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RESEARCH ARTICLE

Sulforaphane protects cortical neurons against 5-*S*-cysteinyl-dopamine-induced toxicity through the activation of ERK1/2, Nrf-2 and the upregulation of detoxification enzymes

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The degeneration of dopaminergic neurons in the substantia nigra has been linked to the formation of the endogenous neurotoxin 5-*S*-cysteinyl-dopamine. Sulforaphane (SFN), an isothiocyanate derived from the corresponding precursor glucosinolate found in cruciferous vegetables has been observed to exert a range of biological activities in various cell populations. In this study, we show that SFN protects primary cortical neurons against 5-*S*-cysteinyl-dopamine induced neuronal injury. Pre-treatment of cortical neurons with SFN (0.01–1 μM) resulted in protection against 5-*S*-cysteinyl-dopamine-induced neurotoxicity, which peaked at 100 nM. This protection was observed to be mediated by the ability of SFN to modulate the extracellular signal-regulated kinase 1 and 2 and the activation of Kelch-like ECH-associated protein 1/NF-E2-related factor-2 leading to the increased expression and activity of glutathione-*S*-transferase (M1, M3 and M5), glutathione reductase, thioredoxin reductase and NAD(P)H oxidoreductase 1. These data suggest that SFN stimulates the NF-E2-related factor-2 pathway of antioxidant gene expression in neurons and may protect against neuronal injury relevant to the aetiology of Parkinson's disease.

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1 Introduction

A hallmark of Parkinson's disease (PD) is the significant degeneration and eventual loss of dopaminergic neurons in the *Substantia nigra pars compacta* region of the basal ganglia [1, 2]. Although the specific vulnerability of this population of neurons remains unclear, evidence suggests that endogenous dopamine may play a role in the pathogenesis. Dopamine is known to undergo oxidation to an *o*-quinone, which may yield neurotoxic species following its reaction with cellular thiols to form the 5-*S*-cysteinyl-dopamine (CysDA) [3–5]. Cysteinyl-dopamine adducts have been reported in human brain tissue and are elevated in the brains of patients suffering from PD [6]. Exposure of neurons to 5-*S*-cysteinyl conjugates of catecholamines leads to neuronal damage [4], increases in oxidative DNA base modification and activation

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Abbreviations: ANOVA, analysis of variance; **ARE,** antioxidant response element; **CysDA,** 5-*S*-cysteinyl-dopamine; **DTNB,** 5,5′-dithiobis(2-nitrobenzoic) acid; **ECL,** electrochemiluminescent; **ERK1/2,** extracellular signal-regulated kinase 1 and 2; **GR,** glutathione reductase; **GSH,** reduced glutathione; **GSSG,** oxidized glutathione; **GST,** glutathione-*S*-transferase; **HRP,** horseradish peroxidase; **Keap1,** Kelch-like ECH-associated protein 1; **MAPK,** mitogen-activated protein kinase; **Nrf2,** NF-E2-related factor-2; **PD,** Parkinson's disease; **PI3-kinase,** phosphoinositide 3-kinase; **PKB,** protein kinase B; **SFN,** sulforaphane; **SSA,** 5-sulfosalicylic acid; **TR,** thioredoxin reductase; **TTBS,** TBS supplemented with 0.05% v/v Tween 20

of caspase-3 activity in neurons [3]. In addition, CysDA may undergo further oxidation to yield new species, such as 7-(2-aminoethyl)-3,4-dihydro-5-hydroxy-2*H*-1,4-benzothiazine-3-carboxylic acid, which have been reported to be potent mitochondrial respiratory complex I inhibitors [7, 8].

There has been much interest in the potential neuroprotective effects of phytochemicals, especially in the context of neurodegenerative disorders such as PD and Alzheimer's disease [4, 9–14]. Sulforaphane (SFN), an aliphatic isothiocyanate, is found in cruciferous vegetables (*i.e.* broccoli, cabbage, watercress and Brussel sprouts) as its precursor glucosinolate [15]. Upon chewing, the glucose moiety of this glucosinolate precursor is enzymatically hydrolysed by the myrosinase into the corresponding isothiocyanate [16]. Glucosinolates that are not hydrolysed can then be degraded by the enteric microflora [17]. Many studies have demonstrated the anticarcinogenic potential of SFN [18], although little is known about the effects of SFN on brain-derived cells. SFN has been reported to protect cortical neurons in mixed primary culture [19] and retinal pigment epithelial cells against photo-oxidative damage [20]. This protection afforded by SFN is thought to be mediated *via* the activation of the NF-E2-related factor-2 (Nrf2) pathway and subsequent upregulations of phase II detoxification enzymes and antioxidant proteins through an enhancer sequence referred to as the electrophile-responsive element or antioxidant responsive element (ARE) [21, 22]. Indeed, SFN has been shown to exert neuroprotective effects against 6-hydroxydopamine, tetrahydrobiopterin and ischaemia/reperfusion through activation of the Nrf-2-electrophile-responsive element/ARE pathway [23, 24].

In this study, we show that SFN is able to protect primary cortical neurons against CysDA-induced injury, by a mechanism involving the increased nuclear translocation of Nrf2 and the increased expression and activity of phase II enzymes such as glutathione-*S*-transferase (GST M1, M3 and M5), glutathione reductase (GR), thioredoxin reductase (TR) and NAD(P)H oxidoreductase 1 (NQO1). We suggest that the activation of the Nrf-2 pathway and subsequent protection exerted by SFN appears also to be mediated by the activation of the extracellular signal-regulated kinase 1 and 2 (ERK1/2) and Akt/protein kinase B (PKB) pathways.

