NF-E2-related Factor-2 (Nrf2) regulates the gene expression of phase II detoxification enzymes and antioxidant proteins through an enhancer sequence referred to as the antioxidant-responsive element (ARE). In this study, we demonstrate that Nrf2 protects neurons in mixed primary neuronal cultures containing both astrocytes (~10%) and neurons (~90%) through coordinate up-regulation of ARE-driven genes. Nrf2−/− neurons in this mixed culture system were more sensitive to mitochondrial toxin (1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine or rotenone)-induced apoptosis compared with Nrf2+/+ neurons. To understand the underlying mechanism of this observed differential sensitivity, we compared the gene expression profiles using oligonucleotide microarrays. Microarray data showed that Nrf2+/+ neuronal cultures had higher expression levels of genes encoding detoxification enzymes, antioxidant proteins, calcium homeostasis proteins, growth factors, neuron-specific proteins, and signaling molecules compared with Nrf2−/− neuronal cultures. As predicted from the microarray data, Nrf2−/− neurons were indeed more vulnerable to the cytotoxic effects of ionomycin- and 2,5-di-(t-butyl)-1,4-hydroquinone-induced increases in intracellular calcium. Finally, adenoviral vector-mediated overexpression of Nrf2 recovered ARE-driven gene expression in Nrf2−/− neuronal cultures and rescued Nrf2−/− neurons from rotenone- or ionomycin-induced cell death. Taken together, these findings suggest that Nrf2 plays an important role in protecting neurons from toxic insult.

The antioxidant-responsive element (ARE) plays an important role in the expression of genes encoding phase II detoxification enzymes and antioxidant proteins such as NAD(P)H: quinone oxidoreductase-1 (NQO1), glutathione S-transferases (GSTs), glutathione-cysteine ligase, and heme oxygenase-1 (1–4). Interestingly, many studies have demonstrated that most of the known ARE-driven genes are transcriptionally regulated by Nrf2-related factor-2 (Nrf2) (5–11).

Nrf2, a basic leucine zipper transcription factor (12), is the principal component leading to ARE-driven gene expression. Recently, Nrf2 target genes were identified by oligonucleotide microarray analysis, and the gene lists suggest that Nrf2 is important in combating electrophiles and reactive oxygen species (11, 13, 14). Many studies have shown that Nrf2 plays a critical role in protecting cells from oxidative stress. Chan et al. reported that Nrf2 protects liver from acetaminophen-induced injury (15) and lung from butylated hydroxytoluene-induced toxicity (16). Cho et al. (17) demonstrated that Nrf2 knockout mice are more sensitive to hyperoxia-induced lung injury. Recently, our laboratory reported that Nrf2−/− primary astrocytes are more susceptible to oxidative stress and inflammation compared with Nrf2+/+ astrocytes (11). Pretreatment of these Nrf2+/+(but not Nrf2−/−) astrocytes with t-butyldihydroquinone, which induces Nrf2 nuclear translocation resulting in coordinate up-regulation of ARE-driven genes, attenuates H2O2- and platelet-activating factor-induced cell death (11). Similarly, t-butyldihydroquinone-mediated protective effects have been shown in rodent and human neuroblastoma cells (18, 19). These observations suggest that the coordinate up-regulation of antioxidant genes is the key to protecting cells from oxidative stress and that Nrf2 is a master regulator of ARE-driven antioxidant gene expression, a process we refer to as programmed cell life (19).

Parkinson’s disease (PD) is a progressive neurodegenerative disease caused by degeneration of dopaminergic neurons in the substantia nigra. Although the underlying mechanism by which dopaminergic neurons degenerate is not clear, oxidative stress has been implicated to play a role in the neuronal cell death associated with PD (20). In support of this, many studies have shown decreased antioxidant levels in PD patients as well as a protective effect of antioxidants in animal models of PD. For example, GSH and coenzyme Q10 have been reported to attenuate oxidative stress and to improve mitochondrial toxin (1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-N,N',N"-tetraacetic acid tetra(acetoxyethyl) ester).
1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPP) used Nrf2 type (neurons (data not shown). In addition, there was no difference in the ratio of cell phosphate-buffered saline; fixed in 4% paraformaldehyde for 20 min; performed according to the manufacturers dUTP nick end labeling (TUNEL; Roche Applied Science) staining were cytotoxicity assay and terminal deoxynucleotidyltransferase-mediated H
determination on HEK293 cells. Nrf2

