

# Nrf2 Activators Provide Neuroprotection Against 6-Hydroxydopamine Toxicity in Rat Organotypic Nigrostriatal Cocultures

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Oxidative stress and inflammation appear to play a critical role in the progression of Parkinson's disease. As a result, there has been growing interest in antioxidant pathways and how these pathways might be exploited to slow the progressive loss of dopamine neurons. One such pathway that has garnered attention recently is mediated by the transcription factor Nrf2 and is integral in orchestrating cells' antiinflammatory defense. Nrf2 controls the inducible expression of numerous antioxidant and phase 2 detoxification genes, such as glutathione S-transferase, heme oxygenase-1, and NAD(P)H:quinone oxidoreductase 1 (NQO1). Once activated, these genes work synergistically to maintain intracellular redox homeostasis. In this study, we test the hypothesis that Nrf2 activation can protect dopaminergic neurons against 6-hydroxydopamine (6-OHDA)-induced toxicity. Treatment of organotypic nigrostriatal cocultures with either tert-butylhydroquinone (tBHQ) or sulforaphane, known activators of Nrf2, mitigated dopaminergic cell loss. The observed protection appeared to be mediated, at least in part, by an increase in antioxidant activity. Simultaneous treatment of cultures with tBHQ and 6-OHDA increased NQO1 expression 17-fold compared with controls. Overall, these results suggest that Nrf2 may play an important role in cellular protection in neurodegenerative diseases and may be a viable therapeutic target in the future. © 2009 Wiley-Liss, Inc.

**Key words:** Nrf2; Parkinson's disease; oxidative stress; neuroprotection; organotypic

Parkinson's disease (PD) is a common neurodegenerative disorder characterized by a progressive loss of dopaminergic neurons in the substantia nigra and subsequent loss of dopaminergic input into the striatum. Breakdown of dopaminergic signaling within the nigrostriatal pathway is believed to underlie many of the motor symptoms associated with PD, such as bradykinesia, muscular rigidity, postural instability, and tremor. Approximately 90% of cases of PD are idiopathic, although some have been linked to known genetic

mutations. The etiology of PD is currently not well understood, although it has been suggested that oxidative stress and inflammation play an important role in its progression. Tissue from PD patients shows abundant evidence of oxidative damage, including increased lipid peroxidation, DNA fragmentation, and protein oxidation (Dexter et al., 1986, 1989; Alam et al., 1997a,b; Floor and Wetzel, 1998; Castellani et al., 2002). Additionally, the presence of activated microglia in the substantia nigra, decreased mitochondrial complex I function, and an increased presence of inflammatory markers and antioxidant enzyme activity supports the role of oxidative stress in mediating dopaminergic neurodegeneration (McGeer et al., 1988; Saggiu et al., 1989; Schapira et al., 1990; Schapira, 1993; Sian et al., 1994; Wullner and Klockgether, 2003).

One mechanism that cells use to neutralize oxidative stress is activation of the basic leucine zipper transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2; Moi et al., 1994). The presence of reactive oxygen and nitrogen species (ROS/RNS) or xenobiotics releases Nrf2 from its actin-bound negative regulator, Keap1 (Itoh et al., 1999; Kang et al., 2004). This allows Nrf2 to translocate to the nucleus, heterodimerize with accessory proteins such as small Maf proteins and Jun, and bind to genes containing an antioxidant response element (ARE) in their promoter (Itoh et al., 1995; Venugopal and Jaiswal, 1998). In turn, several genes that promote cell survival and repair, such as glutathione S-transferase

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(GST), heme oxygenase-1 (HO-1), and NAD(P)H:quinone oxidoreductase 1 (NQO1) are up-regulated.

Both in vitro and in vivo studies have shown that increases in Nrf2 activity can be useful in combating oxidative stress and preventing cell death. Induction of the Nrf2-ARE pathway by *tert*-butylhydroquinone (tBHQ) protects neuronal cell lines and primary cortical cultures from oxidative stress induced by H<sub>2</sub>O<sub>2</sub>, glutamate, dopamine, 6-hydroxydopamine (6-OHDA), and 3-morpholinylsindnonimine (SIN-1; Murphy et al., 1991; Duffy et al., 1998; Hara et al., 2003; Kraft et al., 2004; Cao et al., 2005; Jakel et al., 2005). Additionally, activation of this pathway by sulforaphane, a potent antioxidant found in cruciferous vegetables, has been shown to protect primary cortical cultures against H<sub>2</sub>O<sub>2</sub> and glutamate toxicity (Kraft et al., 2004). Evidence that supports a neuroprotective role for Nrf2 has also been shown when either Nrf2 or Keap1 was overexpressed or knocked out in neuronal cell lines, primary cortical cultures, or mice or when these cells were transfected with a dominant negative form of Nrf2 (Lee et al., 2003; Kraft et al., 2004; Cao et al., 2005; Shih et al., 2005).

Nrf2's ability to mediate a global antioxidant response makes it an interesting therapeutic target for neurodegenerative diseases, such as PD, in which cells are in a chronic state of oxidative stress. In this study, we examined the role of Nrf2 in rescuing dopaminergic neurons from 6-OHDA-induced toxicity in nigrostriatal organotypic cocultures. We found that treatment of organotypic nigrostriatal cocultures with tBHQ or sulforaphane protected against loss of tyrosine hydroxylase immunoreactivity and that simultaneous treatment of cocultures with tBHQ and 6-OHDA significantly increased NQO1 mRNA expression. These results suggest that activation of Nrf2 can protect dopaminergic neurons in a unique model of PD.

## MATERIALS AND METHODS

### Materials

tBHQ and di-*tert*-butylhydroquinone (dtBHQ) were purchased from Fluka-Chemika (Buchs, Switzerland). All other chemicals were purchased from Sigma Chemical Company (St. Louis, MO) unless specifically noted otherwise. tBHQ, dtBHQ, and sulforaphane were dissolved in dimethyl sulfoxide (DMSO; 1:10,000, 1:10,000, and 1:4,000, respectively), with appropriate DMSO vehicle controls.

