain bundle (MFB) on day 12, with a delivery of 2 µL (1 µL/min), using the coordinates: ML, -1.5 mm; AP, -4.0mm; DV, -8.5 mm.

Rat motor function was assessed using the rotarod and open field tests [53, 54]. For the rotarod test, the rotarod speed was increased gradually from 0 to 30 revolutions per minute (rpm) over 3 minutes and maintained at 30 rpm until stopping at 3.5 min, or earlier if the rat fell off the wheel. Criteria were developed to ensure that animals were accustomed to the instrumentation and testing before exposure. Prior to 6-OHDA lesioning, the rats were taken through a training phase during which they were required to complete 2 out of 3 runs at 30 rpm (top speed). For the open field test, the rats were examined before 6-OHDA injection and every five days after lesioning, for a total of 30 days. The animals were placed in a Plexiglas activity box ($100 \times 100 \times 20$ cm^3) with food over the center. Eight infrared beams were set in an X-Y matrix, and the rat's position relative to the beams was monitored at 200 ms intervals with in-house software. The data were used to determine the percentage of the total box area covered (normalized to the area covered by control rats).

2.12. Statistical analysis

Data from measurements of primary neuron viability, mitochondrial O_2 consumption rates, ARE-EGFP fluorescence, GFPu fluorescence, and rat motor function were analyzed via one-way ANOVA followed by Tukey's or Dunnett's multiple comparisons *post hoc* test using GraphPad Prism version 8.0 (La Jolla, CA). Prior to performing these ANOVA analyses, the data were subjected to a square root transformation (neuron viability data) or log transformation (O_2 consumption rate, fluorescence, and open-field test data) to conform to ANOVA assumptions. Neurite length data were subjected to a log transformation to account for skewness in the data. The log-transformed data were analyzed using an approach that accounts for (i) the possibility of multiple neurites arising from a single cell,

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and (ii) comparison across experiments conducted on different days. Log-transformed neurite lengths for multiple treatment groups were compared using a general linear model implemented in the 'Mixed' procedure of SAS Version 9.3 followed by Tukey's multiple comparisons *post hoc* test (Cary, NC). For measurements of mRNA expression, fold-change values were log-transformed, and the transformed data were analyzed using GraphPad Prism 8.0 via a one sample t-test to determine whether the mean of the log(fold-change) was different from the hypothetical value of 0 (corresponding to a ratio of 1). The 'n' values specified in the figure legends represent the number of biological replicates (i.e. independent experiments involving cultures prepared at different times).

3. Results

3.1. Study design

The central hypothesis of this study was that botanical extracts rich in isoflavones can alleviate neurotoxicity elicited by insults linked epidemiologically or genetically to PD. To address this hypothesis we characterized two extracts with high levels of isoflavones, a red clover extract [55] and a soy extract prepared from Tofu soybeans [45, 46], in terms of their ability to interfere with dopaminergic cell death in primary midbrain cultures exposed to two PD stresses: (i) rotenone, an environmental toxicant epidemiologically linked to increased PD risk [22]; and (ii) adenovirus encoding A53T aSyn, a familial mutant form of aSyn that exhibits accelerated fibrillization and enhanced neurotoxicity compared to the wild-type protein [15, 56, 57]. Moreover, we characterized an individual isoflavone constituent of the soy extract, daidzein, and a second isoflavone, equol (produced from daidzein by the intestinal microbiota), to determine their effects on dopaminergic neuron survival in primary midbrain cultures exposed to rotenone or A53T Ad. The extracts and individual isoflavones were examined for their effects on Nrf2 signaling and the UPS, as well as their ability to modulate mitochondrial respiration.

3.2. Effects of a red clover extract on dopaminergic cell death elicited by PD-related insults in primary midbrain cultures

Our first objective was to measure the neuroprotective activity of a red clover extract in a rat primary midbrain culture model. Our rationale for using this model was that these cultures consist of post-mitotic dopaminergic and GABAergic neurons and glial cells (e.g. astrocytes and microglia), and thus they reproduce key features of the midbrain region affected in PD patients [47, 58]. The red clover extract was prepared from dried flowers, as described previously by Pikuni-Blackfeet traditional healers [27], and chemical analysis of the extract via HPLC coupled with UV and MS detection revealed an abundance of isoflavones, including formononetin, biochanin A, and pratensin glycosides, and confirmed the isoflavone-rich nature of the red clover extract (Table 1).

In one set of experiments, primary midbrain cultures were exposed to rotenone in the absence or presence of a red clover extract. The cultures were co-stained for MAP2, a general neuronal marker, and TH, a selective marker of dopaminergic neurons (Fig. 1A), and scored for relative dopaminergic neuron viability by determining the ratio of TH$^+$ neurons to total MAP2$^+$ neurons [15, 27, 42, 47]. Cultures exposed to rotenone alone exhibited a ~20-30% decrease in dopaminergic cell viability, and this decrease was abrogated in cultures exposed to rotenone plus red clover extract (Fig. 1B). Moreover, neurite length measurements revealed a decrease in the lengths of processes extending from MAP2$^+$/TH$^+$ neurons in cultures exposed to rotenone compared to control cultures, consistent with the 'dying back' degeneration of dopaminergic neurites that is thought to precede cell death observed in the brains of PD patients [59, 60], and this decrease in neurite lengths was alleviated in cultures treated with rotenone in the presence of red clover extract (Fig. 1C). These results suggested that polyphenols in the red clover extract interfered with dopaminergic cell death and neurite retraction elicited by rotenone.

In a second set of experiments, primary midbrain cultures were transduced with A53T Ad, previously shown to be selectively toxic to MAP2$^+$/TH$^+$ neurons [15], in the absence or presence of red clover extract. The cultures were co-stained for TH and MAP2 and analyzed for relative dopaminergic cell survival as outlined above. Cultures treated with virus plus extract had a higher percentage of TH$^+$ neurons compared to cultures treated with virus alone (Fig. 1D), suggesting that red clover polyphenols inhibited aSyn-mediated dopaminergic neurotoxicity.

3.3. Effects of a red clover extract on astrocytic Nrf2/ARE signaling

The transcription factor Nrf2 is a master regulator of the cellular antioxidant response [61]. In the cytoplasm, Nrf2 is sequestered by Kelch-like ECH-associated protein 1 (Keap1) [62] and targeted for degradation by the UPS [63]. Under conditions of oxidative stress, the interaction between Nrf2 and Keap1 is disrupted, resulting in Nrf2 translocation to the nucleus where it binds AREs in the regulatory region of its target genes [64, 65]. The Nrf2-mediated response appears to be primarily activated in astrocytes, enabling their production and secretion of glutathione metabolites that are subsequently taken up by neighboring neurons [66, 67].

Evidence suggests that rotenone and aSyn elicit neurotoxicity at least in part by triggering an increase in oxidative stress [9, 68, 69]. Accordingly, we hypothesized that the red clover extract could protect against neurotoxicity elicited by rotenone or A53T aSyn by activating the Nrf2-mediated antioxidant response [70]. To address this hypothesis, primary cortical astrocytes were transduced with a reporter adenovirus encoding EGFP downstream of an enhancer element encompassing two antioxidant response elements (AREs) and the HO-1 minimal promoter [27, 42]. The transduced cultures were incubated in the absence or presence of red clover extract for 24 h and examined for levels of EGFP fluorescence (Fig. 2A). Astrocytes treated with extract exhibited greater fluorescence compared to untreated cells, suggesting that red clover polyphenols activated

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the Nrf2/ARE antioxidant response (Fig. 2B-C). We observed a similar effect in mixed primary cortical cultures consisting of neurons, astrocytes, and microglia, suggesting that the presence of neurons and other glial cell types did not affect the ability of the red clover extract to activate Nrf2 transcriptional activity in astrocytes (Fig. 2D). To confirm that the observed increase in EGFP fluorescence reflected an increase in Nrf2-mediated transcription, we quantified mRNAs encoding Nrf2 target genes by qRT-PCR. In agreement with the results obtained from the EGFP reporter assay, we found that astrocytes treated with red clover extract exhibited an up-regulation of mRNA encoding HO1 or GCLC compared to control cells (Fig. 2E, F).

In subsequent experiments, we assessed whether the red clover extract could activate the Nrf2/ARE pathway in the same cellular context as that in which the extract conferred neuroprotection against toxicity elicited by rotenone or A53T aSyn. To address this question, primary midbrain cultures or astrocytes isolated from these cultures were transduced with the ARE-EGFP reporter adenovirus and incubated in the absence or presence of extract. Surprisingly, isolated astrocytes and neuron-glia cultures from embryonic rat midbrain exhibited similar levels of EGFP fluorescence after incubation with or without extract (Supplementary Fig. 1A, B). In summary, our data indicate that the ability of red clover to activate Nrf2 signaling in cultured astrocytes depends on the brain region from which they were derived (e.g. cortex or midbrain), and the mechanisms of Nrf2 activation mediated by the red clover extract may not be operative in midbrain cultures.

3.4. Mechanism of red clover-mediated Nrf2 activation in cortical astrocytes

Polyphenols have been reported to up-regulate Nrf2 transcriptional activity via various mechanisms. For example, they can trigger Keap1 modification, either by reacting directly with Keap1 regulatory cysteine residues or indirectly by promoting ROS-mediated Keap1 oxidation [71-75]. In turn, Keap1 modification results in disruption of the Nrf2-Keap1 interaction, Nrf2 stabilization, and an increase in Nrf2 nuclear translocation and transcriptional activity. Some polyphenols interfere with the UPS [76-80], and a loss of UPS function has been linked to the activation of Nrf2 and an increase in the expression of its target genes [81-83]. Other polyphenols, including the flavonoid curcumin, have also been shown to induce increased expression of the Nrf2 gene [84-86]. In the next phase of our study, we carried out experiments aimed at elucidating which of these mechanisms could be involved in the activation of Nrf2 signaling in cortical astrocytes by the red clover extract.

To determine whether a pro-oxidant effect was necessary for Nrf2 activation by the red clover extract, we tested the effect of the extract on Nrf2 transcriptional activity in primary cortical astrocytes cultured in the absence or presence of N-acetyl cysteine (NAC), a cell-permeable antioxidant molecule that serves as a ROS scavenger and a glutathione precursor [48, 58]. Astrocytes transduced with the ARE/EGFP reporter virus and treated with red clover extract plus

NAC exhibited similar fluorescence levels compared to transduced astrocytes treated with extract alone (Fig. 3), suggesting that the activation of Nrf2 transcriptional activity by the extract was not affected by treatment with NAC, and, therefore, occurred via a ROS-independent mechanism [48, 58].

To address whether Nrf2 activation by the red clover extract could involve UPS inhibition, we tested the effect of the extract on levels of GFP fluorescence in primary cortical astrocytes transduced with a reporter adenovirus encoding GFPu, an unstable form of GFP that is linked to the CL1 degron [42, 44, 49, 50]. Under physiological conditions, GFPu is efficiently degraded, whereas inhibition of the UPS results in accumulation of GFPu and increased cellular fluorescence. GFPu-expressing astrocytes exhibited greater fluorescence when cultured in the presence versus the absence of the red clover extract (Fig. 4A), suggesting that polyphenols in the extract inhibit UPS activity. As a control, we confirmed that UPS inhibition led to the activation of Nrf2 signaling in our cortical astrocyte model by showing that astrocytes transduced with the ARE-EGFP reporter virus showed an increase in EGFP fluorescence when cultured in the presence of the proteasome inhibitor MG132 (Fig. 4B). Curcumin, a polyphenol previously reported to interfere with UPS function [87], induced an increase in GFP fluorescence in GFPu-expressing astrocytes (Supplementary Fig. 2A) and potently activated the Nrf2 pathway in rat cortical astrocytes, rat mixed cortical or midbrain cultures, and human iPSC-derived astrocytes (Supplementary Fig. 2B), further supporting a link between UPS inhibition and Nrf2 activation.

To assess whether Nrf2 activation by the red clover extract could involve an increase in the expression of the Nrf2 gene, we quantified Nrf2 mRNA in cortical astrocytes via qRT-PCR. The results indicated that Nrf2 mRNA levels were increased by ~25% in cells incubated with extract for 6 h compared to untreated cells (Supplementary Fig. 3).

Collectively, these data suggest that polyphenols in the red clover extract inhibit UPS activity and induce a modest increase in Nrf2 mRNA levels in cortical astrocytes. We infer that both of these effects could account at least in part for the ability of the red clover extract to activate Nrf2 signaling in these cells.