In this study, we raised and tested the hypothesis that de-

- ddCTTAATTGAGGTGACGTT
- GCCATTCGCAGCAAGACT
- GAGCATAGCCCTCGTAGAT
- ACTGACGGCAACATACGCT
- TGGACAGTACGCTTCTG
- ACCACCTCAACA-3

Nrf2-dependent NQO1 Gene Expression in Primary Neuronal

Procedures

EXPERIMENTAL PROCEDURES

Primary Neuronal Culture—Nrf2−/− mice were bred with Nrf2−/+ mice, and primary neuronal cultures were prepared individually. Cer-
elbra corticalis from littermate embryos (gestation day 16) were removed,

- 5'-GGTG-3
- GCCCTCTCTCCTCCC-3

Gene categorization was based on the NetAffex Database. 2

- 5'-GCCAAGTACCCTTGGTTGAA-3
- -GCCAAGTACCCTTGGTTGAA-3

- 5'-GTGTTTGGGAATG-3
- GACAAAAGTCCACCGGAAAA-3

- 5'-GAGCATAGCCCTCGTAGAT-3
- TGGCTACCTTCCCTTACCAA-

Results

Nrf2-dependent NQO1 Gene Expression in Primary Neuronal

Cultures—Initially, we compared the level of expression and enzymatic activity of a known Nrf2-dependent ARE-driven gene, NQO1, in Nrf2−/− and Nrf2−/+ primary neuronal cultures. The expression level of NQO1 was dramatically greater in Nrf2−/− neuronal cultures (Fig. 1A). Similarly, the NQO1 activity of Nrf2−/− neuronal cultures was significantly higher than that of Nrf2−/+ neuronal cultures (Fig. 1B). This corre-
lated with a complete lack of NQO1 histochemical staining in the Nrf2−/+ neuronal cultures (Fig. 1C). It also implies that the lack of Nrf2 significantly reduced the Nrf2-ARE signaling pathway in primary neuronal cultures. It was also noted, as published previously by our laboratory (35), that NQO1 staining was isolated to the astrocytes in this mixed culture system. Differential Sensitivity to Mitochondrial Toxins—To investi-

2 Available at www.affymetrix.com.

 play an important role in protecting dopaminergic neurons not only in human PD patients, but also in animal PD models (21–24). In addition, iron metabolism and ferritin levels are involved in dopaminergic neuronal cell death in PD patients (25, 26). A recent publication demonstrated that selective expression of human ferritin in dopaminergic neurons or administration of an iron chelator prevents 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine-induced death of dopaminergic neurons in vivo (27). Furthermore, superoxide dismutase and glutathi-
one peroxidase have been reported to play a protective role in animal models of PD (28–30). Interestingly, our recent oligonucleotide microarray study with mouse primary astrocytes showed that the expression of many of these protective antioxid-

NQO1 enzymatic activity was determined by a col-

nucleotide microarray study with mouse primary astrocytes

- GATCCTGGTGGGAAGAAAG-3
- -TGGCTACCTTCCCTTACCAA-
- ACCACCTCAACA-3
- TGGGACAGTACGCTTCTG
- AGAGCAGAGAGAGAG-3
- TCTCCTCGCTGGAAAAAG-
- TCTGGTTTGGGAATG-3
- -GCCAAGTACCCTTGGTTGAA-3
- synaptotagmin, 5
- Nrf2 exon 13–1, 5
- 5'-AGTCTTCGCTGTCGGACTA-3'- and 5'-AGGCATCTTTGGGAAATGTGATAGGTGACGTT

NQO1, 5
- 5'-GAGCATAGCCCTCGTAGAT-3
- TGGCCTCTCTCCTCCC-3
- visinin-like 1, 5
- 5'-AAGCTCATGGAGGACCCTGGT-3
- GCLM, 5
- 5'-ACTGACGGCAACATACGCT
- actin, 5
- 5'-GCCCTCTCTCCTCCC-3
- CaM, 5
- 5'-GAGCATAGCCCTCGTAGAT-3
- MPP) or rote-