2 Materials and methods

2.1 Materials

SFN, CelLytic M, 1-chloro-2,4-dinitrobenzene, 5,5′-dithiobis(2-nitrobenzoic) acid (DTNB), reduced glutathione (GSH), oxidized glutathione (GSSG), NADPH and EDTA were all obtained from Sigma (Poole, UK). CysDA was obtained as reported previously [4, 5]. Antibodies used were as follows: pERK1/2, ERK1/2, Akt, pAkt (Ser 473) (New England Biolabs, Hitchin, UK); anti-Nrf-2 (ab31163) and anti-Keap-1 were from Abcam (Cambridge, UK), anti-Nrf-2 (H300) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Wortmannin was purchased from Calbiochem (La Jolla, CA, USA), LY294002, PD98059 and U0126 were purchased from New England Biolabs. Horseradish peroxidase-conjugated goat anti-rabbit secondary antibody was from Sigma, electrochemiluminescent (ECL) reagent and Hyperfilm-ECL were purchased from Amersham Biosciences (Amersham, UK).

2.2 Cell culture and treatment

Primary cultures of mouse cortical neurons were prepared as described previously [3, 12]. Neurons were plated onto Nunc multi-well plates that had been pre-coated overnight with 15 μg/mL poly-L-ornithine (Sigma) and then with 10% heat-inactivated fetal bovine serum (Invitrogen, Paisley, UK) for 2 h. Following removal of the final coating solution cells were plated (10^6/mL) in a serum-free medium composed of a mixture of DMEM and F-12 nutrient (1:1 v/v) supplemented with glucose (33 mM), glutamine (2 mM), sodium bicarbonate (6.5 mM), HEPES (pH 7.4, 5 mM), streptomycin (100 μg/mL) and penicillin (100 U/mL) (all Invitrogen). A mixture of hormones and salts composed of insulin (25 μg/mL), transferrin (100 μg/mL), putrescine (60 μg/mL), progesterone (20 nM) and sodium selenate (30 nM) (all from Sigma) was also added to the culture medium. Cells were cultured at 37°C in a humidified atmosphere of 95% air and 5% CO_2 and after 5–6 days, the vast majority of cells were neuronal (> 98%) with < 2% astrocytes as determined by β-tubulin and GFAP immunocytochemistry, respectively [25]. Toxicity elicited by the neurotoxins was evaluated by Alamar Blue reduction assay. To investigate the protective effect, neurons were pre-treated with SFN (0.01–1 μM) for 24 h. Following pre-treatment with SFN, the medium was removed and neurons were re-incubated with fresh conditioned medium containing cysteinyl-dopamine (100 μM) for a further 24 h prior to assessment of neuronal injury. For the inhibition experiments, primary neurons were treated with U0126 (10 μM), PD98059 (50 μM), wortmannin (100 nM) or LY294002 (50 μM) for 1 h before addition of CysDA (100 μM, 24 h) or SFN (100 nM, 24 h). Following exposure, cultures were washed twice with sterile PBS before the addition of Alamar Blue solution (10% v/v) in DMEM:F12. Plates were returned to the incubator for 2–3 h, before fluorescence was measured (Ex: 540 nm; Em: 612 nm) on a Tecan GENios multiplate reader (Tecan GENios, Theale, UK). Results were expressed as percentage of neuronal injury relative to vehicle-treated cells.

2.3 Immunoblotting

Following exposures either to SFN (0–0.5 μM) or to CysDA (100 μM), neurons were washed with ice-cold D-PBS and lysed on ice using Tris (50 mM), Triton X-100 (0.1%), NaCl (150 mM) and EGTA/EDTA (2 mM), containing

mammalian protease inhibitor cocktail (1:100 dilution), sodium pyrophosphate (1 mM), PMSF (10 μg/mL), sodium vanadate (1 mM) and sodium fluoride (50 mM). Lysed cells were scraped and left on ice to solubilize for 45 min. Lysates were centrifuged at 1000 × *g* for 5 min at 4°C to remove unbroken cell debris and nuclei. Protein concentration in the supernatants was determined by the BCA assay (Thermo Fisher Scientific, Cramlington, UK). Samples were incubated for 5 min at 95°C in boiling buffer (final conc. 62.5 mM Tris, pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol and 0.0025% bromophenol blue). Boiled samples (30 μg/lane) were run on 9–12% SDS-polyacrylamide gels and proteins were transferred onto nitrocellulose membranes (Hybond-ECL®; Amersham) by semi-dry electroblotting (1.5 mA/cm^2). The nitrocellulose membrane was then incubated in a blocking buffer (20 mM Tris, pH 7.5, 150 mM NaCl; TBS) containing 4% w/v skimmed milk powder for 45 min at room temperature followed by 2 × 5 min washes in TBS supplemented with 0.05% v/v Tween 20 (TTBS). Blots were then incubated with either anti-ACTIVE MAPK pAb (1:1000 dilution), anti-phospho-Akt (Ser 473) pAb (1:1000), anti-ERK1/ERK2 pAb (1:1000), anti-Akt pAb (1:1000), anti-Nrf-2 pAb (1:1000) or anti-Keap-1 pAb (1:1000) in TTBS containing 1% w/v skimmed milk powder (antibody buffer) overnight at room temperature on a 3-D rocking table. The blots were washed 2 × 10 min in TTBS and incubated with goat anti-rabbit IgG conjugated to horseradish peroxidase (HRP) (1:1000 dilution) for 60 min. Finally blots were washed 2 × 10 min in TTBS rinsed in TBS and exposed to ECL-reagent® for 1–2 min and developed. Bands were analysed using Bio-Rad Quantity One 1-D Analysis software (Hemel Hempstead, UK). Molecular weights of the bands were calculated from comparison with pre-stained molecular weight markers (MW 10 000–250 000) from Bio-Rad that were run in parallel with the samples. The equal loading and efficient transfer of proteins was confirmed by staining the nitrocellulose with Ponceau Red solution (Sigma).