### Organotypic Nigrostriatal Cocultures

All procedures were approved by the Institutional Animal Care and Use Committee at the University of Maryland School of Medicine, and animals were treated in accordance with the PHS Policy on Humane Care and Use of Laboratory Animals, the National Institutes of Health *Guide for the care and use of laboratory animals*, and the Animal Welfare Act (7 U.S.C. et seq.). Organotypic nigrostriatal cocultures were prepared by using a variation of the technique developed by Gahwiler (1981) and later modified by Plenz and Kitai (1996). Pregnant female Sprague-Dawley rats (Charles River, Wil-

lington, MA) were housed in a light-(12 hr light/12 hr dark) and temperature-controlled environment with food and water ad libitum and monitored for litter delivery. Rat pups 0–2 days old were used for cultures. After rapid decapitation of rat pups, the brains were quickly removed and placed in ice-cold Gey's balanced salt solution with glucose. Coronal sections (500  $\mu$ m) were cut using a vibratome (Vibratome Company, St. Louis, MO), and then the substantia nigra and striatum were carefully dissected from each section with the aid of a stereomicroscope. Single nigral and striatal tissue sections were then paired and attached to glass coverslips coated with poly-D-lysine in a droplet of chicken plasma, which was then coagulated with thrombin (Baxter, Deerfield, IL). Tissue sections were placed  $\sim$ 1 mm from one another. Each coverslip was then placed in a culture tube with semisynthetic media (containing 50% basal medium Eagle, 25% Hanks' balanced salt solution, 25% horse serum, 0.0197% D-glucose, and 0.004% glutamine) and rotated slowly in a roller drum at 35°C in room air and humidity. Media were changed twice per week. At 3 and 27 days in vitro (DIV), mitotic inhibitors (4.4  $\mu$ M cytosine- $\beta$ -D-arabinofuranoside, 4.4  $\mu$ M uridine, and 4.4  $\mu$ M 5-fluorodeoxyuridine) were added to cultures for a period of 24 hr to prevent glial overgrowth. Cultures were on average 26 days old when used in experiments (range 15–48 DIV).

### Cytotoxicity Assay

Viability of dopaminergic neurons was assessed by immunocytochemistry. Cultures were treated with either 100 nM 6-OHDA, 5  $\mu$ M tBHQ, or a combination of 5  $\mu$ M tBHQ and 100 nM 6-OHDA for a period of 16 hr and then stained for tyrosine hydroxylase (TH), the rate-limiting enzyme in dopamine synthesis. We chose to use 5  $\mu$ M tBHQ because this concentration led to a robust elevation in NQO1 while not inappropriately stressing mitochondria in organotypic nigrostriatal cocultures (see under Mitochondrial imaging). To assess cell death, cultures were treated with 15  $\mu$ l propidium iodide (1 mg/ml PI) and incubated for 16 hr, followed by fixation and processing as described below (see under Immunohistochemistry). Experiments with sulforaphane and dtBHQ were conducted in a similar manner (Fig. 1). Cultures used in sulforaphane experiments were treated with 5  $\mu$ M sulforaphane for 48 hr prior to being treated with a combination of 5  $\mu$ M sulforaphane and 100 nM 6-OHDA for 16 hr. Similarly, cultures used in dtBHQ experiments were treated with 50  $\mu$ M dtBHQ for 8 hr prior to being treated with a combination of 50  $\mu$ M dtBHQ and 100 nM 6-OHDA for an additional 16 hr. Two individuals blind to treatment conditions independently performed the cell counts. Slides were masked and randomized prior to counting. Because cultures are slowly rotated, the tissue thins out to a layer that is only one or two cells thick. This allowed all TH<sup>+</sup> or PI<sup>+</sup> somata on the coverslip to be counted without having to sample. Each treatment condition was replicated multiple times within each experiment, and each experiment was repeated multiple times with cultures from different culture dates. All images were taken with a Nikon Eclipse TE300 microscope equipped with a Photometric CoolSnap ES camera (Roper

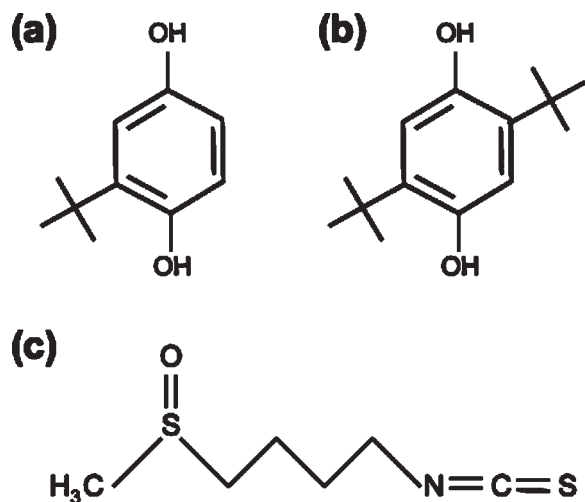


Fig. 1. Chemical structures of tert-butylhydroquinone (tBHQ; **a**), di-tert-butylhydroquinone (dtBHQ; **b**), and sulforaphane (**c**). tBHQ and sulforaphane are both potent activators of Nrf2. dtBHQ is an analog of tBHQ that does not activate the Nrf2-ARE pathway.

Scientific, Ottobrunn, Germany), and montages were assembled in Adobe Photoshop.

#### Immunohistochemistry

Cultures were fixed with 4% paraformaldehyde and then washed and blocked in 0.1 M phosphate-buffered saline (PBS) containing 0.3% Triton X-100, 5% normal goat serum, and 3% H<sub>2</sub>O<sub>2</sub>. Cultures were incubated with mouse anti-TH (1:1,000; Vector, Burlingame, CA) for 24 hr, mouse anti-NeuN (1:1,000; Chemicon, Temecula, CA) for 24 hr, or rabbit anti-nitrotyrosine (1:2,000; Upstate, Lake Placid, NY) for 48 hr. After a second wash, TH and NeuN cultures were incubated with biotinylated goat anti-mouse IgG (1:500; Vector) for 2 hr and then visualized with an ABC Elite Kit and either diaminobenzidine (Sigma) or Vector SG Substrate Kit. Nitrotyrosine cultures were incubated with goat anti-rabbit secondary antibody conjugated with fluorescein for 2 hr. Cultures then underwent a final wash and were mounted onto glass slides with Vectashield Hardmount (Vector) for fluorescent slides or Hydromount (National Diagnostics, Atlanta, GA) for all others. All washes were completed with 0.1 M PBS or distilled H<sub>2</sub>O and all staining steps were performed at room temperature.

#### Nitrotyrosine

Cultures were treated overnight with DMSO (1:10,000), 100 nM 6-OHDA, 5  $\mu$ M tBHQ, or a combination of 5  $\mu$ M tBHQ and 100 nM 6-OHDA, followed by fixation and processing as described above (see under Immunohistochemistry). Nitrotyrosine staining was measured fluorescently. Five random fields within the substantia nigra were imaged at  $\times 20$  (exposure 1,000 msec), and the mean green intensity of each image was determined in SimplePCI software (Hamamatsu Corporation, Sewickly, PA). The values obtained for each slide were then averaged. Slides were masked and

randomized prior to imaging. Each treatment condition was replicated multiple times within each experiment, and each experiment was repeated four times using cultures from different culture dates. All images were taken with a Nikon Eclipse TE300 microscope equipped with a Photometric CoolSnap ES camera (Roper Scientific), and montages were assembled in Adobe Photoshop.