3.5. Neuroprotective effects of a soy extract and individual isoflavones

Our next objective was to examine the neuroprotective activities of an isoflavone-rich soy extract and two individual isoflavones, daidzein and equol (Fig. 5A), against toxicity elicited by PD-related insults in primary midbrain cultures. Previous HPLC analyses of the phytochemical composition of soy extracts have revealed an enrichment in isoflavones including genistein and daidzein (genistein/daidzein ratio consistently around 1.4), and lower levels of glycitein [45, 46]. Cultures treated with rotenone or A53T Ad plus soy extract showed a strong trend towards greater dopaminergic cell viability compared to cultures treated with rotenone or A53T virus alone (Fig. 5B,F). Furthermore, rotenone neurotoxicity was alleviated in midbrain cultures treated with daidzein

(100 nM, with a trend towards a neuroprotective effect at 50 nM) or equol (50 nM) (Fig. 5C-E). An increase in dopaminergic neuron viability was also observed in A53T-expressing cultures treated with daidzein at a concentration as low as 25 nM (Fig. 5G). These data suggest that soy polyphenols and the isoflavones daidzein and equol interfere with neurotoxicity elicited by rotenone and A53T aSyn.

In the next set of experiments, we examined whether the soy extract, daidzein, or equol could activate the astrocytic Nrf2/ARE antioxidant response. To address this question, primary cortical astrocytes were transduced with the ARE/EGFP reporter adenovirus and incubated in the absence or presence of the extract or individual isoflavones. Quantification of EGFP fluorescence revealed that none of the treatments induced an increase in the EGFP signal (Supplementary Fig. 4). However, in contrast to a red clover extract, the soy extract activated the Nrf2 response in human iPSC-derived astrocytes (Fig. 6). Additional analyses revealed that cortical astrocytes transduced with the GFPu reporter virus exhibited a modest increase in GFP fluorescence when treated with equol or the soy extract at a relatively high concentration of 20 µg/mL, but no increase in fluorescence upon incubation with daidzein (Supplementary Fig. 5). These data suggest that (i) the neuroprotection elicited by soy polyphenols, daidzein, and equol may not rely on activation of Nrf2 signaling in cortical astrocytes, whereas a protective antioxidant activity may be induced in other subtypes of astrocytes; and (ii) soy polyphenols and equol, but not daidzein, interfere with UPS function in primary cortical astrocytes.

3.6. Effects of red clover and soy extracts on rotenone-induced impairment of mitochondrial respiration

Several lines of evidence suggest that neurotoxicity elicited by rotenone and aSyn results from various pathogenic events including disruption of mitochondrial respiration and oxidative damage [9, 69, 88-90]. Accordingly, we hypothesized that the red clover and soy extracts might protect dopaminergic neurons against toxicity elicited by rotenone or aSyn by alleviating mitochondrial dysfunction. To address this hypothesis, we developed an assay aimed at measuring O_2 consumption in human SH-SY5Y neuroblastoma cells challenged with rotenone in the absence or presence of extract. To ensure that the cell relied on oxidative phosphorylation as a primary source of energy production, the cultures were maintained in media containing galactose instead of glucose [42, 91, 92]. Cells exposed to rotenone exhibited a ~40% decrease in the rate of cellular O_2 consumption compared to untreated cells, and this decrease was largely reversed in cells pre-treated with extracts prepared from red clover (1 µg/mL) or soy (1 µg/mL), or with daidzein (50 nM) (Fig. 7A). These results suggested that the two extracts and daidzein interfered with rotenone-induced mitochondrial functional deficits.

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A number of polyphenols have been reported to compete with rotenone for ubiquinone binding sites in complex I of the electron transport chain [93]. Accordingly, we hypothesized that the rescue of mitochondrial respiration by the red clover and soy extracts could result from the displacement of rotenone from its binding site in complex I. To address this hypothesis, we developed a competition assay aimed at monitoring the effects of polyphenols on rotenone-mediated deficits in mitochondrial O_2 consumption. Galactose-conditioned SH-SY5Y cells incubated in the presence of rotenone exhibited a decrease in the rate of O_2 consumption compared to untreated cells, and a trend was observed towards a rescue of this inhibitory effect upon addition of red clover extract, but not soy extract (Fig. 7B). These results suggest that the red clover extract, but not the soy extract, preserves mitochondrial function at least in part by displacing rotenone from its binding site in complex I.

3.7. Effects of a soy extract on 6-OHDA-induced motor dysfunction.

To investigate the potential neuroprotective activity of soy isoflavones in an *in vivo* PD model, we tested a soy extract for the ability to alleviate motor deficits in 6-OHDA-treated rats, a classic neurotoxin model of PD. Unilateral, stereotaxic injection of 6-OHDA in the MFB or striatum results in a progressive phenotype involving dopaminergic cell death and depletion of striatal dopamine on the lesioned side [94-96]. The resulting imbalance in striatal dopamine causes pronounced motor deficits. For these experiments, we used another soy extract, Novasoy 400, which is enriched in genistin, daidzein and glycitin. Rats received daily ip injections of extract or control vehicle prior and subsequent to lesioning with 6-OHDA in the right MFB (Fig. 8A). Strikingly, rats injected with soy extract exhibited less pronounced motor defects as monitored by the rotarod and open field tests compared to rats treated with vehicle (Fig. 8B-D). Treatment of lesioned animals with soy extract increased the maximum rotarod speed and the percentage area covered in the open field test by ~1.3- and ~1.6-fold (respectively) compared to lesioned animals treated with vehicle. These results indicate that soy polyphenols alleviate motor dysfunction exhibited by rats exposed to 6-OHDA, potentially by interfering with nigrostriatal degeneration in these animals.

4. Discussion

4.1. Isoflavone-rich extracts and individual isoflavones protect against neurotoxicity elicited by rotenone and A53T aSyn.

The objective of this study was to examine the neuroprotective capacity and mechanisms of action of two isoflavone-rich extracts, prepared from red clover and soy, and the individual isoflavones daidzein and equol, a daidzein metabolite. Results showed that all of the extracts and isoflavones tested alleviated rotenone- and A53T-induced dopaminergic cell death in a primary midbrain culture model relevant to PD (Figs. 2 and 6). These findings are consistent with previous

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data showing a rescue of dopaminergic cell loss in 6-OHDA-lesioned rats supplemented with a red clover diet [97], and the ability of individual isoflavones to attenuate motor and non-motor symptoms of PD induced by exposure to the toxins MPTP and 6-OHDA, or the pro-inflammatory agent LPS [34-36, 98]. Because the red clover and soy extracts examined in this study consist of a complex mixture of isoflavones, we infer that synergistic interactions involving multiple isoflavones could potentially play a role in the extracts' neuroprotective activities [99-101]. At the same time, our observation that daidzein and equol interfered with toxicity elicited by rotenone or A53T aSyn indicates that individual isoflavones can also achieve neuroprotective effects in PD models. Interestingly, equol is a metabolite of daidzein produced by the microbiota of some but not all individuals. It has been suggested that the nature of consumed isoflavones (glycosides vs. aglycones) could influence the production of equol in humans [102], therefore suggesting the importance of the plant material source of isoflavones. Equol has shown health-promoting effects in models of stroke-like injury, osteoporosis, cardiovascular disease, and menopause [103-106] and was recently found to alleviate toxicity elicited by the PD-related toxins MPP$^+$ and 6-OHDA in SH-SY5Y cells [107]. Here, we show that this compound can also achieve neuroprotection in PD models at concentrations similar to those found in the plasma of equol producers [108]. These findings set the stage for future studies of neuroprotective pathways activated by daidzein and equol, with a view towards obtaining insights relevant to the development of personalized therapies accounting for microbiome differences among PD patients.

A number of isoflavones or their metabolites have been shown to penetrate the BBB in Sprague-Dawley rats [109, 110], and equol was found to be highly permeable in an artificial BBB permeability assay [111]. Accordingly, we infer that the isoflavones and isoflavone-rich extracts examined in this study could potentially be of clinical benefit by reducing PD risk or slowing neurodegeneration in the brains of patients. The fact that daidzein and equol interfered with dopaminergic cell death at nanomolar concentrations in our primary midbrain culture model (Fig. 5C-E and G) implies that even a modest accumulation of these compounds in the brain could lead to neuroprotective effects in humans. Importantly, the ability of the extracts and individual isoflavones to mitigate neurotoxicity elicited by rotenone or A53T aSyn implies that these agents could potentially interfere with neurodegeneration in individuals with elevated PD risk triggered by a range of PD-related stresses. Consistent with this idea, we have obtained evidence from an ethnopharmacological study that a red clover extract is used as a form of traditional medicine by the Pikuni-Blackfeet Native Americans to treat PD-related symptoms [27], and additional epidemiological data suggest that the consumption of isoflavones is associated with a reduced risk of neurodegenerative diseases [28, 29].

4.2. A red clover extract activates the Nrf2/ARE antioxidant pathway and interferes with UPS function in cortical astrocytes.

A key result of this study was our finding that a red clover extract, but not a soy extract or the individual isoflavones daidzein and equol, activated Nrf2 signaling in primary cortical astrocytes. There is conflicting evidence in the literature on the ability of isoflavones to activate the Nrf2/ARE pathway and induce the expression of Nrf2-regulated genes. For example, two isoflavone metabolites, tectorigenin and glycitein, were found to up-regulate HO1 and NAD(P)H Quinone Dehydrogenase 1 (NQO1) expression in a Nrf2-dependent manner in cortical astrocytes [112]. Daidzein has been shown to up-regulate HO1 in smooth muscle-derived cells but failed to increase quinone reductase (QR) activity in colon cancer cells [113]. Moreover, daidzein and genistein were found to increase QR activity in different tissues of mice fed a daidzein- or genistein-enriched diet [114]. Interestingly, this study revealed variable effects in term of modulation of glutathione-S-transferase (GST) activity that were dependent on the sex and nature of the tissue studied [114]. These reported differences across various tissues suggest a possible cell type-specific response to isoflavones that could explain the lack of Nrf2 activation by soy and individual isoflavones in cortical astrocytes (Supplementary Fig. 4).

This is the first report of UPS inhibition by a red clover extract (Fig. 4A). A number of polyphenols, including isoflavones, have been shown by our group (Supplementary Fig. 5) and others to interfere with UPS activity [76, 78, 115-117]. Genistein, a soy isoflavone, was reported to inhibit the chymotrypsin-like activity of the proteasome in a cancer cell model, likely via interaction with the proteasomal $\beta5$ subunit [117]. Our observation that a red clover extract interfered with GFPu degradation implies that polyphenols in this extract could induce activation of the Nrf2/ARE pathway by inhibiting UPS function. The concentration at which the extract induced a significant increase in the GFPu signal is higher than the concentration used for the Nrf2 activation, implying that the sensitivity of the GFPu reporter assay is less than that of the Nrf2 assay, and a low level of proteasome inhibition may be sufficient to induce Nrf2 signaling but not sufficient to cause a measurable amount of GFPu accumulation. In contrast, red clover polyphenols apparently do not activate Nrf2-mediated transcription via a mechanism involving redox cycling reactions leading to Keap1 oxidation, given that the antioxidant molecule NAC had no impact on the ability of the red clover extract to activate Nrf2 signaling in cortical astrocytes (Fig. 3) [48, 58, 118]. Finally, our observation that the red clover extract induced a modest up-regulation of Nrf2 mRNA levels (Supplementary Fig. 3) suggests that this is an additional mechanism by which red clover polyphenols could activate Nrf2 signaling, consistent with similar effects reported for quercetin and resveratrol [85, 86].

4.3. Activation of astrocytic Nrf2 signaling by a red clover extract depends on the brain region from which the astrocytes are prepared.