2.4 Preparation of nuclear and cytoplasmic fractions

Preparation of nuclear and cytoplasmic fractions were performed following a method previously described [26] but with some modifications. Briefly, primary cortical neurons were cultured in 60-mm dishes in the absence or presence of SFN 100 nM for 3 h. Cells were gently washed with ice-cold PBS and lysed on ice using a buffer composed of 10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT and 0.5% v/v Nonidet P40 Alternative (Calbiochem) containing mammalian protease inhibitors (Sigma), 50 mM sodium fluoride, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM sodium ortho-vanadate, 5 mM sodium pyrophosphate, 10 μg/mL leupeptin, 2 μg/mL pepstatin A and 1 μg/mL chymostatin. The lysed cells were scraped and left on ice to solubilize for 5 min. The lysates were then centrifuged at 1000 × *g* for 5 min at 4°C and at 2000 × *g* for a further minute. The supernatant containing the cytoplasmic fraction was taken off and the remaining pellet was resuspended in 100 μL of a buffer comprising 20 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 420 mM NaCl, 0.5 mM DTT, 0.2 mM EDTA and 25% v/v glycerol. Samples were homogenized and centrifuged at 15 000 × *g* for 20 min. The supernatant containing the nuclear fraction was retained. Protein concentrations were determined by the BCA assay. Primary antibody against Nrf2 (H300) was obtained from Santa Cruz Biotechnology and used at a dilution of 1:250.

2.5 GSH assay

Total glutathione (GSH+GSSG) levels were determined using the Glutathione Assay Kit from Sigma-Aldrich following the manufacturer's instructions. Briefly, 100 μL of medium was removed from each well and 100 μL of 5% 5-sulfosalicylic acid (SSA) was added. Neuronal cells were washed in PBS, scraped and resuspended in SSA. Samples were then snap-frozen in liquid N_2 and defrosted at 37°C before being centrifuged at 15 000 × *g*. A volume of 10 μL of each supernatant and the relevant GSH standard solutions were transferred to a clear 96-well plate before the reaction mixture composed of 95 mM potassium phosphate buffer, pH 7.0; 0.95 mM EDTA; 48 μM NADPH; 0.031 mg/mL DTNB; 0.115 U/mL GR and 0.24% SSA was added. Levels of total glutathione (GSH+GSSG) were detected using a plate reader set to 412 nm with kinetic read at 1 min intervals for 10 min.

2.6 Measurement of NQO1 and GST isoenzymes activities

Antibodies against the murine NAD(P)H:quinone oxidoreductase 1 (NQO1) and GSTs isoenzymes from the Alpha, Mu and Pi classes were a gift from Professor John D. Hayes (Biomedical Research Centre University of Dundee). Sera for the specific murine NQO1 and the GST subunits A1, A3, M1, M3, M5 and P1 were obtained as described previously [27–29]. Following exposures either to SFN (100 nM) or to CysDA (100 μM), neurons were processed as described above. The blots were probed with the different sera at a concentration of 1:1000 and the reacting primary antibodies were located with an anti-rabbit-HRP-conjugated secondary antibody (Sigma).

2.7 Measurement of antioxidant enzyme activity in cells

To determine the ability of SFN to improve the activity of antioxidant cellular enzymes when challenged with CysDA, cell lysates were prepared as follows. Following exposures, neurons were washed with ice-cold D-PBS and lysed on ice

by using CelLytic M containing mammalian protease inhibitor cocktail (1:100 dilution). Lysed cells were scraped and left on ice to solubilize for 15 min. Lysates were centrifuged at 16 000 × *g* for 15 min at 4°C to remove unbroken cell debris and nuclei. Protein concentrations in the supernatants were determined by the BCA assay (Thermo Fisher Scientific). Total cellular GST activity was measured spectrophotometrically by the method of Habig [30]. Briefly, cell lysates (10 μL) were mixed with 990 μL of 100 mM phosphate buffer pH 6.5 containing 1 mM EDTA, 2 mM GSH, 2 mM 1-chloro-2,4-dinitrobenzene. Absorbance was measured at 340 nm at 30 s intervals over 5 min. GST activity is expressed as nmol × mg *per* protein *per* min. The activity of TR was measured by the reduction of DTNB into 5′-thionitrobenzoic acid (TNB) as described previously [31]. Briefly, 10 μL of the cell lysate was added to 990 μL of reaction mixture composed of 0.25 mM DTNB, 0.24 mM NADPH, 10 mM EDTA, and 100 mM phosphate buffer pH 7.5. The conversion of DTNB into TNB was monitored at 412 nm at 10 s intervals over 1 min. Since several enzymes can reduce DTNB, a specific TR inhibitor was used to determine the reduction of DTNB due only to TR activity. TR activity is expressed as mU × mg *per* protein. One unit of TR causes an increase in A412 of 1.0 *per* min *per* mL (when measured in a non-coupled assay containing DTNB alone) at the condition of pH 7.0 and 25°C. GR activity was measured as described previously [32]. Cell lysates (10 μL) were mixed with 990 μL of 100 mM phosphate buffer, pH 7.5, containing 1 mM EDTA, 2 mM NADPH and 2 mM GSSG. The rate of disappearance of NADPH was monitored at 340 nm for 1 min at 25°C. GR activity is expressed as mU × mg *per* protein. One unit of enzyme activity is defined as the amount of enzyme that causes the oxidation of 1.0 μmol of NADPH at 25°C at pH 7.5.