#### Mitochondrial Imaging

NAD(P)H autofluorescence was monitored using brief (2–10 msec) images taken every 10 sec over the course of the experiment. Experiments were conducted in physiological saline solution (120 mM NaCl, 0.4 mM KH<sub>2</sub>PO<sub>4</sub>, 20 mM HEPES, 5 mM NaHCO<sub>3</sub>, 1.2 mM Na<sub>2</sub>SO<sub>4</sub>, 1.3 mM CaCl<sub>2</sub>, 15 mM glucose, 1 mM MgCl<sub>2</sub>) at 37°C.

#### Quantitative RT-PCR

Cultures were treated overnight with DMSO (1:10,000), 100 nM 6-OHDA, 5  $\mu$ M tBHQ, or a combination of 5  $\mu$ M tBHQ and 100 nM 6-OHDA. Tissue was washed with Dulbecco's PBS without calcium and magnesium, and total RNA was isolated from treated cultures using RNA-Bee according to the manufacturer's instructions (Tel-Test, Inc., Friendswood, TX). RNA was reverse transcribed and PCR amplified using iScript One-Step RT-PCR Kit with SYBR green according to the manufacturer's instructions (Bio-Rad, Hercules, CA) using primers for NQO1 (forward 5'-GCCCGGATATTGTAGCTGAA-3' and reverse 5'-GTG GTGATGGAAAGCAAGGT-3') or  $\beta$ -actin (forward 5'-AGC CATGTACGTAGCCATCC-3' and reverse 5'-CTCTCAG CTGTGGTGGTCAA-3'). Both NQO1 and  $\beta$ -actin amplified an  $\sim 200$ -base-pair fragment. The PCR conditions were 94°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec, with a final extension at 72°C for 5 min. NQO1 C<sub>t</sub> value was determined and subtracted from the  $\beta$ -actin C<sub>t</sub> value, and the fold amplification in test samples compared with control samples was calculated using the formula  $2^{-\Delta\Delta C_t}$ .

#### Data Analysis

Results are presented as mean  $\pm$  SEM. Statistical analysis of raw data was performed with JMP (SAS Corp., Cary, NC). Significance of effects was determined by one-way ANOVA followed by Scheffé's F post hoc or by a Kruskal-Wallis test followed by Mann-Whitney U test post hoc, as appropriate. A statistical probability of  $P < 0.05$  was considered significant.

## RESULTS

### Organotypic Model of Dopaminergic Neuronal Death Induced by 6-OHDA

Organotypic cultures of neuronal tissue provide several advantages over classic cell culture, including preservation of local milieu, superior optical properties, and the ability to be maintained in culture for extended periods. To characterize the population of dopaminergic cells in our nigrostriatal cocultures, we stained untreated cultures against TH, the rate-limiting enzyme in dopa-

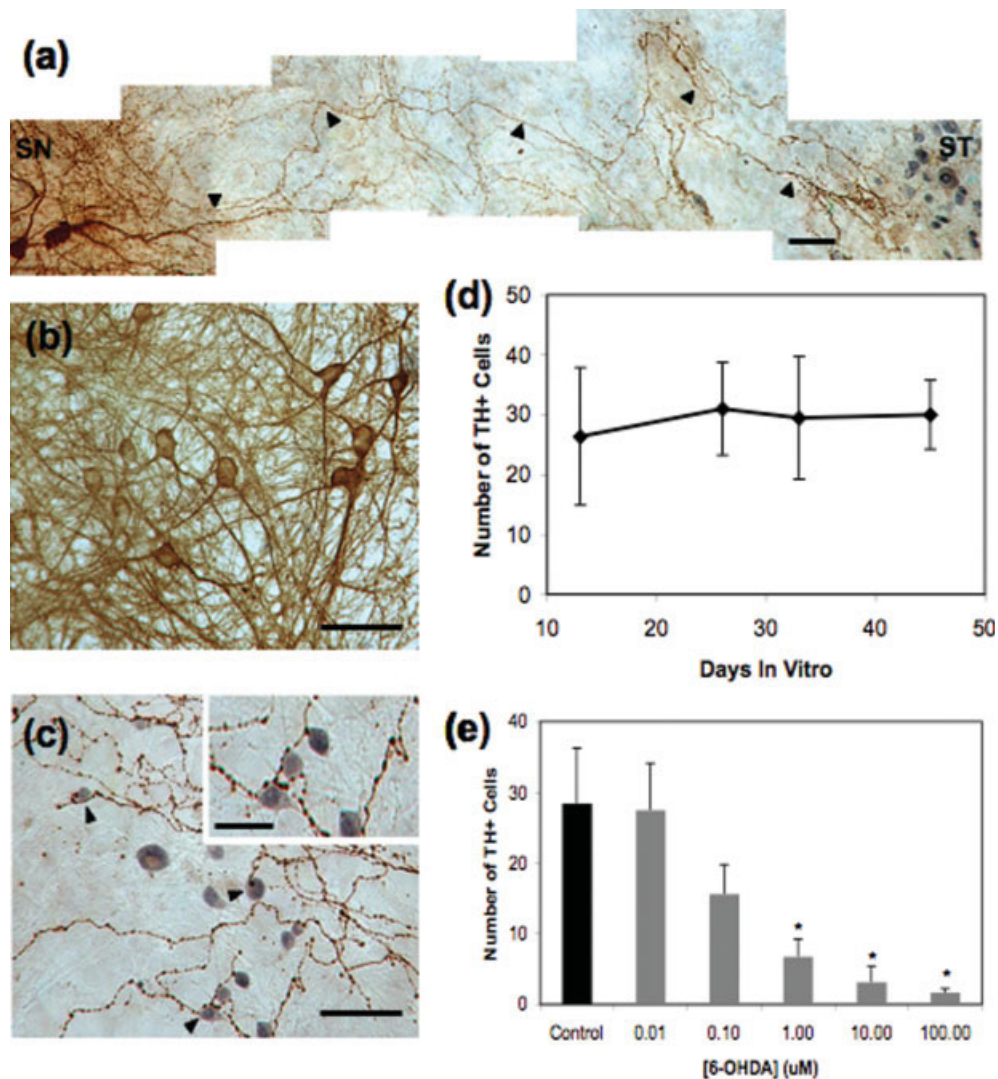


Fig. 2. Establishment of the organotypic nigrostriatal coculture. **a:** Representative image of coculture demonstrating extensive projections extended from dopaminergic neurons in the SN to target cells in the ST. Arrowheads trace the path of a projecting axon. **b:** DA neurons in the SN have large multipolar and fusiform somata with a dense network of projections. **c:** DA neurons in the substantia nigra establish connections with neurons in the ST. Arrowheads indicate