- 5'-GAGCATAGCCCTCGTAGAT-3
- calbindin-28K, 5
- 5'-GAGCATAGCCCTCGTAGAT-3
- GCLC, 5
- 5'-GAGCATAGCCCTCGTAGAT-3
- NQO1, 5
- 5'-GAGCATAGCCCTCGTAGAT-3
- Cav1.3, 5
- 5'-GAGCATAGCCCTCGTAGAT-3
- Nrf2 gene, NQO1, in Nrf2−/+ neuronal cultures (Fig. 1C)

- GAGCATAGCCCTCGTAGAT-3
- hydroxyl radical scavenger, 5
- 5'-GAGCATAGCCCTCGTAGAT-3
- 

8

2 Available at www.affymetrix.com.

Adenoviral Infection—Recombinant adenoviral vectors (Ad-GFP, expressing GFP alone; and Ad-Nrf2, expressing GFP and Nrf2) were

- 5'-GAGCATAGCCCTCGTAGAT-3
- biotinylated cRNA

- 5'-GAGCATAGCCCTCGTAGAT-3
- Nrf2 exon 5, 5
- 5'-GAGCATAGCCCTCGTAGAT-3
- biotinylated cRNA

- 5'-GAGCATAGCCCTCGTAGAT-3
- Nrf2 exon 5, 5
- 5'-GAGCATAGCCCTCGTAGAT-3

- 5'-GAGCATAGCCCTCGTAGAT-3
- biotinylated cRNA

- 5'-GAGCATAGCCCTCGTAGAT-3
- Nrf2 exon 5, 5
- 5'-GAGCATAGCCCTCGTAGAT-3

- 5'-GAGCATAGCCCTCGTAGAT-3
- biotinylated cRNA

- 5'-GAGCATAGCCCTCGTAGAT-3
- Nrf2 exon 5, 5
- 5'-GAGCATAGCCCTCGTAGAT-3

- 5'-GAGCATAGCCCTCGTAGAT-3
- biotinylated cRNA

- 5'-GAGCATAGCCCTCGTAGAT-3
- Nrf2 exon 5, 5
- 5'-GAGCATAGCCCTCGTAGAT-3

- 5'-GAGCATAGCCCTCGTAGAT-3
- biotinylated cRNA

- 5'-GAGCATAGCCCTCGTAGAT-3
- Nrf2 exon 5, 5
- 5'-GAGCATAGCCCTCGTAGAT-3
increased Ca\textsuperscript{2+} potential, increased reactive oxygen species generation, and complex I, resulting in decreased ATP levels, loss of membrane potential, increased sensitivity of neurons to oxidative stress, we treated primary neuronal cultures with the well known mitochondrial toxins MPP\textsuperscript{+} (31) and rotenone (32, 33). These compounds induce neuronal cell death by inhibiting mitochondrial complex I, resulting in decreased ATP levels, loss of membrane potential, increased reactive oxygen species generation, and increased [Ca\textsuperscript{2+}]. As shown in Fig. 2A, Nrf2\textsuperscript{−/−} neuronal cultures were much more sensitive to MPP\textsuperscript{+} or rotenone-induced cytotoxicity. Rotenone (0.1 μM) and MPP\textsuperscript{+} (50 μM) induced 85% cell death (equivalent to 100% neuronal cell death) in Nrf2\textsuperscript{−/−} neuronal cultures (Fig. 2A). The remaining MTS activity is presumably due to the surviving astrocytes. Strikingly, the same concentrations of MPP\textsuperscript{+} and rotenone induced little or no cell death in Nrf2\textsuperscript{+/+} neuronal cultures, respectively (Fig. 2A). This is reflected in the numbers of TUNEL-positive cells in MPP\textsuperscript{+}- or rotenone-treated Nrf2\textsuperscript{−/−} neuronal cultures (Fig. 2B). There were far greater numbers of TUNEL-positive cells in the Nrf2\textsuperscript{−/−} neuronal cultures than in the Nrf2\textsuperscript{+/+} neuronal cultures (Fig. 2B). Double labeling with selective astrocyte or neuronal markers indicated that these apoptotic cells were not astrocytes (data not shown). MPP\textsuperscript{+} and rotenone also preferentially activated the caspase-3 pathway in Nrf2\textsuperscript{−/−} cultured neurons as shown by immunostaining for the cleaved caspase-3 (Fig. 3).