2.8 Statistical analysis

All results are expressed as means ± SD of three separate experiments unless otherwise stated. The statistical evaluation of the results was performed by one-way analysis of variance (ANOVA) followed by a Bonferroni's multiple comparison test using GraphPad InStat version 3.05 (GraphPad Software, San Diego, CA, USA). Significance was defined as $p < 0.05$. Significant changes are indicated as follows: $^{***}p < 0.001$; $^{**}p < 0.01$; $^{*}p < 0.05$ and $a = p < 0.001$; $b = p < 0.01$; $c = p < 0.05$.

3 Results

3.1 SFN protects against CysDA-induced cell death

The toxicity of CysDA (0–500 μM; 24 h) towards primary cortical neurons was assessed with an approximate 50% loss in cell viability observed at 100 μM (Fig. 1A). Control experiments indicated that SFN was not toxic at concentrations up to 10 μM (data not shown). To investigate the protective effect of SFN (0.01–1 μM), neurons were pretreated with SFN (0.01–1 μM) for 24 h, prior to addition of CysDA (100 μM) (Fig. 1B). The protection afforded by SFN was observed to peak at a concentration of 100 nM (30.6 ± 1.7%; $p < 0.001$) with both higher and lower concentrations providing a lower but significant level of protection. SFN (10 μM) did not show any significant protection.

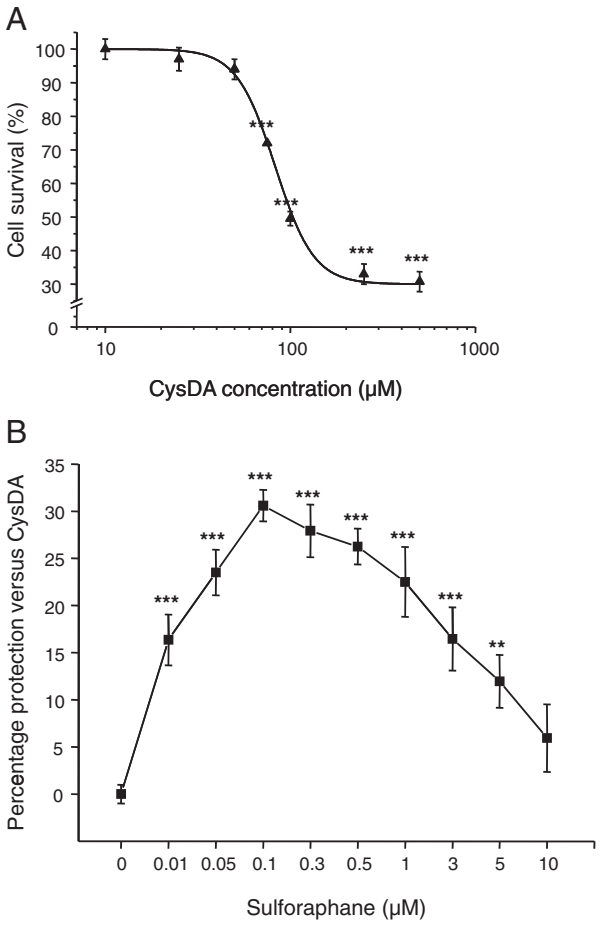

Figure 1. (A) CysDA induces toxicity on primary cortical neurons. 5–6 days *in vitro* primary cortical neurons were exposed to CysDA (10–500 μM) or vehicle for 24 h. After 24 h, cell viability was determined by Alamar blue reduction. (B) Protective effects of SFN against CysDA-induced neuronal injury. Following pretreatment with SFN (0.01–10 μM) for 24 h, primary cortical neurons were exposed to CysDA (100 μM) for an extra 24 h before assessment of viability by the Alamar Blue test. Results are expressed as mean ± SD of quadruplicate wells from single experiments repeated three times with similar results. $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$ indicate significant decrease in cortical neuron viability as analysed by one-way ANOVA followed by Bonferroni's multiple comparison test.