NeuN<sup>+</sup> cells (blue) receiving DA input (TH, brown). **Inset** is a magnified image of TH<sup>+</sup> projections synapsing onto a NeuN<sup>+</sup> cell. **d:** Cocultures maintain a stable number of TH<sup>+</sup> neurons up to 50 DIV (n = 3–5 cultures/time point) and demonstrate a concentration-dependent sensitivity to the PD toxin 6-OHDA (e; n = 3–6 cultures/concentration; \*P < 0.05). Values represent mean ± SEM. Scale bars = 100 μm in a,inset; 200 μm in b,c.

mine synthesis. The TH<sup>+</sup> cells displayed attributes characteristic of nigral dopamine neurons (Grace and Onn, 1989). Cells were multipolar or fusiform and had large, darkly stained somata with diffuse dendritic arborization (Fig. 2B). Further examination revealed an extensive network of TH<sup>+</sup> dendrites and axonal projections that branch out from the substantia nigra to cells in the striatum (Fig. 2A). Dopaminergic projections within the striatum had small varicosities reminiscent of synaptic boutons, and we often found these projections encircling NeuN<sup>+</sup> cells in the striatum (Fig. 2C). Additionally, cultures maintained a stable number of TH<sup>+</sup> cells between 14 and 50 DIV (Fig. 2D).

To characterize our model further, we assessed its response to 6-OHDA. The toxicity of 6-OHDA was evaluated by using immunocytochemistry. Cultures were treated with 0.01, 0.10, 1, 10, or 100 μM 6-OHDA for 16 hr and then labeled for TH immunoreactivity. 6-OHDA was toxic to TH<sup>+</sup> cells in a concentration-dependent manner (Fig. 2E). In subsequent experiments, we chose to treat cultures with 100 nM 6-OHDA because this concentration resulted in only a moderate loss of TH<sup>+</sup> cells, leaving both injured and healthy cells in culture. Qualitatively, injured cells had a slightly reduced somatic size, pruned dendritic branches, and shorter axonal projections but still retained strong TH<sup>+</sup>

staining. Additionally, 6-OHDA toxicity was demonstrated to be specific for dopamine neurons in the substantia nigra at this concentration. Cultures were stained with the general neuronal marker NeuN, and the number of NeuN-positive cells in the striatum was not significantly different in control and 6-OHDA-treated cultures (data not shown).

**TABLE I. Increasing Concentrations of tBHQ Negatively Affect Mitochondria in Organotypic Nigrostriatal Cocultures<sup>†</sup>**

tBHQ	NAD(P)H reduction ( $\Delta F/F$ )
3 $\mu$ M	0.000 $\pm$ 0.000
10 $\mu$ M	0.0252 $\pm$ 0.003*
30 $\mu$ M	0.0760 $\pm$ 0.007*
100 $\mu$ M	0.1109 $\pm$ 0.013*

<sup>†</sup>Values represent mean  $\pm$  SEM (n = 29).

\* $P < 0.05$ .

### Nrf2 Activators Confer Neuroprotection

tBHQ is a known activator of the Nrf2-ARE pathway and can induce a greater than 30-fold increase in ARE activation in primary cortical cultures (Jakel et al., 2005). However, tBHQ can also directly affect mitochondria (Okubo et al., 2003). Thus, to avoid using a concentration of tBHQ that could adversely affect cellular energy metabolism, imaging of NAD(P)H autofluorescence was used to determine the level of tBHQ that disturbs cellular redox state in our culture system (Schuchmann et al., 2001). We found that concentrations greater than 5  $\mu$ M led to a decrease in NAD(P)H autofluorescence, indicative of respiratory uncoupling (Table 1). A concentration of 5  $\mu$ M tBHQ negligibly affected mitochondria and was assessed for Nrf2 activation by measuring NQO1 mRNA expression (via quantitative RT-PCR) in both rat PC12 cell line and organotypic nigrostriatal cocultures. At 5  $\mu$ M, tBHQ elicited a fivefold increase in NQO1 expression, without stressing mitochondria.

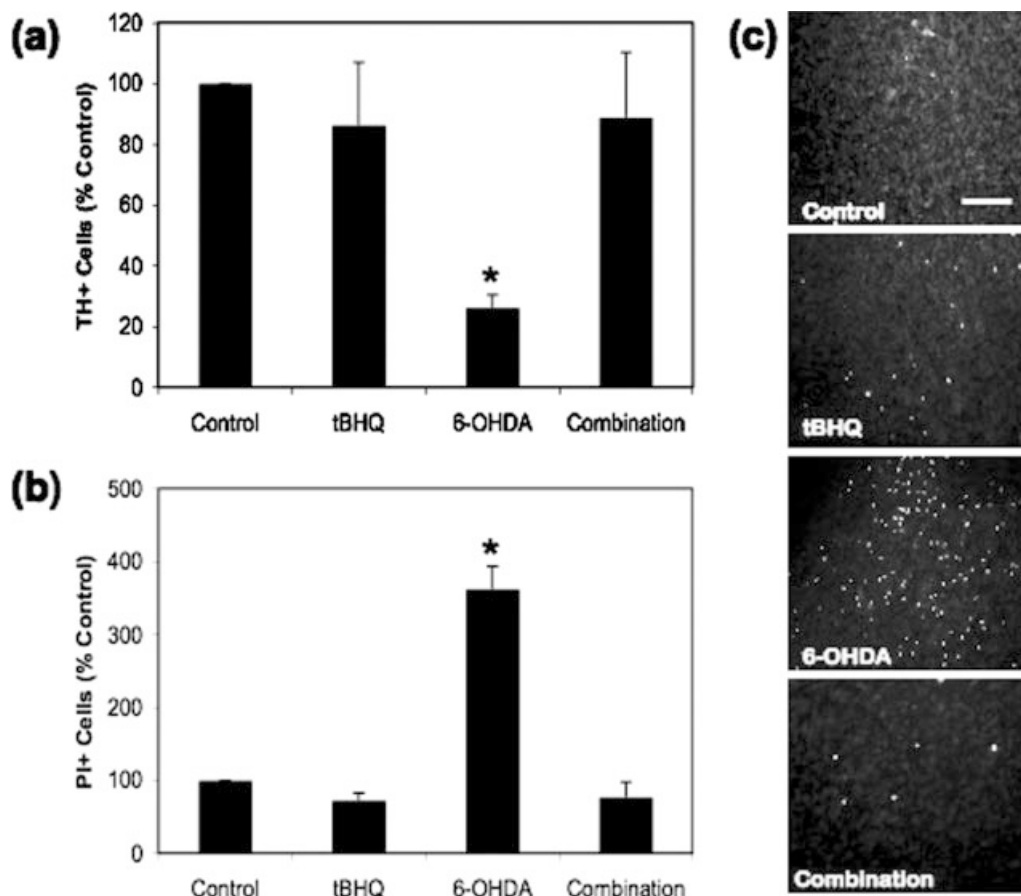


Fig. 3. tBHQ confers protection against 6-OHDA-induced cytotoxicity. **a:** Treatment of cultures with 5  $\mu$ M tBHQ for 16 hr protected TH<sup>+</sup> cells against 6-OHDA-induced toxicity (n = 17–31 cultures/treatment group; \* $P < 0.05$ ). **b:** Similarly, propidium iodide staining was significantly reduced when cultures were treated with tBHQ