Identification of Nrf2 Target Genes—To identify the Nrf2 target genes conferring this observed neuroprotection, we performed oligonucleotide microarray analysis on Nrf2\textsuperscript{−/−} and Nrf2\textsuperscript{+/−} primary neuronal cultures. A total of 142 increased and 175 decreased genes were identified using rank analysis (R ≥ 4, R ≤ −4), followed by cutoff values for cv (cv < 1.0, cv > −1.0) and -fold change (-fold ≥ 1.3, -fold ≤ −1.3). The major functional categories of Nrf2 target genes in primary neuronal cultures are 1) detoxification/antioxidant/reducing potential, 2) calcium homeostasis, 3) growth factors, 4) signaling, 5) receptor/channel/carrier protein, 6) neuron-specific proteins, and 7) defense/immune/inflammation (Table I). Although many of the classical Nrf2-dependent ARE-driven genes such as NQO1, GSTs, and GCLC were increased by Nrf2 in primary neuronal cultures, the final list of Nrf2 target genes in these neuronal cultures was quite distinct from those of other cell types (11, 13). The gene classifications of calcium homeostasis, receptor/
channel/carrier protein, and neuron-specific proteins, for instance, were unique to the primary neuronal cultures.

**Verification of Microarray Data—**RT-PCR and Western blot analysis for selected genes were performed to verify the microarray data. The differences in expression levels by RT-PCR (Fig. 4A) correlated well with the microarray data (Fig. 4B). In addition, Western blot analysis showed that the Nrf2-/- neuronal cultures had higher GST Mu1 and GCLC protein levels (Fig. 4C), and the total GSH level in Nrf2-/- neuronal cultures was significantly higher than that in Nrf2+/+ neuronal cultures (Fig. 4D).

**Important Role of Nrf2 in Calcium Homeostasis—**Our microarray (Fig. 5B and Table I) and RT-PCR (Fig. 5A) data show that many genes playing an important role in calcium homeostasis were increased in an Nrf2-dependent manner in primary neuronal cultures. This gene cluster includes visinin-like 1 (NVP-1), calbindin-28K (Calb1), synaptotagmin-1 (Syt1), hippocalcin (Hpca), synaptotagmin-5, nucleobindin-2, calmodulin III, and ryanodine receptor-3 (Fig. 5A and B and Table 1). Because these genes have been reported to play an important role in Ca\(^{2+}\)-mediated neuronal cell death (37, 38), we treated neuronal cultures with ionomycin or 2,5-di-(t-butyl)-1,4-hydroquinone (dtBHQ) to challenge the cells with an increase in intracellular calcium. Ionomycin and dtBHQ increase [Ca\(^{2+}\)], by inducing influx of Ca\(^{2+}\) into cells and by inhibiting endoplasmic reticulum Ca\(^{2+}\)-ATPase activity, respectively. In regular neurobasal medium with 1.8 mM Ca\(^{2+}\), ionomycin and dtBHQ induced more cell death in Nrf2-/- neuronal cultures compared with Nrf2+/+ neuronal cultures (Fig. 5C). Pretreatment with the selective calcium chelator BAPTA-AM partially protected dtBHQ-treated neuronal cultures (both Nrf2-/- and Nrf2+/+ neuronal cultures) (data not shown). Interestingly, BAPTA-AM alone showed cytotoxic effects in Nrf2-/- neuronal cultures, suggesting that a reduced calcium-buffering capacity can make Nrf2-/- neuronal cultures more sensitive to capacitative calcium entry (39). Again, as seen with rotenone and MPP\(^+\), the neurons (but not the astrocytes) accounted for the cell death in these cultures.