Another important outcome of our study was the finding that astrocytic Nrf2-mediated transcription was activated by a red clover extract in astrocytes prepared from rat cortex (Fig. 2C,D), but not in astrocytes prepared from rat midbrain or in mixed midbrain cultures (Suppl. Fig. 1). These observations are consistent with previous data showing that astrocytes from different brain regions differ in terms of their molecular profiles and their responses to different stimuli [119-126]. For example, midbrain and cortical astrocytes were found to express different levels of glial glutamate transporter-1 (GLT-1) in response to corticosteroid stimulation [127]. The data presented here support previous findings relating to the heterogeneity of glial cells [119, 124, 128] by showing that astrocytes prepared from different brain regions respond differently to inducers of the Nrf2-mediated antioxidant response. Another explanation could be that red clover polyphenols undergo metabolic reactions that result in a loss of the extract's ability to modulate the Nrf2/ARE pathway in midbrain but not cortical astrocytes. our data showing that the polyphenol curcumin can induce up-regulation of Nrf2-mediated transcription in mixed midbrain, mixed cortical, and iPSC-derived astrocytic cultures (Supplementary Fig. 2B) suggest that it (i) activates Nrf2 signaling via another mechanism than the red clover extract; or (ii) is resistant to metabolic reactions that interfere with Nrf2 activation by red clover polyphenols in mixed midbrain cultures.

Collectively, our results suggest that the ability of isoflavone-rich extracts to activate astrocytic Nrf2 signaling varies in astrocytes and mixed cultures prepared from different brain regions. Our observation that the red clover and soy extracts exhibited opposite patterns of Nrf2 activation in rat cortical astrocytes versus human iPSC-derived astrocytes (Fig. 2, 6 and Supplementary Fig. 4A) further highlights the point that isoflavone-rich extracts induce up-regulation of the astrocytic, Nrf2-mediated transcription in a cell type-specific manner. Uncovering mechanisms underlying these brain region-specific responses has the potential to advance our understanding of the role of astrocytes in neuroprotection and enhance the impact of medicines designed to target the Nrf2/ARE pathway.

Finally, because the red clover extract failed to activate Nrf2 signaling in primary midbrain cultures, we infer that the protective effects against toxicity elicited by rotenone or A53T aSyn did not involve up-regulation of astrocytic, Nrf2-mediated transcription under our experimental conditions. Nevertheless, the extracts could potentially alleviate dopaminergic cell death in the midbrains of rodents exposed to PD-related insults via a mechanism involving activation of Nrf2 signaling, based on the premise that midbrain astrocytes are likely exposed to a different environment *in vivo* than in the primary midbrain cultures studied here.

4.4. Isoflavone-rich extracts rescue mitochondrial dysfunction.

O_2 consumption experiments revealed that a red clover extract and a soy extract alleviated rotenone-induced deficits in mitochondrial electron transport in SH-SY5Y neuroblastoma cells (Figure 8). These findings are also consistent with previous data showing that soy isoflavones mitigated brain mitochondrial oxidative stress triggered by the amyloid-β peptide in rat models of AD [129, 130]. Based on evidence that the neurotoxic effects of rotenone exposure and aSyn over-expression are related to mitochondrial functional deficits [9, 69, 88-90], we infer that a rescue of these deficits by the red clover and soy extracts could play a key role in their ability to protect against these insults. Our finding that the red clover extract, but not the soy extract, preserved mitochondrial function at least in part by displacing rotenone from its binding site in complex I suggests that red clover polyphenols can protect against rotenone neurotoxicity by interfering with the toxicant's ability to disrupt mitochondrial electron transport. Previous studies revealed that isoflavones such as genistein interact with the electron transport chain [131], and genistein and daidzein were found to associate with the F_0F_1-ATPase/ATP synthase [132] and/or modulate NADH:ubiquinone oxidoreductase activity [133]. Our observation that the soy extract, in contrast to the red clover extract, failed to displace rotenone from its complex I binding site (Fig. 7B) implies that only specific isoflavones rescue mitochondrial functional deficits via interactions with electron transport chain components. In addition to engaging in such interactions, isoflavones in both the red clover and soy extracts could ameliorate mitochondrial dysfunction via other mechanisms, including potentially up-regulating the transcriptional co-activator PGC1-alpha, a master regulator of mitochondrial biogenesis [134].

4.5. An isoflavone-enriched soy extract protects against 6-OHDA toxicity.

Treatment of 6-OHDA-lesioned rats with Novasoy 400, a 40% (w/v) isoflavone standardized soy extract enriched in genistin, daidzein and glycitin, led to an amelioration of 6-OHDA-induced motor dysfunction (Fig. 8). These findings suggest that soy isoflavones can mitigate nigrostriatal degeneration induced by the neurotoxin, potentially by rescuing mitochondrial dysfunction and activating protective antioxidant pathways in astrocytes, as seen in our cellular models of PD. Consistent with this idea, isoflavones such as genistein and equol have been shown to cross the BBB to reach target cell types [109, 111]. The ability of a standardized soy extract to improve motor function in 6-OHDA-treated rats is in agreement with other published studies showing neuroprotective activities of individual soy isoflavones in animal models of PD. For example, the abundant soy isoflavone genistein was shown to increase the survival of neurons in the *substantia nigra* of rodents exposed to 6-OHDA [37] or MPTP [34], and the daidzein metabolite equol protected *Caenorhabditis elegans* against toxicity elicited by MPP+ [107]. Future studies will be focused on

examining neuroprotective effects of Novasoy 400 via immunohistochemical analysis of brain sections from rats exposed to 6-OHDA and other PD-related insults.

5. Conclusion

In conclusion, isoflavone-rich red clover and soy extracts, and the individual isoflavones daidzein and equol, were found to alleviate neurotoxicity elicited by insults linked epidemiologically or genetically to PD. Equol showed potent neuroprotective activity at levels similar to those in the plasma of equol-producers, suggesting that there may be opportunities for the development of individualized therapies targeting patients with differences in the gut microbiome. Although both the red clover and soy extracts interfered with neurotoxic effects of PD-related insults, they differed in their abilities to modulate astrocytic Nrf2 signaling and rescue mitochondrial respiration, highlighting the important role of the extracts' polyphenolic composition and potential synergies among their constituents in determining their neuroprotective mechanisms. Based on evidence that a number of isoflavones or their metabolites can penetrate the BBB [109, 110], our findings suggest that the extracts and isoflavones examined in this study have the potential to lower the risk of PD or slow disease progression in humans. Studies found that supplementation at 60 mg or 100 mg total isoflavone equivalent per day for 10 to 12 weeks resulted in improved cognitive functions [31, 135], suggesting that isoflavone supplementation can be achieved and leveraged to promote brain health in patients. An important area of future research will be to determine if isoflavones interact with existing PD medications, and if their supplementation triggers adverse effects in patients. For example, a number of animal studies have raised concerns over the toxicity profile resulting from long-term or high-dose intake of isoflavones (e.g. 150 mg/kg body weight of genistein) [136], but a phase I clinical trial reported a well-tolerated supplementation in healthy women [137]. A better understanding of the risks is needed, and would greatly benefit ongoing efforts to develop safe and efficacious dietary interventions for PD patients.

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References

1. Dauer, W. and S. Przedborski, *Parkinson's disease: mechanisms and models.* Neuron, 2003. **39**(6): p. 889-909.
2. Shulman, J.M., P.L. De Jager, and M.B. Feany, *Parkinson's disease: genetics and pathogenesis.* Annu Rev Pathol, 2011. **6**: p. 193-222.
3. Dawson, T.M. and V.L. Dawson, *Molecular pathways of neurodegeneration in Parkinson's disease.* Science, 2003. **302**(5646): p. 819-22.
4. Massano, J. and K.P. Bhatia, *Clinical approach to Parkinson's disease: features, diagnosis, and principles of management.* Cold Spring Harb Perspect Med, 2012. **2**(6): p. a008870.
5. Wang, J.Y., et al., *Dual effects of antioxidants in neurodegeneration: direct neuroprotection against oxidative stress and indirect protection via suppression of glia-mediated inflammation.* Curr Pharm Des, 2006. **12**(27): p. 3521-33.
6. Sanders, L.H. and J. Timothy Greenamyre, *Oxidative damage to macromolecules in human Parkinson disease and the rotenone model.* Free Radic Biol Med, 2013. **62**: p. 111-20.
7. Banerjee, R., et al., *Mitochondrial dysfunction in the limelight of Parkinson's disease pathogenesis.* Biochim Biophys Acta, 2009. **1792**(7): p. 651-63.
8. Youle, R.J. and A.M. van der Bliek, *Mitochondrial fission, fusion, and stress.* Science, 2012. **337**(6098): p. 1062-5.
9. Betarbet, R., et al., *Chronic systemic pesticide exposure reproduces features of Parkinson's disease.* Nature Neuroscience, 2000. **3**(12): p. 1301-6.
10. Spillantini, M.G., et al., *Alpha-synuclein in Lewy bodies.* Nature, 1997. **388**(6645): p. 839-40.
11. Dawson, T.M., H.S. Ko, and V.L. Dawson, *Genetic animal models of Parkinson's disease.* Neuron, 2010. **66**(5): p. 646-61.
12. Rochet, J.C., B.A. Hay, and M. Guo, *Molecular insights into Parkinson's disease.* Prog Mol Biol Transl Sci, 2012. **107**: p. 125-88.
13. Block, M.L., L. Zecca, and J.S. Hong, *Microglia-mediated neurotoxicity: uncovering the molecular mechanisms.* Nat Rev Neurosci, 2007. **8**(1): p. 57-69.
14. Conway, K.A., et al., *Acceleration of oligomerization, not fibrillization, is a shared property of both alpha-synuclein mutations linked to early-onset Parkinson's disease: implications for pathogenesis and therapy.* Proc Natl Acad Sci U S A, 2000. **97**(2): p. 571-6.
15. Ysselstein, D., et al., *Effects of impaired membrane interactions on alpha-synuclein aggregation and neurotoxicity.* Neurobiol Dis, 2015. **79**: p. 150-163.
16. Greenbaum, E.A., et al., *The E46K mutation in alpha -synuclein increases amyloid fibril formation.* J. Biol. Chem., 2005. **280**(9): p. 7800-7807.
17. Li, J., V.N. Uversky, and A.L. Fink, *Effect of familial Parkinson's disease point mutations A30P and A53T on the structural properties, aggregation, and fibrillation of human a-synuclein.* Biochemistry, 2001. **40**: p. 11604-11613.
18. Singleton, A.B., et al., *alpha-Synuclein locus triplication causes Parkinson's disease.* Science, 2003. **302**(5646): p. 841.
19. Menegon, A., et al., *Parkinson's disease, pesticides, and glutathione transferase polymorphisms.* Lancet, 1998. **352**(9137): p. 1344-6.
20. Butterfield, P.G., et al., *Environmental antecedents of young-onset Parkinson's disease.* Neurology, 1993. **43**(6): p. 1150-8.
21. Gorell, J.M., et al., *The risk of Parkinson's disease with exposure to pesticides, farming, well water, and rural living.* Neurology, 1998. **50**(5): p. 1346-50.
22. Tanner, C.M., et al., *Rotenone, Paraquat and Parkinson's Disease.* Environ Health Perspect, 2011. **119**(6): p. 866-872.
23. Conway, K.A., et al., *Kinetic stabilization of the a-synuclein protofibril by a dopamine-a-synuclein adduct.* Science, 2001. **294**: p. 1346-1349.
24. Mirzaei, H., et al., *Identification of rotenone-induced modifications in alpha-synuclein using affinity pull-down and tandem mass spectrometry.* Anal Chem, 2006. **78**(7): p. 2422-31.

25. Jankovic, J. and W. Poewe, *Therapies in Parkinson's disease.* Curr Opin Neurol, 2012. **25**(4): p. 433-47.

26. Lawrence, A.D., A.H. Evans, and A.J. Lees, *Compulsive use of dopamine replacement therapy in Parkinson's disease: reward systems gone awry?* Lancet Neurol, 2003. **2**(10): p. 595-604.

27. de Rus Jacquet, A., et al., *Pikuni-Blackfeet traditional medicine: Neuroprotective activities of medicinal plants used to treat Parkinson's disease-related symptoms.* J Ethnopharmacol, 2017. **206**: p. 393-407.

28. Tang, M.X., et al., *Effect of oestrogen during menopause on risk and age at onset of Alzheimer's disease.* Lancet, 1996. **348**(9025): p. 429-32.

29. Kim, H., et al., *Attenuation of neurodegeneration-relevant modifications of brain proteins by dietary soy.* Biofactors, 2000. **12**(1-4): p. 243-50.

30. Song, Y.J., et al., *The Effect of Estrogen Replacement Therapy on Alzheimer's Disease and Parkinson's Disease in Postmenopausal Women: A Meta-Analysis.* Front Neurosci, 2020. **14**: p. 157.