(n = 4–6 cultures/treatment group; \* $P < 0.001$ ). **c:** Representative images of propidium iodide staining. Note the dramatic difference in PI<sup>+</sup> staining in cultures treated with 6-OHDA alone compared with cultures treated with both 6-OHDA and tBHQ. Values represent mean  $\pm$  SEM. Scale bar = 400  $\mu$ m.

To determine whether tBHQ was able to protect dopaminergic cells against 6-OHDA-mediated toxicity, nigrostriatal cocultures were treated with 5  $\mu$ M tBHQ, 100 nM 6-OHDA, or a combination of both tBHQ and 6-OHDA overnight. Treatment of cultures with tBHQ conferred protection against 6-OHDA-induced toxicity (Fig. 3A). Cultures treated with a combination of tBHQ and 6-OHDA maintained a comparable number of TH<sup>+</sup> cells compared with control cultures (6-OHDA: 40%  $\pm$  6.9% media control, tBHQ + 6-OHDA: 89%  $\pm$  21.5% DMSO control,  $n = 28$  and 21, respectively;  $P < 0.05$ ). tBHQ and DMSO alone were not significantly toxic at the concentrations used (tBHQ: 86%  $\pm$  20.7% DMSO control, DMSO: 100%  $\pm$  21.0% media control;  $n = 17$  and 10, respectively). To confirm that the loss of TH immunoreactivity observed was due to cell death and not just a loss of TH activity, cell death was assessed with propidium iodide (PI). Cultures treated with 100 nM 6-OHDA alone demonstrated abundant PI staining (362%  $\pm$  31.0% DMSO control,  $n = 4$ ;  $P < 0.05$ ). This increase in cell death was dramatically reduced when cultures were treated with a combination of 5  $\mu$ M tBHQ and 100 nM 6-OHDA (76.7%  $\pm$  20.9% DMSO control,  $n = 6$ ; Fig. 3B). Again, tBHQ and DMSO alone had no effect on cytotoxicity at the concentrations used (tBHQ: 72%  $\pm$  10.3% DMSO control, DMSO: 71%  $\pm$  19.1% media control,  $n = 4$  for both tBHQ and DMSO).

To determine whether the tBHQ-induced neuroprotection was mediated by induction of the Nrf2-ARE pathway, we exposed cultures to either sulforaphane, a known activator of the Nrf2 pathway that is structurally distinct from tBHQ, or dtBHQ, an inactive analog of tBHQ (Fig. 1). Cultures were pretreated with 5  $\mu$ M sulforaphane for 48 hr and then exposed to a combination of 5  $\mu$ M sulforaphane and 100 nM 6-OHDA overnight. Cultures pretreated with sulforaphane showed significant protection against 6-OHDA-induced toxicity, maintaining numbers of TH-immunoreactive cells similar to those of controls (101%  $\pm$  16.9% DMSO control,  $n = 12$ ,  $P < 0.05$ ; Fig. 4A). Sulforaphane and DMSO alone had no significant effect on cell viability at the concentrations used (sulforaphane: 144%  $\pm$  25.7% DMSO control, DMSO: 100%  $\pm$  21.0% media control;  $n = 12$  for both sulforaphane and DMSO). Again, cultures exposed to only 100 nM 6-OHDA had strong PI staining (357%  $\pm$  78.4% control,  $n = 11$ ,  $P < 0.05$ ). This increase in cell death was mitigated when cultures were pretreated with sulforaphane for 48 hr (70.0%  $\pm$  15.7% control,  $n = 11$ ; Fig. 4B). Cultures were also assessed after treatment with dtBHQ, an inactive analog of tBHQ (Jakel et al., 2005). Pretreatment of cultures with 50  $\mu$ M dtBHQ for 8 hr did not confer protection against 6-OHDA-induced toxicity, suggesting that the protection observed with tBHQ and sulforaphane treatment was mediated by activation of the Nrf2-ARE pathway (37%  $\pm$  7.0% DMSO control,  $n = 12$ ; Fig. 5).

Formation of nitrotyrosine conjugates is a hallmark of oxidative damage, particularly in dopaminergic degen-

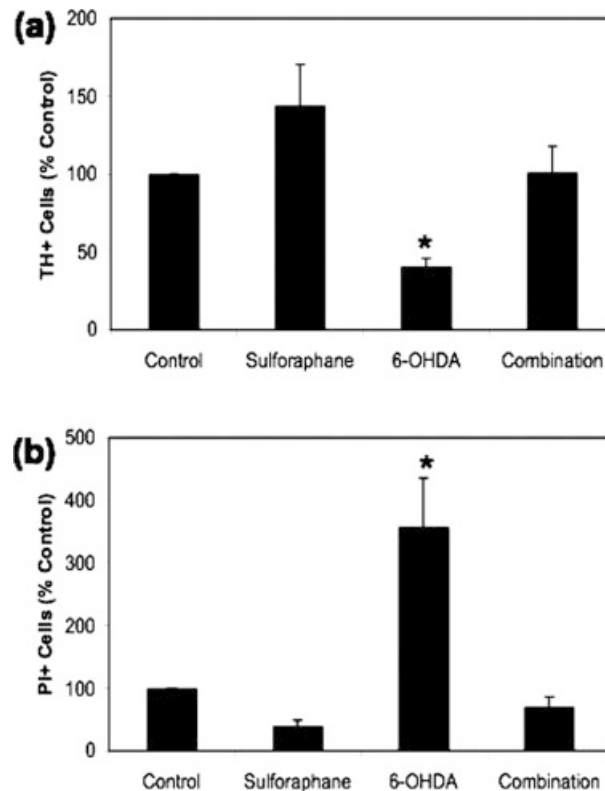


Fig. 4. Sulforaphane confers protection against 6-OHDA-induced cytotoxicity. **a:** Pretreatment of cultures with 5  $\mu$ M sulforaphane for 48 hr protected TH<sup>+</sup> cells against 6-OHDA toxicity ( $n = 12$ –31 cultures/treatment group;  $*P < 0.05$ ). **b:** Similarly, propidium iodide staining was dramatically reduced in cultures pretreated with sulforaphane for 48 hr compared with those treated with 100 nM 6-OHDA alone ( $n = 8$ –11 cultures/treatment group;  $*P < 0.05$ ). Values represent mean  $\pm$  SEM.