**Nrf2-dependent ARE-driven Gene Expression—**To confirm that Nrf2 is important for ARE-driven gene expression and neuroprotection, we infected Nrf2-/- neuronal cultures with recombinant adenoviral vectors (Ad-GFP and Ad-Nrf2). As shown in Fig. 6 (A–C), both cell types (astrocytes and neurons) were infected by adenoviral vectors (both Ad-GFP and Ad-Nrf2) as evidenced by GFP (infection marker) and MAP2 (neuron-specific marker) staining (Fig. 6B) and GFAP (astrocyte-specific marker) staining (Fig. 6C). Astrocytes were, however, preferentially infected by the adenovirus such that virtually all astrocytes were infected, whereas only 10–20% of the neurons were infected (Fig. 6C). Infection of Nrf2-/- neuronal cultures with Ad-Nrf2 increased Nrf2 immunostaining (Fig. 6D) and its downstream targets such as GST Mu1 (Fig. 6A), GCLC (Fig. 7B), and NQO1 (Fig. 7C). RT-PCR experiments also showed that the expression levels of these genes were dramatically increased by Ad-Nrf2 in Nrf2-/- neuronal cultures (NQO1, 23.6-fold increase; GST Mu1, 5.6-fold increase; and GCLC, 15.4-fold increase) (Fig. 7D). Ad-GFP itself did not change the expression levels of these genes (data not shown).

**Nrf2 Protects Nrf2-/- Neurons—**Finally, we tested whether overexpression Nrf2 can protect Nrf2-/- neurons from oxidative stress. Infected neuronal cultures were treated with several doses of rotenone and ionomycin, and we observed that Ad-Nrf2-infected Nrf2-/- neuronal cultures were more resistant to these neurotoxins compared with Ad-GFP-infected Nrf2-/- neuronal cultures (Fig. 8, A and B). At the highest dose of ionomycin (Fig. 8, C and D) or rotenone (data not shown), only the neurons infected with Ad-Nrf2 survived, whereas in cultures treated with the lowest doses (0.02 \(\mu\)M rotenone or 1 \(\mu\)M ionomycin), all or most of the Nrf2-/- neurons in the Ad-Nrf2-infected cultures (both uninfected or infected) survived. In the case of rotenone (0.02 \(\mu\)M), this effect was very dramatic, with >70% of the Nrf2-/- neurons being killed in the Ad-GFP-infected cultures and absolutely no neuronal cell death in the Ad-Nrf2-infected cultures.

**DISCUSSION**

In this study, we have shown that ARE-driven gene expression is dependent on Nrf2 in primary neuronal cultures and that Nrf2-/- neurons are more sensitive to mitochondrial toxin-mediated apoptosis. To identify Nrf2 target genes conferring observed neuroprotection, we performed oligonucleotide microarray analysis. Microarray data showed that Nrf2 is important for the expression of many protective genes such as 1) ARE-driven detoxification and antioxidant genes, 2) calcium homeostasis genes, 3) growth factors, 4) signaling proteins, 5) receptor/channel/carrier proteins, 6) neuron-specific genes, and 7) defense/immune/inflammation genes. Based on the microarray data, we tested the hypothesis that Nrf2-/- neurons are more susceptible to the cytotoxic effect of increased \([Ca^{2+}]_{i}\) and found that ionomycin and dtBHQ induce more cell death in Nrf2-/- neurons compared with Nrf2+/+ neurons. Finally, we demonstrated that overexpression of Nrf2 rescues ARE-driven gene expression in Nrf2-/- neuronal cultures and rescues Nrf2-/- neurons from rotenone- or ionomycin-induced cell death. Taken together, these findings suggest that Nrf2 plays an important role in protecting neurons from toxic insult.