31. Duffy, R., H. Wiseman, and S.E. File, *Improved cognitive function in postmenopausal women after 12 weeks of consumption of a soya extract containing isoflavones.* Pharmacol Biochem Behav, 2003. **75**(3): p. 721-9.

32. Uddin, M.S. and M.T. Kabir, *Emerging Signal Regulating Potential of Genistein Against Alzheimer's Disease: A Promising Molecule of Interest.* Front Cell Dev Biol, 2019. **7**: p. 197.

33. Lu, C., et al., *Neuroprotective Effects of Soy Isoflavones on Scopolamine-Induced Amnesia in Mice.* Nutrients, 2018. **10**(7).

34. Liu, L.X., et al., *Neuroprotective effects of genistein on dopaminergic neurons in the mice model of Parkinson's disease.* Neurosci Res, 2008. **60**(2): p. 156-61.

35. Kyuhou, S., *Preventive effects of genistein on motor dysfunction following 6-hydroxydopamine injection in ovariectomized rats.* Neurosci Lett, 2008. **448**(1): p. 10-4.

36. Chen, H.Q., et al., *Protective effect of isoflavones from Trifolium pratense on dopaminergic neurons.* Neurosci Res, 2008. **62**(2): p. 123-30.

37. Baluchnejadmojarad, T., et al., *Neuroprotective effect of genistein in 6-hydroxydopamine hemi-parkinsonian rat model.* Phytother Res, 2009. **23**(1): p. 132-5.

38. Mercer, L.D., et al., *Dietary polyphenols protect dopamine neurons from oxidative insults and apoptosis: investigations in primary rat mesencephalic cultures.* Biochem Pharmacol, 2005. **69**(2): p. 339-45.

39. Li, R., et al., *Puerarin attenuates neuronal degeneration in the substantia nigra of 6-OHDA-lesioned rats through regulating BDNF expression and activating the Nrf2/ARE signaling pathway.* Brain Research, 2013. **1523**: p. 1-9.

40. Fang, Y.Z., S. Yang, and G. Wu, *Free radicals, antioxidants, and nutrition.* Nutrition, 2002. **18**(10): p. 872-9.

41. Kim, D.O. and C.Y. Lee, *Comprehensive study on vitamin C equivalent antioxidant capacity (VCEAC) of various polyphenolics in scavenging a free radical and its structural relationship.* Crit Rev Food Sci Nutr, 2004. **44**(4): p. 253-73.

42. de Rus Jacquet, A., et al., *Lumbee traditional medicine: Neuroprotective activities of medicinal plants used to treat Parkinson's disease-related symptoms.* J Ethnopharmacol, 2017. **206**: p. 408-425.

43. Alam, J., et al., *Nrf2, a Cap'n'Collar transcription factor, regulates induction of the heme oxygenase-1 gene.* J Biol Chem, 1999. **274**(37): p. 26071-8.

44. Tian, Z., et al., *Autophagic-lysosomal inhibition compromises ubiquitin-proteasome system performance in a p62 dependent manner in cardiomyocytes.* PLoS One, 2014. **9**(6): p. e100715.

45. Wu, Q., et al., *LC/UV/ESI-MS analysis of isoflavones in Edamame and Tofu soybeans.* J Agric Food Chem, 2004. **52**(10): p. 2763-9.

46. Shen, D., et al., *Chromatographic fingerprints and quantitative analysis of isoflavones in Tofu-type soybeans.* Food Chemistry, 2012. **130**(4): p. 1003-1009.

47. Strathearn, K.E., et al., *Neuroprotective effects of anthocyanin- and proanthocyanidin-rich extracts in cellular models of Parkinsons disease.* Brain Research, 2014. **1555**: p. 60-77.

48.   Liu, F., et al., *Methionine sulfoxide reductase A protects dopaminergic cells from Parkinson's disease-related insults.* Free Radic Biol Med, 2008. **45**(3): p. 242-55.

49.   Bence, N.F., R.M. Sampat, and R.R. Kopito, *Impairment of the ubiquitin-proteasome system by protein aggregation.* Science, 2001. **292**(5521): p. 1552-5.

50.   Bence, N.F., E.J. Bennett, and R.R. Kopito, *Application and analysis of the GFPu family of ubiquitin-proteasome system reporters.* Methods Enzymol, 2005. **399**: p. 481-90.

51.   Olofinsae, S.A., B.O. Ibeh, and J. Ahluwalia, *Increased oxygen consumption observed in phorbol 12-myristate 13-acetate stimulated human cultured promonocytic U937 cell lines treated with calcitriol and retinoic acid.* Asian Pac J Trop Med, 2014. **7S1**: p. S272-7.

52.   Herst, P.M. and M.V. Berridge, *Cell surface oxygen consumption: a major contributor to cellular oxygen consumption in glycolytic cancer cell lines.* Biochim Biophys Acta, 2007. **1767**(2): p. 170-7.

53.   Walls, M.K., et al., *Structural and biochemical abnormalities in the absence of acute deficits in mild primary blast-induced head trauma.* J Neurosurg, 2016. **124**(3): p. 675-86.

54.   Ambaw, A., et al., *Acrolein-mediated neuronal cell death and alpha-synuclein aggregation: Implications for Parkinson's disease.* Mol Cell Neurosci, 2018. **88**: p. 70-82.

55.   Wu, Q., M. Wang, and J.E. Simon, *Determination of isoflavones in red clover and related species by high-performance liquid chromatography combined with ultraviolet and mass spectrometric detection.* J Chromatogr A, 2003. **1016**(2): p. 195-209.

56.   Polymeropoulos, M.H., et al., *Mutation in the alpha-synuclein gene identified in families with Parkinson's disease.* Science, 1997. **276**(5321): p. 2045-7.

57.   Conway, K.A., J.D. Harper, and P.T. Lansbury, *Accelerated in vitro fibril formation by a mutant alpha-synuclein linked to early-onset Parkinson disease.* Nat Med, 1998. **4**(11): p. 1318-20.

58.   Liu, F., et al., *Mechanisms of DJ-1 neuroprotection in a cellular model of Parkinson's disease.* J Neurochem, 2008. **105**(6): p. 2435-53.

59.   Burke, R.E. and K. O'Malley, *Axon degeneration in Parkinson's disease.* Exp Neurol, 2013. **246**: p. 72-83.

60.   Cheng, H.C., C.M. Ulane, and R.E. Burke, *Clinical progression in Parkinson disease and the neurobiology of axons.* Ann Neurol, 2010. **67**(6): p. 715-25.

61.   Surh, Y.J., J.K. Kundu, and H.K. Na, *Nrf2 as a master redox switch in turning on the cellular signaling involved in the induction of cytoprotective genes by some chemopreventive phytochemicals.* Planta Med, 2008. **74**(13): p. 1526-39.

62.   Itoh, K., et al., *Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain.* Genes Dev, 1999. **13**(1): p. 76-86.

63.   Kobayashi, A., et al., *Oxidative stress sensor Keap1 functions as an adaptor for Cul3-based E3 ligase to regulate proteasomal degradation of Nrf2.* Mol Cell Biol, 2004. **24**(16): p. 7130-9.

64.   Itoh, K., et al., *An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements.* Biochem Biophys Res Commun, 1997. **236**(2): p. 313-22.

65.   Rushmore, T.H., M.R. Morton, and C.B. Pickett, *The antioxidant responsive element. Activation by oxidative stress and identification of the DNA consensus sequence required for functional activity.* J Biol Chem, 1991. **266**(18): p. 11632-9.

66.   Shih, A.Y., et al., *Coordinate regulation of glutathione biosynthesis and release by Nrf2-expressing glia potently protects neurons from oxidative stress.* J Neurosci, 2003. **23**(8): p. 3394-406.

67.   Baxter, P.S. and G.E. Hardingham, *Adaptive regulation of the brain's antioxidant defences by neurons and astrocytes.* Free Radic Biol Med, 2016. **100**: p. 147-152.

68.   Sherer, T.B., et al., *Mechanism of toxicity in rotenone models of Parkinson's disease.* J. Neurosci., 2003. **23**(34): p. 10756-10764.

69.   Dryanovski, D.I., et al., *Calcium entry and alpha-synuclein inclusions elevate dendritic mitochondrial oxidant stress in dopaminergic neurons.* J Neurosci, 2013. **33**(24): p. 10154-64.

70.   Gan, L., et al., *Astrocyte-specific overexpression of Nrf2 delays motor pathology and synuclein aggregation throughout the CNS in the alpha-synuclein mutant (A53T) mouse model.* J Neurosci, 2012. **32**(49): p. 17775-87.

71.   Erlank, H., et al., *Polyphenols activate Nrf2 in astrocytes via H2O2, semiquinones, and quinones.* Free Radic Biol Med, 2011. **51**(12): p. 2319-27.

72.   Satoh, T., et al., *Carnosic acid, a catechol-type electrophilic compound, protects neurons both in vitro and in vivo through activation of the Keap1/Nrf2 pathway via S-alkylation of targeted cysteines on Keap1.* J Neurochem, 2008. **104**(4): p. 1116-31.

73.   Satoh, T., S.R. McKercher, and S.A. Lipton, *Nrf2/ARE-mediated antioxidant actions of pro-electrophilic drugs.* Free Radic Biol Med, 2013. **65**: p. 645-57.

74.   Magesh, S., Y. Chen, and L. Hu, *Small molecule modulators of Keap1-Nrf2-ARE pathway as potential preventive and therapeutic agents.* Med Res Rev, 2012. **32**(4): p. 687-726.

75.   Kumar, H., et al., *Natural product-derived pharmacological modulators of Nrf2/ARE pathway for chronic diseases.* Nat Prod Rep, 2014. **31**(1): p. 109-39.

76.   Chang, T.L., *Inhibitory effect of flavonoids on 26S proteasome activity.* J Agric Food Chem, 2009. **57**(20): p. 9706-15.

77.   Yang, H., J.A. Zonder, and Q.P. Dou, *Clinical development of novel proteasome inhibitors for cancer treatment.* Expert Opin Investig Drugs, 2009. **18**(7): p. 957-71.

78.   Shen, M., T.H. Chan, and Q.P. Dou, *Targeting tumor ubiquitin-proteasome pathway with polyphenols for chemosensitization.* Anticancer Agents Med Chem, 2012. **12**(8): p. 891-901.

79.   Murakami, A., *Modulation of protein quality control systems by food phytochemicals.* J Clin Biochem Nutr, 2013. **52**(3): p. 215-27.

80.   Pettinari, A., et al., *Effect of polyphenolic compounds on the proteolytic activities of constitutive and immuno-proteasomes.* Antioxid Redox Signal, 2006. **8**(1-2): p. 121-9.

81.   Yamamoto, N., et al., *Proteasome inhibition induces glutathione synthesis and protects cells from oxidative stress: relevance to Parkinson disease.* J Biol Chem, 2007. **282**(7): p. 4364-72.

82.   Chen, J. and R.F. Regan, *Increasing expression of heme oxygenase-1 by proteasome inhibition protects astrocytes from heme-mediated oxidative injury.* Curr Neurovasc Res, 2005. **2**(3): p. 189-96.

83.   Dreger, H., et al., *Protection of vascular cells from oxidative stress by proteasome inhibition depends on Nrf2.* Cardiovasc Res, 2010. **85**(2): p. 395-403.

84.   Khor, T.O., et al., *Pharmacodynamics of curcumin as DNA hypomethylation agent in restoring the expression of Nrf2 via promoter CpGs demethylation.* Biochem Pharmacol, 2011. **82**(9): p. 1073-8.

85.   Tanigawa, S., M. Fujii, and D.X. Hou, *Action of Nrf2 and Keap1 in ARE-mediated NQO1 expression by quercetin.* Free Radic Biol Med, 2007. **42**(11): p. 1690-703.

86.   Rubiolo, J.A., G. Mithieux, and F.V. Vega, *Resveratrol protects primary rat hepatocytes against oxidative stress damage: activation of the Nrf2 transcription factor and augmented activities of antioxidant enzymes.* Eur J Pharmacol, 2008. **591**(1-3): p. 66-72.

87.   Jana, N.R., et al., *Inhibition of proteasomal function by curcumin induces apoptosis through mitochondrial pathway.* J Biol Chem, 2004. **279**(12): p. 11680-5.

88.   Nakamura, K., et al., *Direct Membrane Association Drives Mitochondrial Fission by the Parkinson Disease-associated Protein {alpha}-Synuclein.* J Biol Chem, 2011. **286**(23): p. 20710-26.