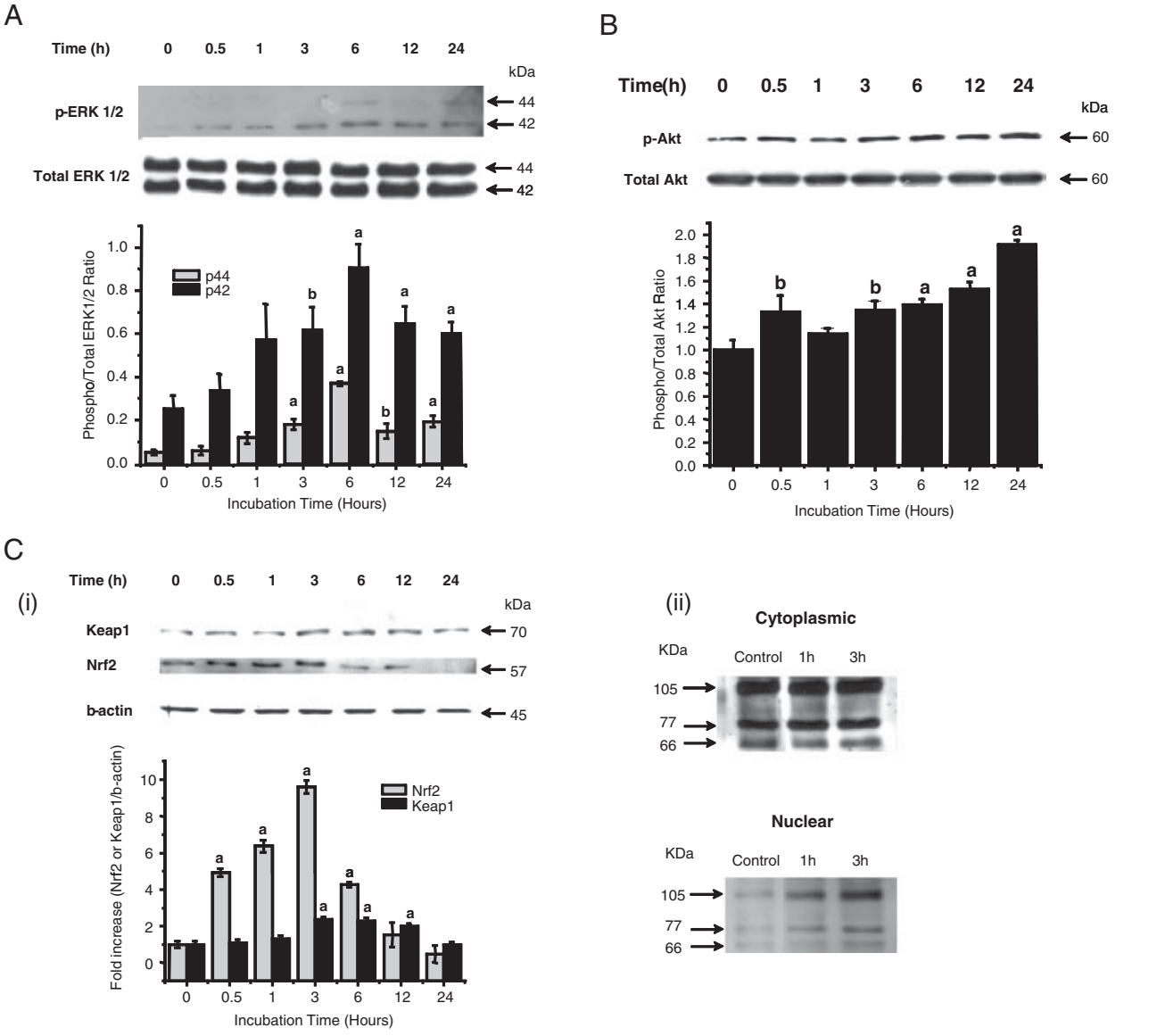

Figure 2. Time-course activation of ERK1/2, Akt/PKB, Nrf2 and Keap1 in primary cortical neurons. (A) Western blot time-course analysis of ERK1/2 phosphorylation in cortical neurons. Proteins were extracted at the indicated time points following SFN (0.1 μM) treatment. Crude homogenates (30 μg) were immunoblotted with antibodies that detect endogenous levels of ERK1/2 MAP kinase only when activated by phosphorylation at Thr 202 and Tyr 204. (B) Western blot time-course analysis of Akt/PKB phosphorylation in cortical neurons. Proteins were extracted at the indicated time points following SFN (0.1 μM) treatment. Crude homogenates (30 μg) were immunoblotted with antibodies that detect endogenous levels of Akt/PKB only when activated by phosphorylation at Ser^{473} (C) (i) Western blot time-course analysis of Nrf2 and Keap1 activation in cortical neurons. Proteins were extracted at the indicated time points following SFN (0.1 μM) treatment. Crude homogenates (30 μg) were immunoblotted with antibodies that detect endogenous levels of total Nrf2 and Keap1 proteins. (ii) Cytoplasmic and nuclear lysates were run on polyacrylamide gels and blotted for Nrf2 (Santa Cruz; H300). Three strongly immunoreactive bands all increased in intensity in the nuclear fraction of primary neurons. Data obtained from immunoblot experiments were analysed using Bio-Rad Quantity One 1-D Analysis software. Each column represents the mean ± SD of three independent experiments. $a = p < 0.001$; $b = p < 0.01$; $c = p < 0.05$ represent significant increase relative to vehicle-treated cells.

3.2 SFN stimulates Akt and ERK phosphorylation and activates the Nrf2 pathway

Experiments aimed at studying the effect of SFN on cortical neurons were undertaken in order to assess the time course of activation of ERK, Akt, Nrf2 and Kelch-like ECH-associated protein 1 (Keap1). To investigate the phosphorylation state of ERK1/2 and Akt/PKB, phospho-specific antibodies were employed that recognize the dually phosphorylated motif pTEpY within activated ERK1/2, and the phosphory-

lated Ser^{473} of Akt/PKB respectively. Incubation of cortical neurons with SFN (100 nM) at different times (0–24 h) revealed an increase in phosphorylation states above basal levels in two bands corresponding to phospho-ERK1 (44 kDa) and phospho-ERK2 (42 kDa) (Fig. 2A) and phospho-Akt (Ser^{473}) (Fig. 2B). Indeed, levels of p44 and p42 extracellular signal-regulated kinases (ERK1/2) were found to be rapidly modified, reaching significant activation after 3 h of SFN 100 nM treatment. In contrast to ERK1/2, active Akt levels were observed to be rapidly elevated but reached a maximum after 24 h (Fig. 2B). Parallel blots were run and probed with antibodies that detected total levels of ERK1/2 and total levels of Akt/PKB, demonstrating no modification in the total amount of proteins and an equal amount of loading. Assessment of the other MAPKs (*i.e.* JNK and p38) did not reveal any modification in their phosphorylation state under these experimental conditions (data not shown).

The release of Nrf2 from Keap1 following SFN treatment was closely correlated with ERK1/2 activation, although Nrf2 released prior to ERK1/2 activation (Fig. 2Ci). To strengthen the evidence linking isothiocyanate to Nrf2 activation and translocation, we sought to determine the effect of SFN on the cellular localization of Nrf2 by analysing both cytoplasmic and nuclear fractions by immunoblotting (Fig. 2Cii). The antibody used to probe Nrf2 proteins detected three immunoreactive bands in both cytoplasmic and nuclear fractions. The intensity of all the three bands (105, 77 and 66 kDa) increased in the nuclear fraction after 1 and 3 h, but not in the cytoplasmic fraction, suggesting that Nrf2 is targeted to the nucleus in presence of SFN (Fig. 2Cii).