eration and in PD. Treatment of cultures with 100 nM 6-OHDA alone significantly increased nitrotyrosine staining in the SN compared with controls (control: 33.33  $\pm$  2.45 a.u., 6-OHDA: 64.79  $\pm$  5.98 a.u.,  $n = 6$ –13 cultures/treatment group;  $P < 0.01$ ; Fig. 6). This effect was diminished when cultures were treated with both 6-OHDA and tBHQ (39.29  $\pm$  1.72 a.u.), suggesting that treatment of cultures with Nrf2 activators is able to alleviate oxidative stress in the SN.

To begin to delineate the mechanism underlying tBHQ- and sulforaphane-mediated neuroprotection, we measured NQO1 mRNA levels by quantitative RT-PCR. NQO1 is one of the major enzymes up-regulated by the Nrf2-ARE pathway. Cultures were treated overnight with DMSO (1:10,000), 5  $\mu$ M tBHQ, 100 nM 6-OHDA, or a combination of both 5  $\mu$ M tBHQ and 100 nM 6-OHDA. Treatment with tBHQ alone led to a five-fold increase in NQO1 mRNA levels compared with controls (5.65-  $\pm$  1.20-fold compared with DMSO vehicle-treated control cultures,  $n = 3$ ;  $P < 0.05$ ; Fig. 7). Surprisingly, treatment of cultures with both tBHQ and 6-OHDA led to an almost 18-fold increase in NQO1

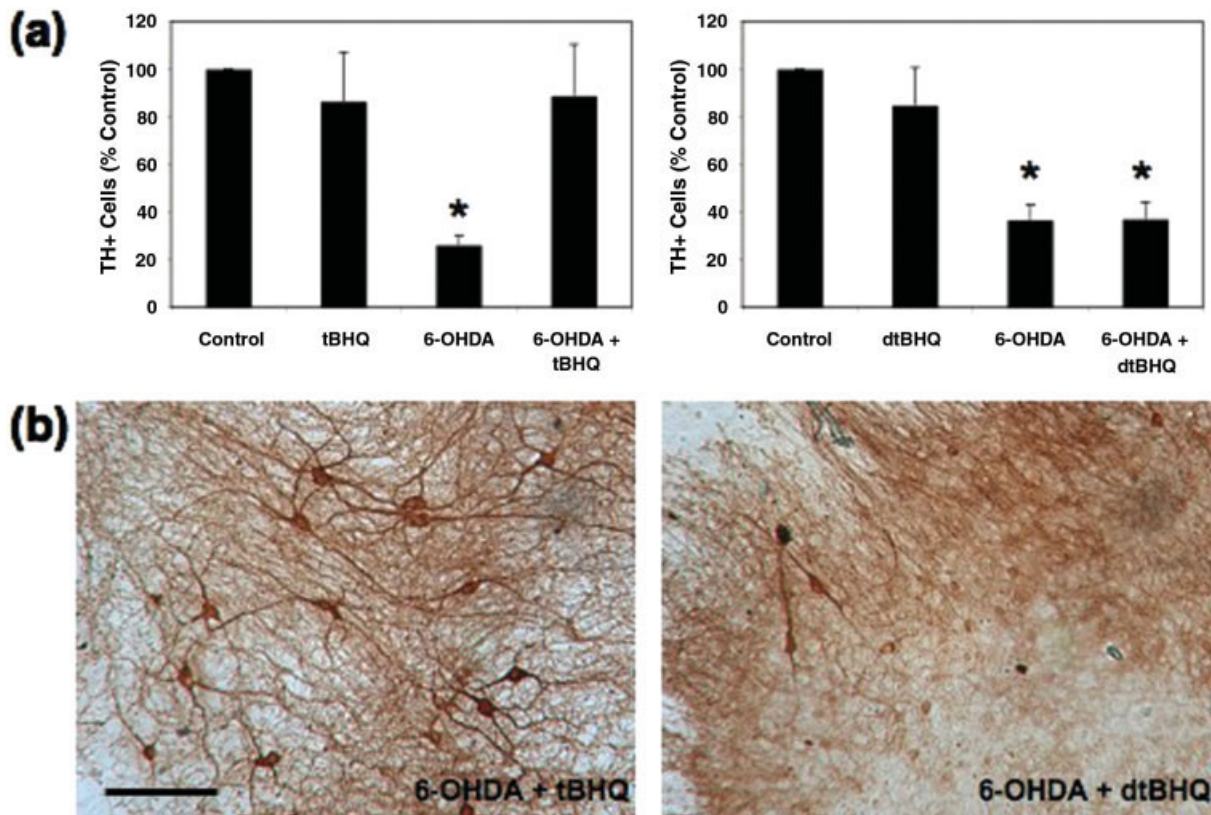


Fig. 5. dtBHQ does not protect TH<sup>+</sup> cells against 6-OHDA induced cytotoxicity. **a:** Pretreatment of cultures with 50  $\mu$ M dtBHQ for 8 hr did not protect cells against 6-OHDA-induced toxicity ( $n = 10$ –31 cultures/treatment group;  $*P < 0.05$ ). Values represent mean  $\pm$  SEM. Figure at left is identical to Figure 3a. **b:** Representative images of dopaminergic neurons in the substantia nigra of cultures

treated with either tBHQ + 6-OHDA (left) or dtBHQ + 6-OHDA (right). Note the dramatic loss of neuronal cell bodies and projections in the dtBHQ + 6-OHDA-treated cultures compared with tBHQ + 6-OHDA-treated cultures. Scale bar = 400  $\mu$ m. [Color figure can be viewed in the online issue, which is available at [www.interscience.wiley.com](http://www.interscience.wiley.com).]