89.   Kamp, F., et al., *Inhibition of mitochondrial fusion by alpha-synuclein is rescued by PINK1, Parkin and DJ-1.* EMBO J, 2010. **29**(20): p. 3571-89.

90.   Devi, L., et al., *Mitochondrial import and accumulation of alpha-synuclein impair complex I in human dopaminergic neuronal cultures and Parkinson disease brain.* J Biol Chem, 2008. **283**(14): p. 9089-100.

91.   Marroquin, L.D., et al., *Circumventing the Crabtree effect: replacing media glucose with galactose increases susceptibility of HepG2 cells to mitochondrial toxicants.* Toxicol Sci, 2007. **97**(2): p. 539-47.

92.   Swiss, R. and Y. Will, *Assessment of mitochondrial toxicity in HepG2 cells cultured in high-glucose- or galactose-containing media.* Curr Protoc Toxicol, 2011. **Chapter 2**: p. Unit2 20.

93. Lagoa, R., et al., *Complex I and cytochrome c are molecular targets of flavonoids that inhibit hydrogen peroxide production by mitochondria.* Biochim Biophys Acta, 2011. **1807**(12): p. 1562-72.

94. Bove, J., et al., *Toxin-induced models of Parkinson's disease.* NeuroRx, 2005. **2**(3): p. 484-94.

95. Schober, A., *Classic toxin-induced animal models of Parkinson's disease: 6-OHDA and MPTP.* Cell Tissue Res, 2004. **318**(1): p. 215-24.

96. Terzioglu, M. and D. Galter, *Parkinson's disease: genetic versus toxin-induced rodent models.* FEBS J, 2008. **275**(7): p. 1384-91.

97. Datla, K.P., et al., *Short-term supplementation with plant extracts rich in flavonoids protect nigrostriatal dopaminergic neurons in a rat model of Parkinson's disease.* J Am Coll Nutr, 2007. **26**(4): p. 341-9.

98. Mirahmadi, S.M., et al., *Soy isoflavone genistein attenuates lipopolysaccharide-induced cognitive impairments in the rat via exerting anti-oxidative and anti-inflammatory effects.* Cytokine, 2018. **104**: p. 151-159.

99. Carey, A.N., et al., *Stilbenes and Anthocyanins Reduce Stress Signaling in BV-2 Mouse Microglia.* J Agric Food Chem, 2013. **61**(25): p. 5979-86.

100. Williamson, E.M., *Synergy and other interactions in phytomedicines.* Phytomedicine, 2001. **8**(5): p. 401-9.

101. Zhao, L., Z. Mao, and R.D. Brinton, *A select combination of clinically relevant phytoestrogens enhances estrogen receptor beta-binding selectivity and neuroprotective activities in vitro and in vivo.* Endocrinology, 2009. **150**(2): p. 770-83.

102. Setchell, K.D. and C. Clerici, *Equol: history, chemistry, and formation.* Journal of Nutrition, 2010. **140**(7): p. 1355S-62S.

103. Schreihofer, D.A. and L. Redmond, *Soy phytoestrogens are neuroprotective against stroke-like injury in vitro.* Neuroscience, 2009. **158**(2): p. 602-9.

104. Ma, Y., J.C. Sullivan, and D.A. Schreihofer, *Dietary genistein and equol (4', 7 isoflavandiol) reduce oxidative stress and protect rats against focal cerebral ischemia.* Am J Physiol Regul Integr Comp Physiol, 2010. **299**(3): p. R871-7.

105. Mathey, J., et al., *Modulation of soy isoflavones bioavailability and subsequent effects on bone health in ovariectomized rats: the case for equol.* Osteoporos Int, 2007. **18**(5): p. 671-9.

106. Jackman, K.A., O.L. Woodman, and C.G. Sobey, *Isoflavones, equol and cardiovascular disease: pharmacological and therapeutic insights.* Curr Med Chem, 2007. **14**(26): p. 2824-30.

107. Johnson, S.L., et al., *Equol, a Blood-Brain Barrier Permeable Gut Microbial Metabolite of Dietary Isoflavone Daidzein, Exhibits Neuroprotective Effects against Neurotoxins Induced Toxicity in Human Neuroblastoma SH-SY5Y Cells and Caenorhabditis elegans.* Plant Foods Hum Nutr, 2020.

108. Setchell, K.D., N.M. Brown, and E. Lydeking-Olsen, *The clinical importance of the metabolite equol-a clue to the effectiveness of soy and its isoflavones.* Journal of Nutrition, 2002. **132**(12): p. 3577-84.

109. Chang, H.C., et al., *Mass spectrometric determination of Genistein tissue distribution in diet-exposed Sprague-Dawley rats.* Journal of Nutrition, 2000. **130**(8): p. 1963-70.

110. Prasain, J.K., et al., *Identification of puerarin and its metabolites in rats by liquid chromatography-tandem mass spectrometry.* J Agric Food Chem, 2004. **52**(12): p. 3708-12.

111. Johnson, S.L., et al., *Polyphenol Microbial Metabolites Exhibit Gut and Blood(-)Brain Barrier Permeability and Protect Murine Microglia against LPS-Induced Inflammation.* Metabolites, 2019. **9**(4).

112. Park, J.S., et al., *Antioxidant mechanism of isoflavone metabolites in hydrogen peroxide-stimulated rat primary astrocytes: critical role of hemeoxygenase-1 and NQO1 expression.* J Neurochem, 2011.

113. Siow, R.C., et al., *Cardiovascular targets for estrogens and phytoestrogens: transcriptional regulation of nitric oxide synthase and antioxidant defense genes.* Free Radic Biol Med, 2007. **42**(7): p. 909-25.

114. Froyen, E.B., et al., *Regulation of phase II enzymes by genistein and daidzein in male and female Swiss Webster mice.* Journal of Medicinal Food, 2009. **12**(6): p. 1227-37.

115. Dreger, H., et al., *Nrf2-dependent upregulation of antioxidative enzymes: a novel pathway for proteasome inhibitor-mediated cardioprotection.* Cardiovasc Res, 2009. **83**(2): p. 354-61.

116. Dreiseitel, A., et al., *Inhibition of proteasome activity by anthocyanins and anthocyanidins.* Biochem Biophys Res Commun, 2008. **372**(1): p. 57-61.

117. Kazi, A., et al., *Inhibition of the proteasome activity, a novel mechanism associated with the tumor cell apoptosis-inducing ability of genistein.* Biochem Pharmacol, 2003. **66**(6): p. 965-76.

118. Li, H.Y., S.Y. Wu, and N. Shi, *Transcription factor Nrf2 activation by deltamethrin in PC12 cells: Involvement of ROS.* Toxicol Lett, 2007. **171**(1-2): p. 87-98.

119. Bachoo, R.M., et al., *Molecular diversity of astrocytes with implications for neurological disorders.* Proc Natl Acad Sci U S A, 2004. **101**(22): p. 8384-9.

120. Kipp, M., et al., *Brain-region-specific astroglial responses in vitro after LPS exposure.* J Mol Neurosci, 2008. **35**(2): p. 235-43.

121. Zamanian, J.L., et al., *Genomic analysis of reactive astrogliosis.* J Neurosci, 2012. **32**(18): p. 6391-410.

122. Khakh, B.S. and M.V. Sofroniew, *Diversity of astrocyte functions and phenotypes in neural circuits.* Nature Neuroscience, 2015. **18**(7): p. 942-52.

123. Castelo-Branco, G., et al., *Ventral midbrain glia express region-specific transcription factors and regulate dopaminergic neurogenesis through Wnt-5a secretion.* Mol Cell Neurosci, 2006. **31**(2): p. 251-62.

124. Yeh, T.H., et al., *Microarray analyses reveal regional astrocyte heterogeneity with implications for neurofibromatosis type 1 (NF1)-regulated glial proliferation.* Glia, 2009. **57**(11): p. 1239-49.

125. Lozzi, B., et al., *Regionally Distinct Astrocytes Display Unique Transcription Factor Profiles in the Adult Brain.* Front Neurosci, 2020. **14**: p. 61.

126. Batiuk, M.Y., et al., *Identification of region-specific astrocyte subtypes at single cell resolution.* Nat Commun, 2020. **11**(1): p. 1220.

127. Zschocke, J., et al., *Differential promotion of glutamate transporter expression and function by glucocorticoids in astrocytes from various brain regions.* J Biol Chem, 2005. **280**(41): p. 34924-32.

128. Oberheim, N.A., S.A. Goldman, and M. Nedergaard, *Heterogeneity of astrocytic form and function.* Methods Mol Biol, 2012. **814**: p. 23-45.

129. Ding, J., et al., *Soy isoflavone attenuates brain mitochondrial oxidative stress induced by beta-amyloid peptides 1-42 injection in lateral cerebral ventricle.* J Neurosci Res, 2013. **91**(4): p. 562-7.

130. Feng, J.F., et al., *Antagonizing effects of soybean isoflavones on beta-amyloid peptide-induced oxidative damage in neuron mitochondria of rats.* Basic Clin Pharmacol Toxicol, 2012. **111**(4): p. 248-53.

131. Salvi, M., et al., *Interaction of genistein with the mitochondrial electron transport chain results in opening of the membrane transition pore.* Biochim Biophys Acta, 2002. **1556**(2-3): p. 187-96.

132. Zheng, J. and V.D. Ramirez, *Inhibition of mitochondrial proton F0F1-ATPase/ATP synthase by polyphenolic phytochemicals.* Br J Pharmacol, 2000. **130**(5): p. 1115-23.

133. Fang, N. and J.E. Casida, *Anticancer action of cube insecticide: correlation for rotenoid constituents between inhibition of NADH:ubiquinone oxidoreductase and induced ornithine decarboxylase activities.* Proc Natl Acad Sci U S A, 1998. **95**(7): p. 3380-4.

134. Rasbach, K.A. and R.G. Schnellmann, *Isoflavones promote mitochondrial biogenesis.* J Pharmacol Exp Ther, 2008. **325**(2): p. 536-43.

135. File, S.E., et al., *Eating soya improves human memory.* Psychopharmacology (Berl), 2001. **157**(4): p. 430-6.

136. Yu, J., et al., *Isoflavones: Anti-Inflammatory Benefit and Possible Caveats.* Nutrients, 2016. **8**(6).

137.   Pop, E.A., et al., *Effects of a high daily dose of soy isoflavones on DNA damage, apoptosis, and estrogenic outcomes in healthy postmenopausal women: a phase I clinical trial.* Menopause, 2008. **15**(4 Pt 1): p. 684-92.

Table 1. HPLC-TOF-MS analysis of a red clover extract.