CysDA (100 μM; 24 h) caused a significant decrease in the phosphorylation state of ERK1/2 (Fig. 3A), and Akt/PKB (Fig. 3B). However, pre-treating the cells with SFN (0.05–0.5 μM), protected against these reductions and indeed led to an increase in the phosphorylation ratio of both ERK1/2 and Akt/PKB above basal levels, with the maximum of activation being reached at 100 nM (Figs. 3A and B). To determine whether these kinases are involved in SFN-driven cytoprotection, the pharmacological inhibitors of MEK–ERK1/2 (U0126 and PD98059) and phosphoinositide 3-kinase (PI3-kinase)–Akt signalling (wortmannin and LY294002) were used. Pre-treating the primary neurons with U0126, wortmannin and LY294002 effectively inhibited the protection afforded by SFN against CysDA toxicity (Fig. 3C).

Inhibiting MEK-ERK1/2 with U0126 (10 μM) decreased the protection afforded by SFN by $22 \pm 9\%$ ($p < 0.001$). Such inhibition was not observed when the neurons were pre-treated with PD98059 (50 μM). Inhibiting PI3-kinase activity with wortmannin and LY294002 was observed to decrease the protection afforded by SFN by 17.9 ± 2 and $46.5 \pm 5\%$, respectively. Inhibition of the PI3-kinase pathway appeared to be more effective in the presence of LY294002 than wortmannin.

3.3 SFN activates GST, GR, TR and NAD(P)H oxidoreductase 1 enzymes in cultured neurons

Since TR, GST, GR and NAD(P)H oxidoreductase 1 (NQO1) enzymes interact with a whole range of important cellular pathways and play a crucial role in the detoxification of reactive oxygen species and electrophiles, we investigated the ability of SFN to modulate their activities in neurons. CysDA did not show any ability to modulate the activities of the four enzymes (Figs 4A–C and 5A). However, SFN was

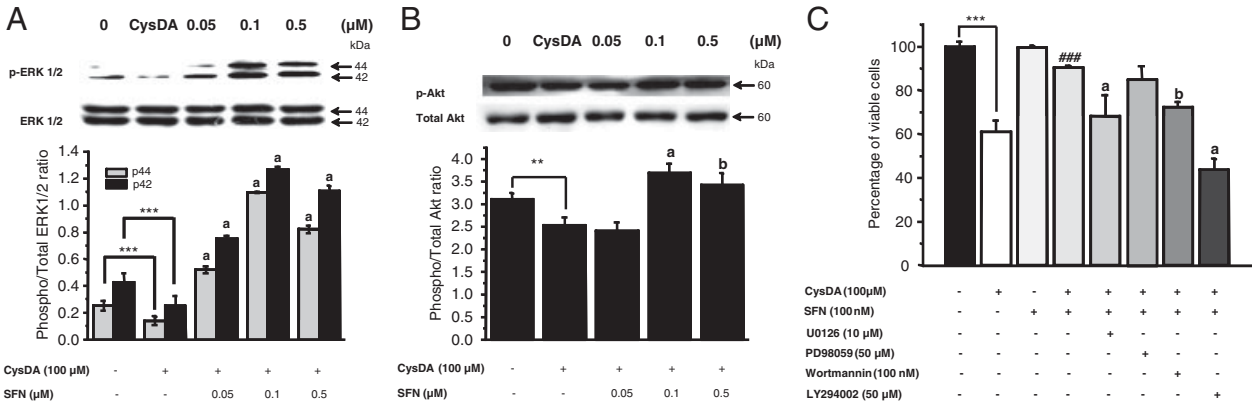

Figure 3. Protection of primary cortical neurons by SFN is mediated through ERK1/2 and Akt/PKB activation. Crude homogenates (30 μg) prepared from primary cortical neurons exposed to vehicle (DMSO), CysDA (100 μM) or SFN (0.05–0.5 μM, 24 h pre-treatment)+CysDA (100 μM; 24 h) were immunoblotted with an antibody that recognizes (A) the dually phosphorylated region of the active form of ERK1 and ERK2 (pERK1/2), or (B) an antibody that specifically recognizes phosphorylated Akt/PKB (pAkt). Data obtained from immunoblots were analysed using Bio-Rad Quantity One 1-D Analysis software. Values are presented as means ± SD from four independent experiments. Data were analysed by one-way ANOVA followed by Bonferroni's multiple comparison test. $a = p < 0.001$ and $b = p < 0.01$ with respect to control, $^{***}p < 0.001$ and $^{**}p < 0.01$ with respect to CysDA. (C) Effect of the MAPK and PI3-kinase pharmacological inhibitors on the cytoprotection afforded by SFN. Primary cortical neurons were treated with either SFN 100 nM or CysDA 100 μM in the presence or absence of pharmacological inhibitors of MEK-ERK1/2 (U0126, 10 μM and PD98059, 50 μM) and PI3-kinase/Akt (LY294002, 50 μM and wortmannin, 100 nM) for 24 h. The assessment of the cytoprotection was conducted as described in Section 2.

observed to significantly increase GR, GST, TR and NQO1 activities both in the absence and presence of CysDA.