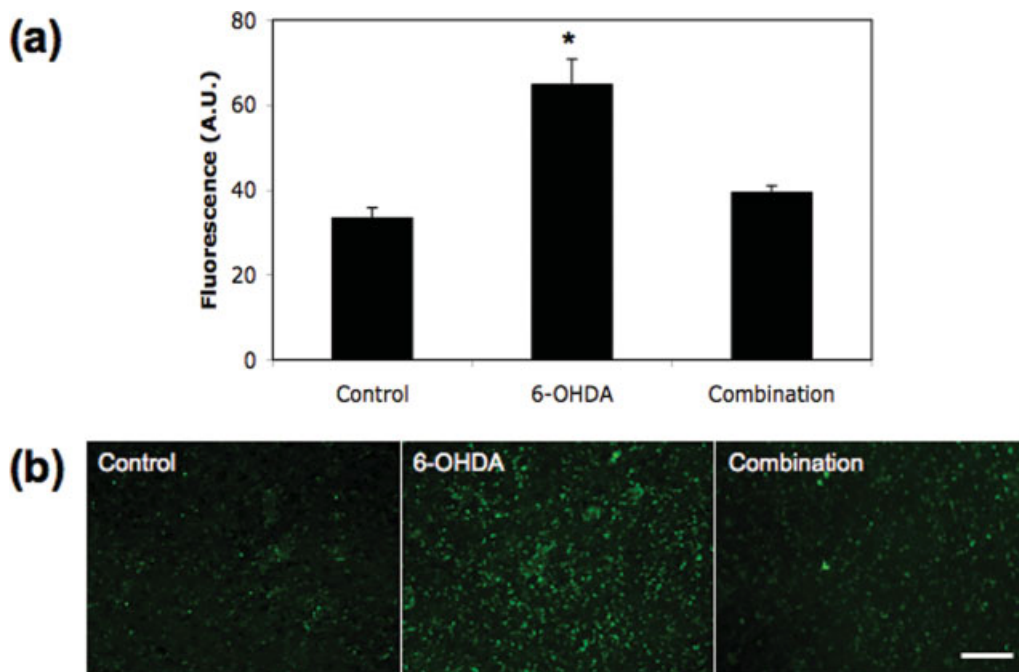


Fig. 6. Simultaneous treatment of cultures with tBHQ and 6-OHDA significantly decreases nitrotyrosine. **a:** Treatment with 100 nM 6-OHDA alone led to a significant increase in nitrotyrosine staining in the substantia nigra compared with controls ( $n = 6$ –13 cultures/treatment group;  $*P < 0.01$ ). tBHQ + 6-OHDA treatment mitigated this effect. Values represent mean  $\pm$  SEM in arbitrary units (A.U.).

**b:** Representative images of nitrotyrosine staining. Note the dramatic difference in cultures treated with 6-OHDA alone compared with cultures treated with tBHQ + 6-OHDA. Scale bar = 200  $\mu$ m. [Color figure can be viewed in the online issue, which is available at [www.interscience.wiley.com](http://www.interscience.wiley.com).]

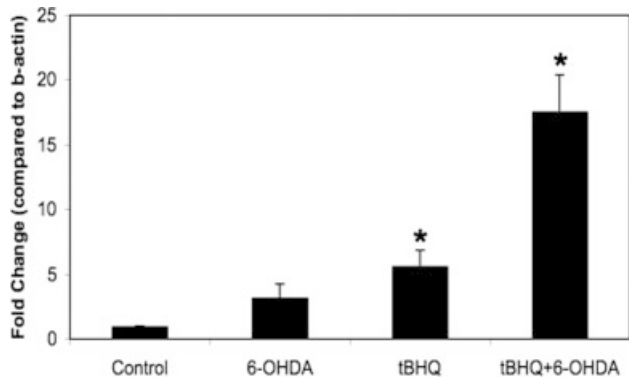


Fig. 7. Simultaneous treatment of cultures with tBHQ and 6-OHDA significantly increases NQO1 mRNA expression. Cultures were treated overnight with 100 nM 6-OHDA, 5  $\mu$ M tBHQ, or a combination of 5  $\mu$ M tBHQ and 100 nM 6-OHDA. tBHQ treatment led to a significant increase in NQO1 mRNA levels compared with controls. tBHQ + 6-OHDA treatment led to a significant increase in NQO1 mRNA levels compared with all other treatment groups. Fold change was determined by comparing NQO1 mRNA levels with  $\beta$ -actin mRNA levels ( $n = 3$  cultures/treatment group; \* $P < 0.05$ ). Values represents mean  $\pm$  SEM.

mRNA levels compared with controls and was significantly higher than that in all other groups (17.57-  $\pm$  2.84-fold compared with control cultures,  $n = 3$ ;  $P < 0.05$ ). Treatment of cultures with 6-OHDA alone, however, did not significantly increase NQO1 mRNA levels in comparison with all other groups (3.21-  $\pm$  1.03-fold compared with vehicle-treated control cultures,  $n = 3$ ).

## DISCUSSION

This study provides evidence to support the neuroprotective function of Nrf2 in a unique model of PD: the organotypic nigrostriatal coculture. This is the first report to demonstrate that activators of Nrf2 can almost completely protect against dopaminergic cell loss without pretreatment. These results support the idea that Nrf2 may be a viable therapeutic target for the treatment of PD.

### Organotypic Nigrostriatal Cocultures: A Model for Studying Parkinson's Disease Neurotoxins

This is the first study to examine the ability of Nrf2 activators to confer protection in dopaminergic neurons in vitro. This is due partially to the lack of an appropriate model in which to study dopaminergic degeneration and the effect of potential therapeutics on the degenerative process. Primary cell culture systems typically utilize either neuronal cell lines or dissociated cells. Although these models are convenient and easy to manipulate, they enable investigators only to study isolated cells over a short period of time (hours to days). In contrast, in vivo models of PD exhibit many of the pathological features of PD and allow investigators to study dopaminergic degeneration within the context of a

mature adult brain over much longer periods of time. However, it is much more challenging to study the time course and mechanisms underlying dopamine cell loss in in vivo models because of their complexity. Additionally, they are more difficult to control and are often labor-intensive and expensive (Testa et al., 2005). Organotypic nigrostriatal cocultures offer a useful intermediate tool for studying dopaminergic degeneration by combining advantages of both classic cell culture and animal models into one system. Specifically, they maintain much of the native milieu, have nigrostriatal synaptic connections, and survive for over 2 months, making long-term studies on neurodegeneration and aging feasible, and may ultimately allow a more accurate picture of these processes to be constructed (Plenz and Kitai, 1996, 1998).

Organotypic nigrostriatal cocultures treated overnight with 6-OHDA exhibited many of the same characteristics as well-established acute models of PD, including a concentration-dependent and specific loss of dopaminergic neurons in the substantia nigra and a loss of dopaminergic input to the striatum (for review see Betarbet et al., 2002). Thus, the data presented here demonstrate the potential that this unique culture system has as a model for the study of PD and neurodegeneration, particularly in addressing questions related to mechanisms and intercellular relationships.