Retention Time (min)	m/z positive mode	m/z negative mode	Formula	Identification
3.38	193.042	191.0188	C6H8O7	citric acid
10.27	166.08582		$C_9H_{11}NO_2$	phenylalanine
12.11	205.09673		$C_{11}H_{12}N_2O_2$	tryptophan
12.84	433.13409	431.11977	$C_{21}H_{20}O_{10}$	cosmosiin
13.6	360.10794	358.09205	$C_{18}H_{17}NO_7$	cis and trans clovamide
13.93	360.10748	358.09348	$C_{18}H_{17}NO_7$	cis and trans clovamide
14.45	465.10325	463.0894	$C_{21}H_{20}O_{12}$	quercetin-3-*O*-β-glucoside
14.82	551.10326	549.09	$C_{24}H_{22}O_{15}$	quercetin-3-*O*-β-malonylglucoside
14.9	449.10856		$C_{21}H_{20}O_{11}$	unknown flavonoid glycoside
15.55	565.11947		$C_{25}H_{24}O_{15}$	3-methyl quercetin-7-*O*-β-glucoside malonate
16.33	549.12436		$C_{25}H_{24}O_{14}$	pratensein-7-*O*-β-glucoside-6''-*O*-malonate
17.85	547.10891		$C_{25}H_{22}O_{14}$	irilone-4'-*O*-β-glucoside-6''-*O*-malonate
18.14	533.12919	1063.23790 (2M-H)	$C_{25}H_{24}O_{13}$	biochanin A 7-*O*-β-glucoside-6''-*O*-malonate
18.71	269.08129	267.06598	$C_{16}H_{12}O_4$	formononetin

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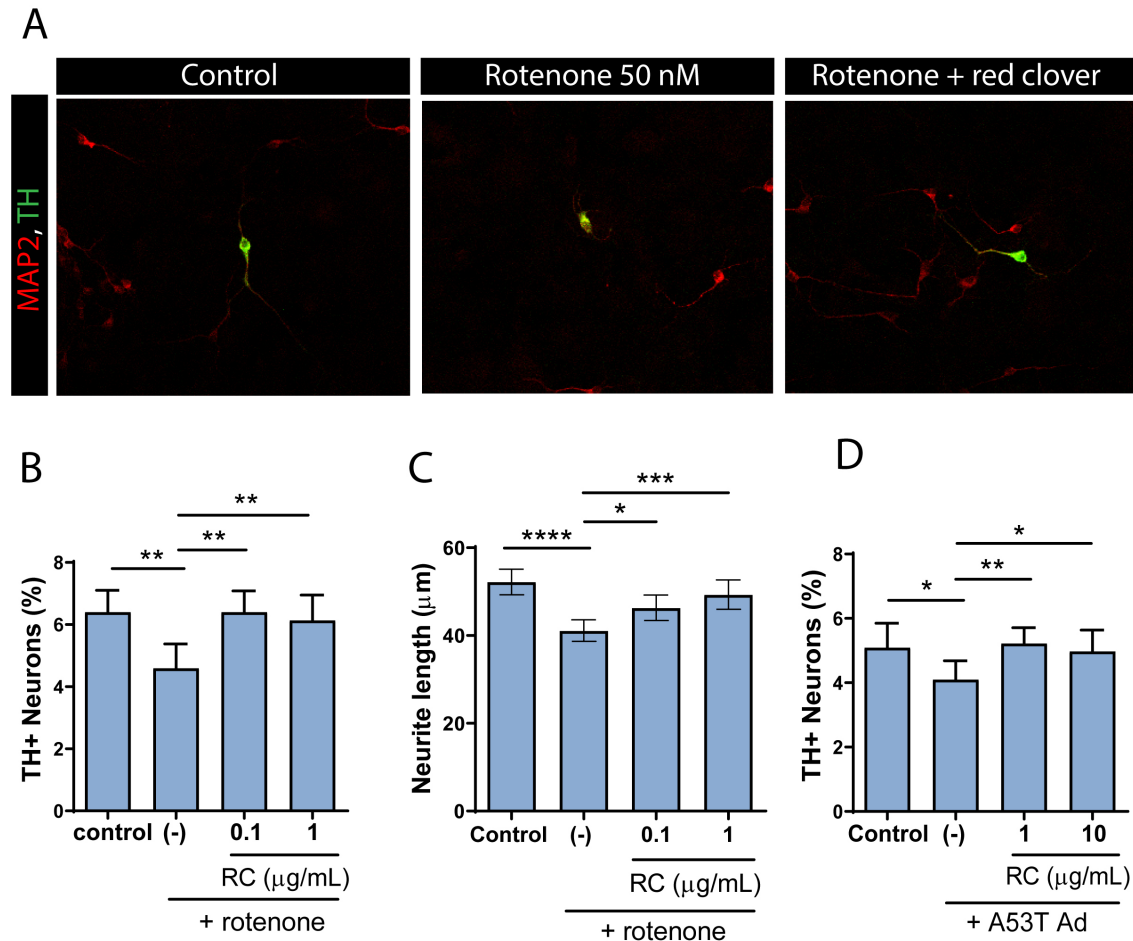
Figure 1. A red clover extract alleviates rotenone and A53T neurotoxicity. (A) Confocal images of immunostained dopaminergic neurons in primary midbrain cultures show expression of the pan-neuronal marker MAP2 (red) and the marker for dopaminergic neurons TH (green). Images show a protective effect of the red clover (RC) extract against rotenone-induced neurite retraction. (B-D) Cultures were treated with 50 nM rotenone for 24 h (B, C) or A53T Ad (MOI 15) for 72 h (D) in the absence or presence of RC. Control cells were incubated in the absence of rotenone, A53T or RC. The cells were stained with antibodies specific for MAP2 and TH and scored for relative dopaminergic cell viability (B, D), or neurite length (C). The data are presented as the mean ± SEM; the neurite length data are presented as the mean value ± 95% confidence limits after back-transformation of log-transformed data as outlined in the Methods; n = 3; **p≤0.01, ****p≤0.0001; one-way ANOVA followed by Tukey's multiple comparisons *post hoc* test.