Having demonstrated induction of the GST by SFN, cortical neurons were probed with sera raised against the Alpha, Mu and Pi classes of GST. Treatment of the neurons with CysDA (100 μM, 24 h) did not affect the activity of the GST subunits, although a small activation was observed with the GST M5 isoform (Fig. 5B). However, treating the neurons with SFN (100 nM, 24 h) induced the activation of the GST enzymes Mu class, with the GST M1 isoform being the most regulated (Fig. 5B). No modulation was observed with the GST P1 isoenzyme. The Alpha classes (GST A1 and A3) were found difficult to analyse due to their low constitutive expression in the brain. Total intracellular levels of GSH (GSH and GSSG) were assessed after treatment either with SFN or CysDA for 6 h. Treatment with CysDA (100 μM) decreased the levels of total GSH by 20-fold ($p<0.001$). However, pre-treatment of cortical neurons with SFN 100 nM 24 h before CysDA adjunction completely restored total GSH levels (Fig. 5C). Extracellular levels of GSH were low and were not altered by treatments (not shown)

4 Discussion

Recently, the neuroprotective properties of a host of phytochemical components have been identified [9, 13, 14, 33]. Increasingly, the beneficial effects of these dietary-derived

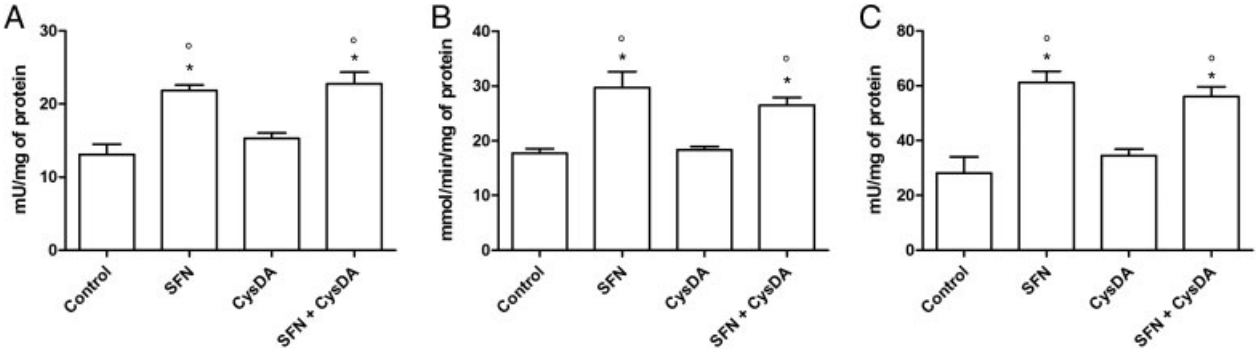

Figure 4. (A) Effect of SFN treatment on GR activity in neurons. Cells were treated with 0.1 μM SFN and after 24 h were exposed to 100 μM CysDA for 24 h. GR activity was measured as described in Section 2. (B) Effect of SFN treatment on GST activity in neurons. Cells were treated with 0.1 μM SFN and after 24 h were exposed to 100 μM CysDA for 24 h. GST activity was measured as described in Section 2. (C) Effect of SFN treatment on TRX activity in neurons. Cells were treated with 0.1 μM SFN and after 24 h were exposed to 100 μM CysDA for 24 h. TRX activity was measured as described under in Section 2. Values are presented as means ± SEM from four independent experiments. Data were analysed by one-way ANOVA followed by Bonferroni's multiple comparison test. $^{*}p<0.05$ with respect to control, $^{\circ}p<0.05$ with respect to CysDA.

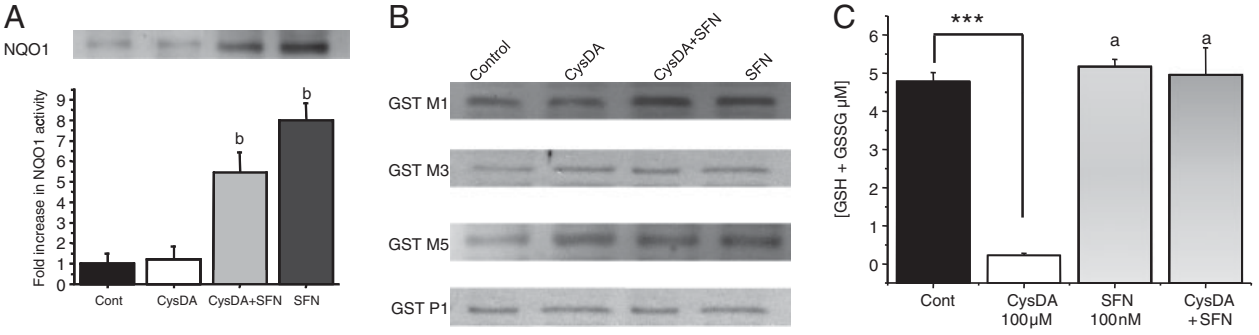

Figure 5. Regulation of NQO1 (A) and GST isoforms (B) in neurons. Level expression of NQO1 and class Alpha, Mu and Pi GST subunits were examined in primary neurons by Western blotting. Crude homogenates (30 μg) were immunoblotted with antibodies that detect endogenous levels of NQO1 and GST A1, A3, M1, M3, M5 and P1. Cross reacting primary antibodies were located with a HRP-labelled secondary antibody against rabbit IgG and the complexes made visible using enhanced chemiluminescence. The identity of the immunoreactive GST subunits and NQO1 is shown in the left-hand margin. (C) SFN protects neuronal cells against CysDA induced toxicity by restoring GSH levels. Levels of total GSH/GSSG were assessed by using a Glutathione assay kit from Sigma-Aldrich. SFN protected neuronal cells against CysDA induced toxicity by restoring GSH levels. Values are presented as means ± SD from three independent experiments. Data were analysed by one-way ANOVA followed by Bonferroni's multiple comparison test. $a = p<0.001$, $b = p<0.01$ with respect to control; $^{***}p<0.001$ with respect to CysDA.