### Nrf2 Activators Protect Without Pretreatment

The present study demonstrates that activators of the Nrf2-ARE pathway can protect dopaminergic neurons against oxidative stress in an organotypic model of PD. Treatment of cocultures with 6-OHDA alone resulted in a significant loss of TH<sup>+</sup> cells and an increase in nitrotyrosine staining. Quite remarkably, these effects were almost completely mitigated when cultures were *simultaneously* treated with 6-OHDA and tBHQ, a known activator of Nrf2. Several groups have shown that activation of Nrf2 prior to administration of a toxin, such as 6-OHDA or MPP<sup>+</sup>, can protect both neuronal cell lines and primary cortical cultures against various forms of oxidative stress (Hara et al., 2003; Kraft et al., 2004; Cao et al., 2005; Jakel et al., 2005). Additionally, increased Nrf2 activity has been shown to mediate protection against dopaminergic cell loss in rat models of PD using either 6-OHDA or MPTP (Burton et al., 2006; Jakel et al., 2007). All of these studies provide important evidence to support the neuroprotective role of Nrf2 and its potential as a therapeutic target. However, both the in vitro and in vivo studies bolster Nrf2 activity prior to administering the toxin. Clinically, it is critical to demonstrate that Nrf2 can be protective when activated after the start of a lesion, because approximately half of the DA neurons have already been lost by the time patients are diagnosed with PD. We have shown that concurrent treatment with a known Nrf2 activator and 6-OHDA can still lead to significant protection against dopaminergic cell loss. Future studies will extend

this work to a paradigm that more closely mimics the slow, degenerative process seen in PD patients by developing a chronic low-dose model using organotypic nigrostriatal cocultures. The longevity of this culture system makes this type of study possible and will allow us to determine whether Nrf2 activation can still render protection after the start of a lesion.

To date, no other study has shown that exposure to Nrf2 activators can protect dopaminergic neurons against an oxidative insult *in vitro* without pretreatment. It is quite possible that this difference can be attributed to the different systems used in each study. As an explant culture, organotypic nigrostriatal cocultures are able to maintain a cytoarchitecture and an intercellular milieu that is similar to what cells experience *in vivo* during the culturing process. Thus the presence of interneurons and glia, which are not found in cell lines and primary cortical cultures, could have provided additional support to neurons (e.g., neurotrophic factors, buffering ROS/RNS) that helped lead to their survival. Unlike primary culture systems in which cells are typically introduced to an oxidative insult shortly after being cultured (days), organotypic cocultures were not exposed to 6-OHDA for at least 2 weeks after being cultured. It is possible that this "extra" time *in vitro* allowed neurons to mature fully and return to a stable state, allowing them to mount a more robust antioxidant response once exposed to the toxin. This idea is supported by the finding that resident microglia become activated during the culturing process and remain so for ~7 DIV before returning to a resting state, suggesting that recently cultured tissue has a heightened sensitivity to homeostatic challenges (Hailer et al., 1996; Mertsch et al., 2001).

tBHQ and sulforaphane are known inducers of Nrf2; however, they also affect various other cellular processes. tBHQ can induce release of  $Ca^{2+}$  from internal stores, stimulate mitogen-activated protein kinase (MAPK) pathways, and induce apoptosis via cytochrome p450 1A1 in hepatic cancer cells (Robinson et al., 1992; Yu et al., 1997; Gharavi and El Kadi, 2005). Stimulation of MAPK pathways by tBHQ is commonly associated with cell growth and proliferation, two processes that support cell survival and neuroprotection. MAPKs also activate the Nrf2-ARE pathway by direct phosphorylation of Nrf2, further supporting a role in cell survival (Zipper and Mulcahy, 2000). Similarly, sulforaphane has also been shown to affect a number of other cellular mechanisms, including inducing cell cycle arrest and apoptosis in cancer cell lines, activating checkpoint 2 kinase, and inhibiting tubulin polymerization and histone deacetylase activity (for review see Myzak and Dashwood, 2006). Many of the studies that showed toxic or carcinogenic effects of either compound used high concentrations (up to 100 times more concentrated) in very different models (*in vitro* and *in vivo* cancer models). Thus it is likely that the diverse effects that both tBHQ and sulforaphane have in a cell is dependent on both the concentration used and the system being studied. Although we have not ruled out the possibility that these

processes may be involved in the present study, we plan to explore further their impact on neuroprotection in the future.

One question that remains unanswered is how Nrf2 is mediating the observed protection. Although it is well established that Nrf2 activation leads to the up-regulation of numerous antioxidant and phase II enzymes (for review see, e.g., Lee et al., 2005; Zhang, 2006), the cellular population(s) that mediates this phenomenon remains unclear. Over the past several years, a number of interesting reports have suggested that glia, particularly astrocytes, play a significant role in the protection of neurons from oxidative stress. It has been shown that the Nrf2-ARE pathway is preferentially activated in astrocytes compared with neurons (Murphy et al., 1991; Johnson et al., 2002) and that neurons found proximal to astrocytes were more likely to show ARE activity than those located more distally (Johnson et al., 2002). This suggests that activation of ARE in astrocytes leads to the production and secretion of some factor(s) that allows activation of the Nrf2-ARE pathway within neurons, mediating protection against oxidative insults. In support of this idea, Shih and colleagues (2003) have shown that Nrf2 can regulate the synthesis and release of GSH in astrocytes and that this phenomenon is sufficient to protect neurons against oxidative stress. However, the role that Nrf2 and astrocytes may play in neuronal survival remains controversial. Several groups have shown that increasing Nrf2 activity in reduced systems where glia are absent, such as primary neuronal cultures or neuronal cell lines, can still protect cells against oxidative insults (Hara et al., 2003; Lee et al., 2003; Cao et al., 2005). In support of this, Ramsey et al. (2007) found strong Nrf2 staining in nuclei of the remaining dopaminergic neurons within the substantia nigra of patients with PD and observed only minimal Nrf2 staining in surrounding glia. It is likely that Nrf2 activity in both neurons and glia contributes to neuronal survival in disease states. The unique combination of cells found within organotypic nigrostriatal cocultures provides an ideal system with which to examine this relationship between neurons and glia. In the future, we want to explore further how Nrf2 localization is regulated, how it differs in various cellular populations, and how these differences may contribute to neuronal protection.

In summary, we have demonstrated that simultaneous administration of 6-OHDA and a known activator of Nrf2 (tBHQ) is able to protect dopaminergic neurons *in vitro*. These results support the neuroprotective role of Nrf2 and its potential as a therapeutic target. Additionally, this report helps to establish organotypic nigrostriatal cocultures as a unique model of dopaminergic degeneration for the study of PD.

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