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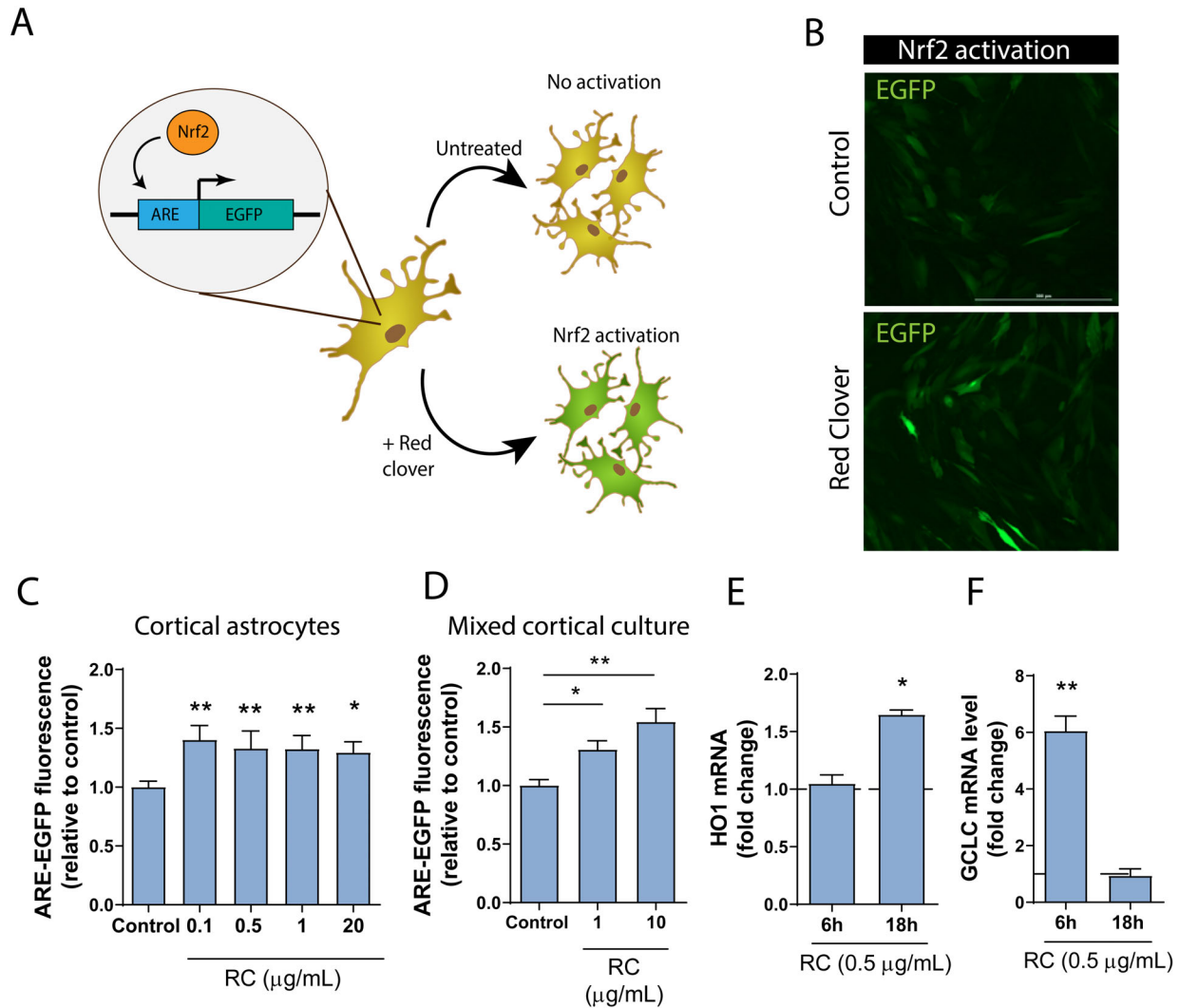
Figure 2. A red clover extract activates the Nrf2/ARE antioxidant response in primary cortical astrocytes. (A-B) Primary cortical astrocytes were transduced with an ARE-EGFP reporter adenovirus for 48 h and incubated in the presence of red clover (RC) extract for 24 h. Control cells were transduced with the reporter virus and incubated in the absence of extract. (C-D) Quantification of EGFP fluorescence intensity in astrocytes show an increase in EGFP fluorescence in cortical astrocytes (C) or mixed cortical cultures (D) treated with an extract prepared from RC. (E-F) To validate that the observed increase in EGFP fluorescence resulted in increased transcription of Nrf2 target genes, cortical astrocytes were incubated in absence or presence of RC extract, and the mRNA levels of heme oxygenase 1 (HO1) (E) and glutamate-cysteine ligase catalytic subunit (GCLC) (F) were measured by qRT-PCR. Scale bar in (B): 300 μm. The data are presented as the mean ± SEM; n = 2 (E), n = 3 (D,F), n = 4 (C); *$p \leq 0.05$, **$p \leq 0.01$; one-way ANOVA with Dunnett's multiple comparison *post hoc* test (C,D); *$p \leq 0.05$, **$p \leq 0.01$ versus a predicted ratio of 1; log transformation followed by one-sample t-test (E,F).
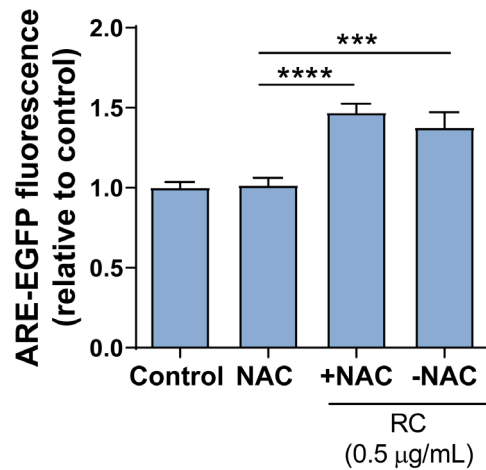
Figure 3. Red clover-mediated activation of Nrf2 transcription is ROS-independent. Primary cortical astrocytes were transduced with an ARE-EGFP reporter adenovirus for 48 h and incubated with a red clover (RC) extract for 24 h in the absence or presence of NAC (1 mM). Control cells were transduced with the reporter virus, and incubated in the absence of extract or NAC. The graph shows the fluorescence intensity quantified post-treatment. The data are presented as the mean ± SEM; n = 6; ***p≤ 0.001; ***p≤ 0.001; one-way ANOVA with Dunnett's multiple comparisons *post hoc* test.
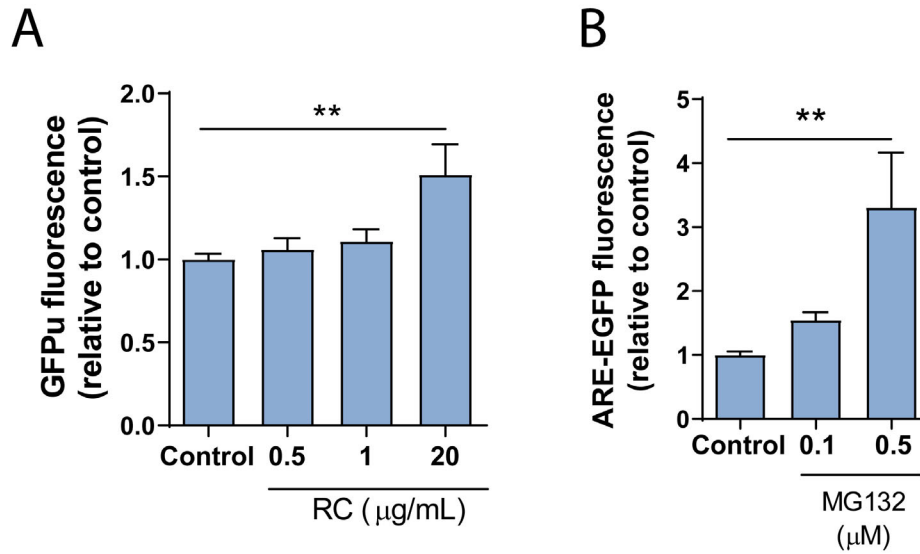
Figure 4. Red clover polyphenols inhibit the UPS. (A) Primary cortical astrocytes were transduced with a reporter adenovirus encoding the UPS substrate GFPu for 48 h, in the absence or presence of RC, and fluorescence intensity was quantified. (B) Primary cortical astrocytes were transduced with an ARE-EGFP reporter adenovirus for 48 h and incubated with the proteasome inhibitor MG132 for 24 h. Control cells for both panels were transduced with reporter virus and incubated in the absence of extract. The data are presented as the mean ± SEM; n = 3 or 4; **p≤ 0.01; one-way ANOVA with Dunnett's multiple comparisons *post hoc* test.
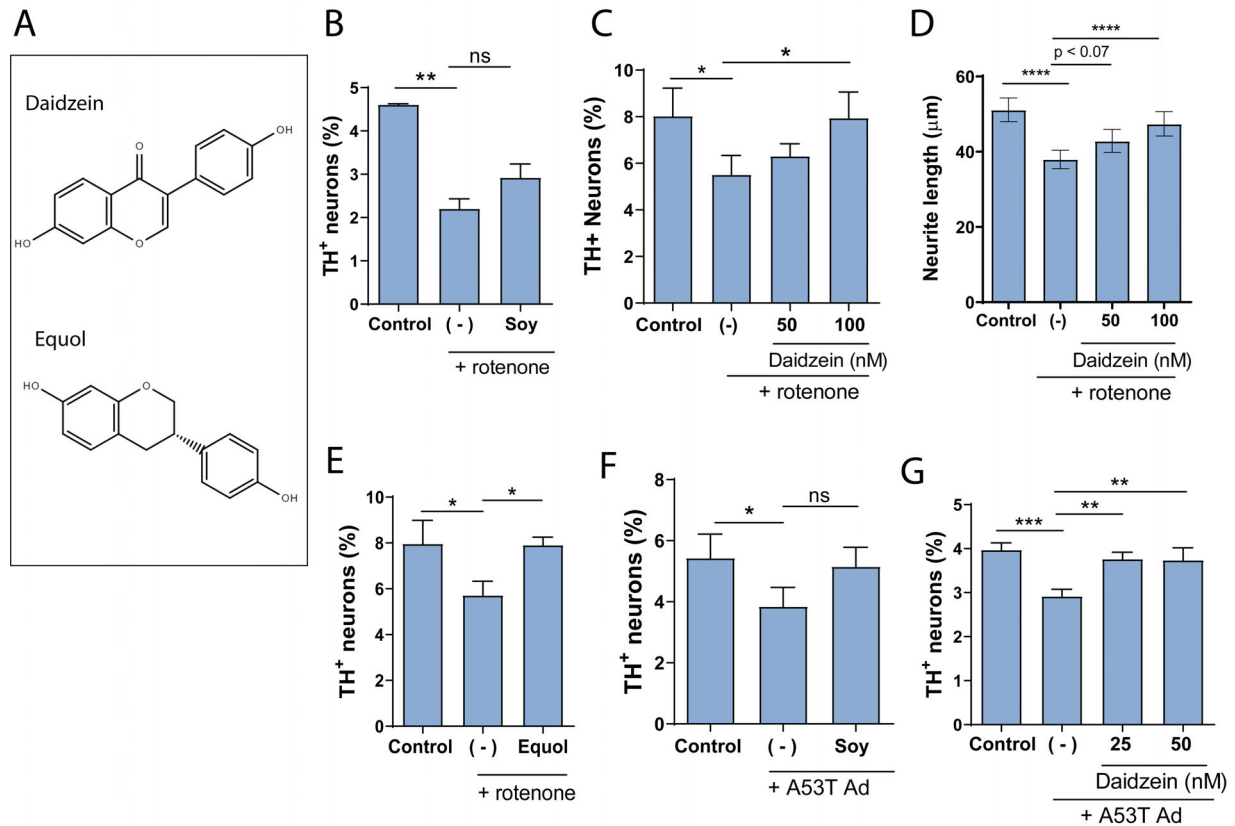
Figure 5. Soy isoflavones alleviate rotenone and A53T neurotoxicity. (A) Molecular structures of the isoflavones daidzein and equol. (B-G) Primary midbrain cultures were treated with rotenone (50 nM or 100 nM) for 24 h (B-E) or A53T Ad (MOI 15) for 72 h (F,G) in the absence or presence of a soy extract (1 µg/mL, B, F), daidzein (25 to 100 nM, C, D, G) or equol (50 nM, E). Control cells were incubated in the absence of rotenone, A53T or extract/compound. The cells were stained with antibodies specific for MAP2 and TH, and scored for relative dopaminergic cell viability. The data are presented as the mean ± SEM; the neurite length data are presented as the mean value ± 95% confidence limits after back-transformation of log-transformed data as outlined in the Methods; n = 3 or 4; *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$; one-way ANOVA followed by Tukey's multiple comparisons *post hoc* test. In panels (B) and (F), a statistically significant neuroprotective effect is observed in cultures treated with soy plus insult versus insult alone when the data are analyzed via ANOVA followed by the Newman-Keuls *post hoc* test ($p \leq 0.05$).
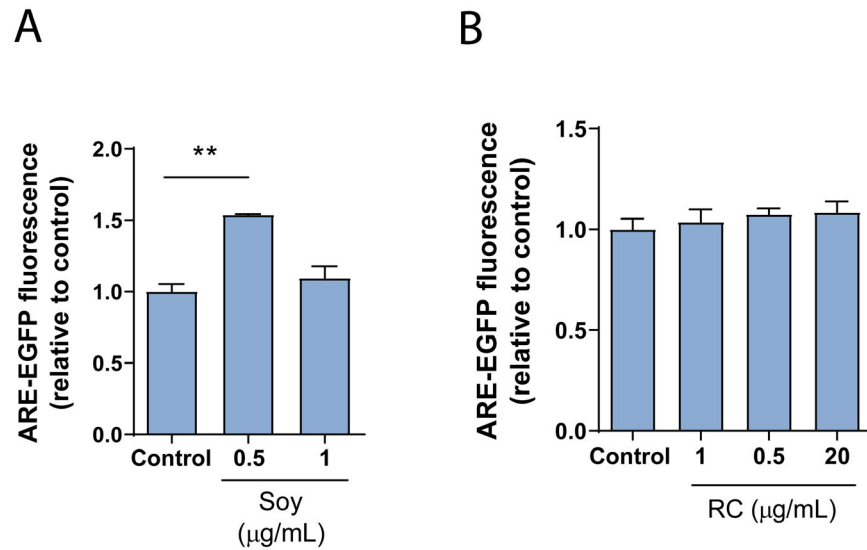
Figure 6. An extract prepared from soy but not red clover activates the Nrf2/ARE antioxidant response in hiPSC-derived astrocytes. (A,B) Human iPSC-derived astrocytes were transduced with an ARE-EGFP reporter adenovirus for 48 h and incubated in the presence of soy (A) or red clover (RC) (B) extract for 24 h. Control cells were transduced with the reporter virus and incubated in the absence of extract. The data are presented as the mean ± SEM; n = 3 or 4; **$p \leq 0.01$; one-way ANOVA with Dunnett's multiple comparisons *post hoc* test.
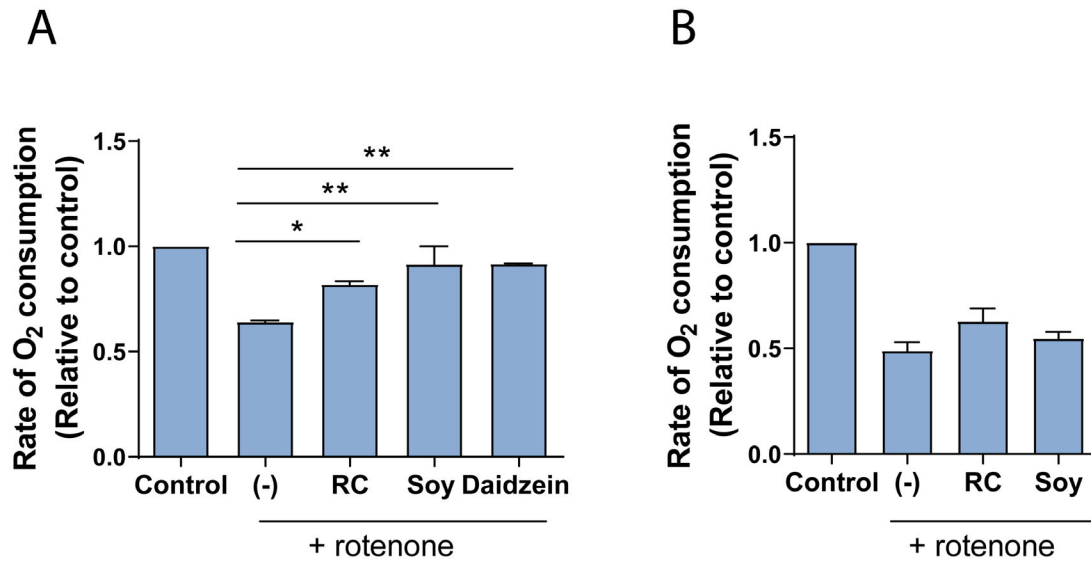
Figure 7. Isoflavone-rich extracts prevent rotenone-induced loss of mitochondrial function.
(A) Galactose-conditioned SH-SY5Y cells were incubated in the absence or presence of an extract prepared from red clover (1 μg/mL, RC), soy (1 μg/mL), or daidzein (50 nM) for 19 h and then exposed to rotenone (30 nM) in the absence or presence of extract for 5 h. The cells were then analyzed for their O2 consumption rates. (B) Alternatively, the rate of O_2 consumption was measured immediately after adding rotenone (50 nM) followed by the RC extract (1 μg/mL) or soy extract (1 μg/mL). The O_2 consumption rate was measured with a Clark type oxygen electrode attached to a voltmeter. The data are presented as the rate of O_2 consumption; mean ± SEM, n = 2 or 3; *p≤0.05, **p≤0.01; one-way ANOVA with Dunnett's multiple comparisons *post hoc* test (A). In panel (B), a statistically significant protective effect is observed in cultures treated with RC plus rotenone versus rotenone alone when the data are analyzed via ANOVA followed by the Holm-Sidak post hoc test (p≤ 0.05).
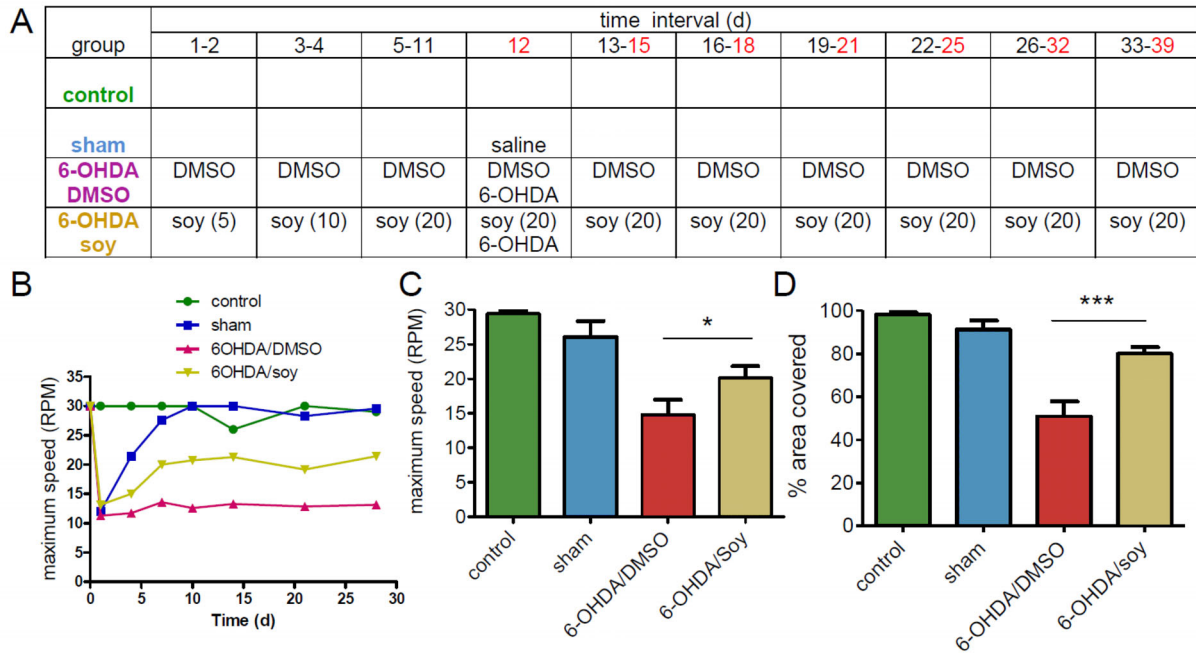
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| group | time interval (d) |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1-2 | 3-4 | 5-11 | 12 | 13-15 | 16-18 | 19-21 | 22-25 | 26-32 | 33-39 |
| control | | | | | | | | | | |
| sham | | | | saline | | | | | | |
| 6-OHDA DMSO | DMSO | DMSO | DMSO | DMSO 6-OHDA | DMSO | DMSO | DMSO | DMSO | DMSO | DMSO |
| 6-OHDA soy | soy (5) | soy (10) | soy (20) | soy (20) 6-OHDA | soy (20) | soy (20) | soy (20) | soy (20) | soy (20) | soy (20) |