compounds have been shown to be dependent on their ability to regulate intracellular signalling pathways, such as the MAP kinase cascade [10, 34, 35]. We show that SFN, an aliphatic isothiocyanate, derived from cruciferous vegetables [15] protects neurons against neuronal injury induced by the endogenous neurotoxin CysDA *via* the activation of pro-survival neuronal signalling. 5-*S*-Cysteinyl conjugates of catecholamines, in particular CysDA and its derivative, 7-(2-aminoethyl)-3,4-dihydro-5-hydroxy-2*H*-1,4-benzothiazine-3-carboxylic acid [36], have been shown to be strongly neurotoxic [3, 8, 37, 38] and have been linked to the underlying neurodegeneration observed in PD. Such species have been reported in the human brain [6, 39] and may be generated by other reactive species, such as peroxynitrite [4]. We found that the protection exerted by SFN against this neurotoxin was linked to an activation of ERK1/2, an associated release of Nrf2 from Keap1 and a subsequent increase in the expression and activity of specific detoxifying phase I and II enzymes.

These effects are in agreement with previous studies which have reported the ability of SFN to induce several classes of genes implicated in detoxifying reactive oxygen species and electrophiles [40]. Furthermore, SFN has also been shown to protect against both hydrogen peroxide and glutamate neurotoxicity *via* an activation of the Nrf-2 pathway [19]. The transcription factor NF-E2-related factor-2 (Nrf2), which binds to the ARE with high affinity, plays a central role in the upregulation of genes implicated in the regulation of the cellular redox status and the protection of the cell from oxidative insult and has been observed to be upregulated following SFN exposure [19, 41]. Under basal conditions, Nrf2 interacts with a cytosolic repressor protein Keap1 limiting Nrf2-mediated gene expression [42]. In cells exposed to oxidative stress, Nrf2 is released from Keap1 and translocates to the nucleus, where it activates ARE-dependent transcription of phase II and antioxidant defence enzymes, such as GST, GSH peroxidase and heme oxygenase-1 [43]. GST are a complex multi-gene family of isoenzymes comprising at least four distinct classes of protein designated Alpha, Mu, Pi and microsomal GST, which are involved in the biotransformation of a wide spectrum of endogenous and xenobiotic electrophiles [44]. In our study we observed that GST M1, M3 and M5 were activated in the presence of SFN, whereas GST P1 was not. This agrees with previous observations where primary cortical neurons were challenged with either mitochondrial complex I inhibitors or increased concentrations of intracellular calcium [45].

Protection afforded by SFN against this endogenous neurotoxin was observed to occur in the nanomolar to low micromolar range. However, the potential bioactivity of such a compound may be restricted to the extent of their metabolism and by processes occurring at the blood–brain barrier. Although assessment of precise brain concentrations of SFN have proved difficult, previous studies have reported high levels (mM) of cellular SFN accumulation in mammalian cells and appreciable levels of SFN and its metabolites are known to be excreted in the urine of humans who consume broccoli [46–48]. Following oral administration, SFN and its metabolites reach peak plasma concentrations of 0.80 μM after 1 h indicating a rapid absorption, compatible with its small molecular size [49, 50]. In addition, systemically administered SFN has been shown to reduce infarct volume following focal cerebral ischaemia in rodents and increase mRNA and protein levels of the Nrf2-responsive gene haem oxygenase 1 (HO-1), suggesting that it may enter the brain and exert biological activity [51]. We observed that low nanomolar concentrations of SFN are capable of activating Nrf-2 and subsequent increased expression of the enzymes GST, GR, TR and NAD(P)H oxidoreductase 1. Our data also suggest that the release of Nrf2 by SFN may also involve the MAPK (ERK1/2) pathway, in particular ERK2, as inhibition of the MEK-ERK1/2 pathway with pharmacological inhibitors reduced the protective effects of SFN. Moreover, the use of inhibitors of the PI3-kinase pathway showed strong reduction in SFN-driven protection. Collectively, these data suggest that protection afforded by SFN is mediated through a MAPK and PI3-kinase-dependent signalling. Previous studies investigating the involvement of the extracellular regulated kinase (ERK) pathway contribution to the ARE-driven gene expression reported that the ARE reporter gene activity stimulated by the mono-functional inducers *ter*-butylhydroquinone and the isothiocyanate SFN was mediated at least in part by ERK2 in human and mouse hepatoma cell lines. These compounds were found to cause an increase in the phosphorylation of ERK2 in hepatoma cells, and the MEK1 inhibitor PD98059 could attenuate this activation [52]. Although we have not demonstrated direct modulation or implication of Nrf2 by either ERK or Akt, previous experiments have demonstrated an implication of such pathways by using either luciferase reporter assays [19, 26] or by immunocytochemistry [53]. For example, the pharmacological inhibitors of MEK and PI3-kinase/Akt, which are upstream kinases responsible for phosphorylation of ERK1/2 and Akt, attenuated the nuclear localization of Nrf2 induced by EGCG in MCF10A cells [53]. To date, three major signal transduction pathways have been implicated in regulation of the ARE, which include those mediated by the MAPK cascades, PI3-kinase, and PKC [38]. It is possible, however, that all of these signalling pathways may play some role in regulating the ARE because there is the capacity for cross-talk to occur between these kinases. For example, it has been demonstrated that PI3-kinase can activate PKC isoenzymes [54], and in turn that PKC isoenzymes can activate the MAPK cascades [55, 56]. Since kinases have been implicated in the transduction of signals in neurodegenerative disorders, such as Alzheimer's disease or PD [57, 58], it is of interest to design new drugs able to downregulate pro-apoptotic proteins and enhance pathways triggering neuronal survival through the activation of phase II detoxification enzymes.

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