Figure 8. A soy extract alleviates motor deficits in 6-OHDA-lesioned rats. (A) Table outlining the experimental design. Numbers in the '6-OHDA/soy' row refer to the amount of soy extract injected (mg/kg). Times shown in red at the top refer to days when motor tests were performed. (B,C) Graphs showing maximum rotarod speeds, expressed as average values at different times (B) or the overall average from all time points (C). (D) Graph showing % area covered in the open field test. The data in (C) and (D) are presented as the mean ± SEM; *p<0.05, ***p<0.001; one-way ANOVA followed by Tukey's multiple comparisons *post hoc* test.
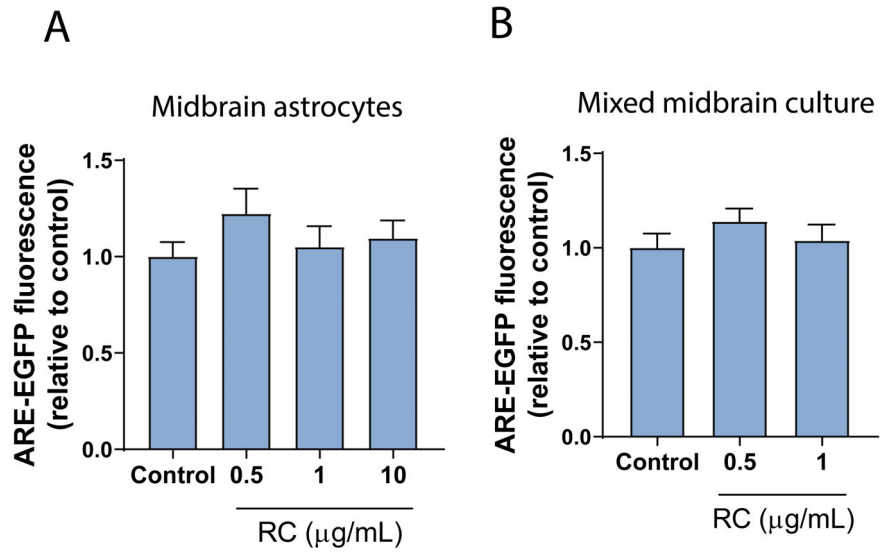
Supplementary Figure 1. A red clover extract fails to activate Nrf2 transcription in midbrain astrocytes. Primary midbrain astrocytes (A) or mixed midbrain cultures (B) were transduced with an ARE-EGFP reporter adenovirus for 48 h and incubated in the presence of red clover (RC) extract for 24 h. Control cells were transduced with the reporter virus and incubated in the absence of extract. Quantification of EGFP fluorescence intensity does not show any increase in EGFP fluorescence in RC-treated astrocytes vs control. The data are presented as the mean ± SEM; n = 6 (A) or n = 3 (B).
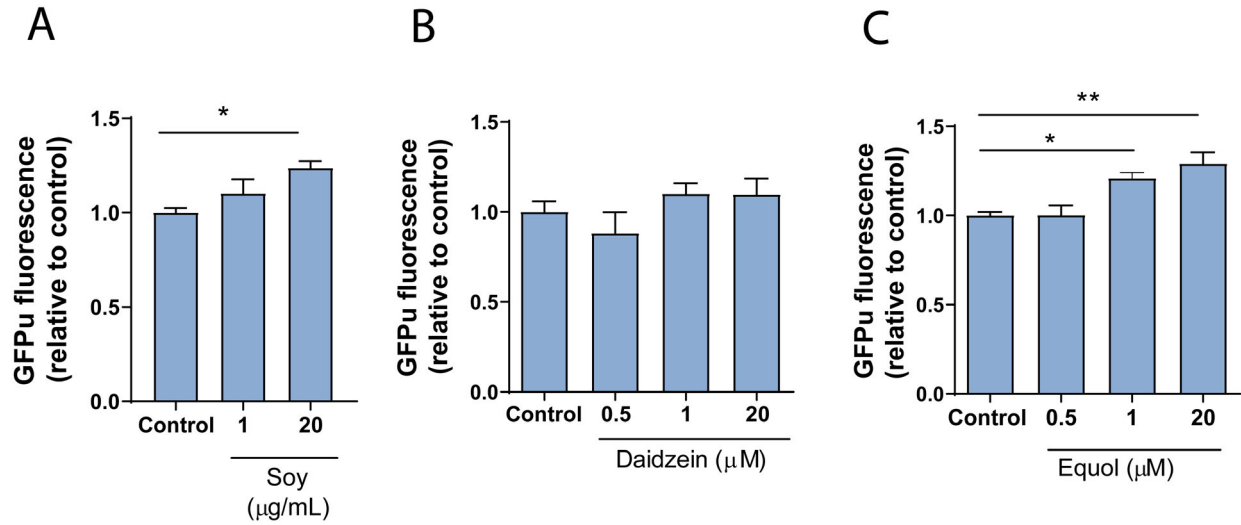
31
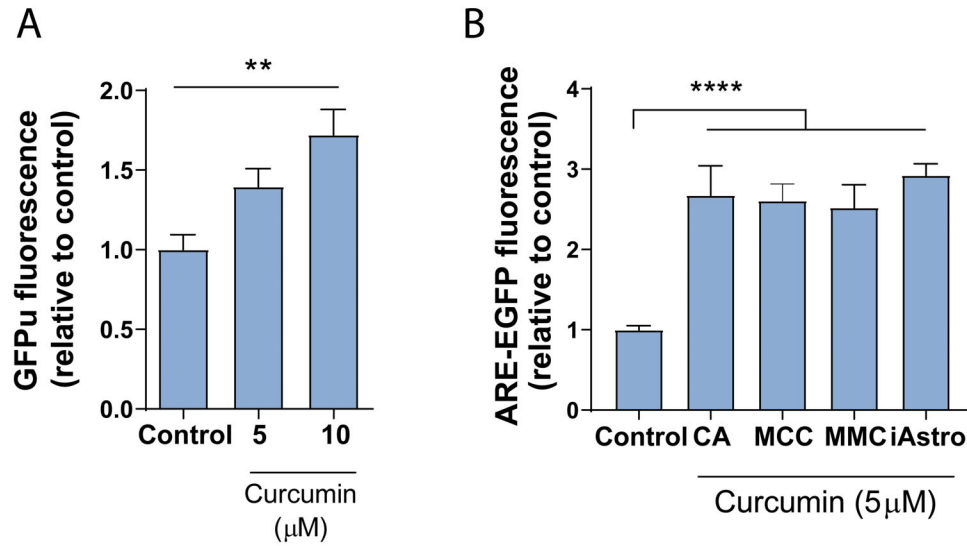
Supplementary Figure 2. Curcumin inhibits the UPS and activates Nrf2 transcription. (A) Primary cortical astrocytes were transduced with a reporter adenovirus encoding the UPS substrate GFPu for 48 h, in the absence or presence of curcumin, and fluorescence intensity was quantified. (B) Primary cortical astrocytes (CA), mixed cortical cultures (MCC), mixed midbrain cultures (MMC), or iPSC-derived astrocytes (iAstro) were transduced with an ARE-EGFP reporter adenovirus for 48 h and incubated in the presence of curcumin for 24 h. Control cells for both panels were transduced with reporter virus and incubated in the absence of extract. The data are presented as the mean ± SEM; n = 3; **$p \leq 0.01$, ****$p \leq 0.0001$; one-way ANOVA followed by Dunnett's multiple comparisons *post hoc* test.
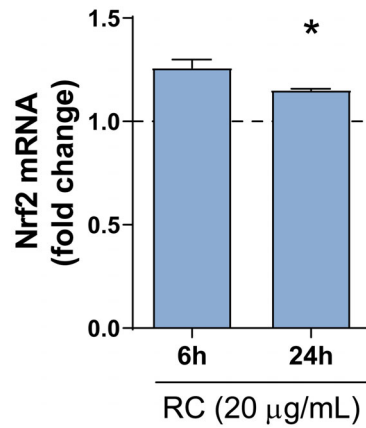
Supplementary Figure 3. A red clover extract induces a modest upregulation of Nrf2 expression. Primary cortical astrocytes were incubated in the absence (control) or presence of red clover (RC) extract (20 μg/mL) for 6 h and 24 h, and Nrf2 mRNA levels were determined by qRT-PCR. The data are presented as the mean ± SEM; n = 2; *p≤0.05 versus a predicted ratio of 1; log transformation followed by one-sample t-test.
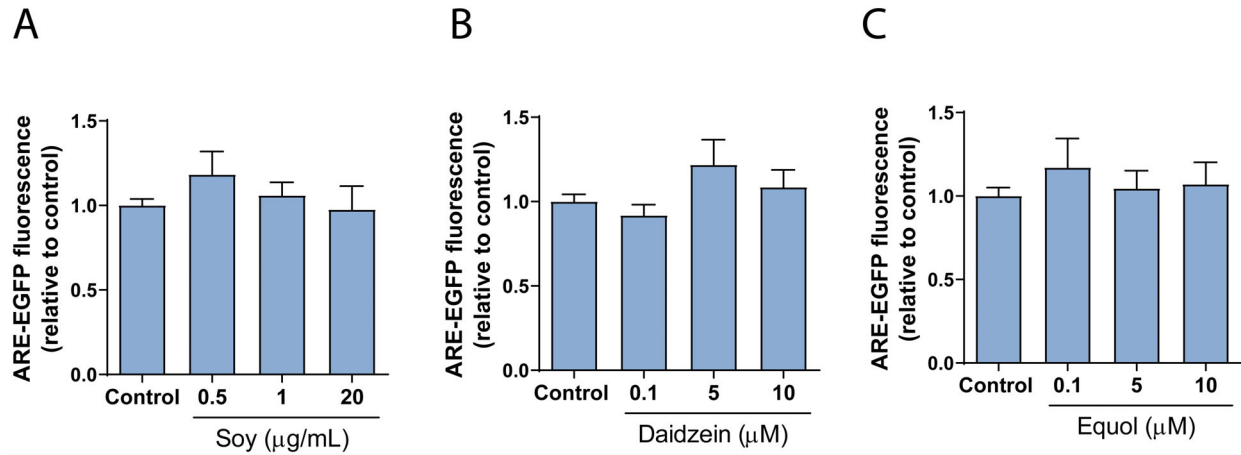
Supplementary Figure 4. A soy extract and soy isoflavones do not activate the Nrf2-mediated response. Primary cortical astrocytes were transduced with an ARE-EGFP reporter adenovirus for 48 h and incubated in the presence of soy extract or compound for 24 h. Control cells were transduced with the reporter virus and incubated in the absence of extract. Graphs show no increase in EGFP fluorescence in astrocytes treated with soy extract (A), daidzein (B) or equol (C). The data are presented as the mean ± SEM; n = 6 (C); n = 7 (B); n = 9 (A); one-way ANOVA followed by Dunnett's multiple comparisons *post hoc* test.

Supplementary Figure 5. A soy extract and equol but not daidzein modulate the UPS. Primary cortical astrocytes were transduced with a reporter adenovirus encoding the UPS substrate GFPu for 48 h, in the absence or presence of soy (A), daidzein (B) or equol (C), and fluorescence intensity after treatment was quantified. The data are presented as the mean ± SEM; n = 4 (A, C), n = 7 (B); *p≤0.05, **p≤0.01; one-way ANOVA followed by Dunnett's multiple comparisons *post hoc* test.