In this study, we used mitochondrial toxins (i.e. MPP\(^+\) and rotenone), a calcium ionophore (ionomycin), and an endoplasmic reticulum Ca\(^{2+}\)-ATPase inhibitor (dtBHQ) to investigate the neuroprotective role of Nrf2. Rotenone and MPP\(^+\), which are commonly used in animal and cell culture models of PD,
inhibit mitochondria complex I, resulting in increased reactive oxygen species production, increased [Ca\(^{2+}\)], and eventually neuronal cell death via apoptosis (31–33). Therefore, the cells’ antioxidant potential and calcium-buffering capacity are very important in protecting neurons from the harmful effects of these neurotoxins. In our study, Nrf2 \(-/\) primary neurons were very sensitive to mitochondrial toxins. In addition, Nrf2 \(-/\) neurons were more susceptible to increased [Ca\(^{2+}\)], induced by
ionomycin or dtBHQ, suggesting an important role for Nrf2 in the maintenance of calcium homeostasis as well as antioxidant potential. In support of a role of Nrf2 in calcium homeostasis, our microarray analysis identified several calcium-binding proteins and calcium sensors that play an important role in neuroprotection against increased [Ca$^{2+}$]. For example, hippocalcin protects neuronal cell lines NSC-34 and Neuro-2a from ionomycin- or thapsigargin-induced cell death by interacting with neuronal apoptosis inhibitory protein (37). Calbindin offers striatal neurons protection against quinolic acid-induced excitotoxic insults (38). Based on these observations, it would appear that Nrf2-driven calcium homeostasis genes significantly contribute to the observed neuroprotective effect.

Previously, our laboratory showed that coordinate up-regulation of ARE-driven genes by t-butylhydroquinone protects human neuroblastoma cells from H$_2$O$_2$-induced apoptosis and identified ARE-driven genes by oligonucleotide microarray analysis (19). Furthermore, we reported that Nrf2 plays an important role in ARE-driven gene expression and protection in mouse primary astrocytes (11). Until now, small intestine (13), primary astrocytes (11), liver (40), and primary neurons (this study) from Nrf2$^{-/-}$ mice have been used for microarray analysis, and three studies identified Nrf2 target genes (small intestine, primary astrocytes, and primary neurons). In these three studies, only five genes were commonly increased by Nrf2 (NQO1, GST Alpha4, GST Mu1, GST Mu3, and malic enzyme), suggesting that these genes are ubiquitously expressed by an Nrf2-dependent manner. In contrast, the lists of Nrf2 target genes are very tissue/cell-specific. For example, genes encoding detoxification and drug-metabolizing enzymes are increased by Nrf2 in small intestine (13). In addition to detoxification genes, many antioxidant and immune-related genes are increased by Nrf2 in primary astrocytes (11). This study with primary neurons revealed unique Nrf2 target gene clusters such as calcium homeostasis and neuron-specific genes. This tissue-specific pattern of Nrf2 target genes suggests a specialized function of Nrf2 depending on the tissue or cell. For example, small intestine-specific Nrf2 target genes (i.e. detoxification) may be important for chemoprevention in forestomach (41, 42). Astrocyte-specific Nrf2 target gene sets (i.e. antioxidants) may play a role in antioxidant and anti-inflammatory effects in brain (11). Finally, neuron-specific Nrf2 target genes (i.e. calcium homeostasis; this study) may

**Fig. 4. Verification of microarray data.** A, total RNA was isolated from primary neuronal cultures, and cDNA was synthesized for PCR amplification. **GST A4**, GST Alpha4; **PAFAH**, platelet-activating factor acetylhydrolase. B, the corresponding averages of the signal values from the microarray data are listed. C, Western blot analysis for GST Mu1, GCLM, and GCLC was performed. D, total GSH levels (GSH + GSSG) were measured as described under "Experimental Procedures." Data bars represent means ± S.E. (n = 6). *, genes called "increased" in the microarray analysis; †, significantly different from Nrf2$^{-/-}$ neuronal cultures by Student's t test (p < 0.05).

**Fig. 5. Important role of Nrf2 in Ca$^{2+}$ homeostasis.** A, total RNA was isolated from primary neuronal cultures, and cDNA was synthesized for PCR amplification. NVP-1, visinin-like 1; Calb1, calbindin-28K; Syt1, synaptotagmin-1; Hpcal, hippocalcin. B, the corresponding averages of the signal values from the microarray data are listed. C, primary neuronal cultures were treated with vehicle (0.01% Me$_2$SO), ionomycin (1 µM), or dtBHQ (200 µM). After 24 h, MTS cytotoxicity assay was performed as described under "Experimental Procedures." Data bars represent means ± S.E. (n = 4). *, genes called "increased" in the microarray analysis; †, significantly different from the corresponding vehicle-treated cultures by Student's t test (p < 0.05); ‡, significantly different from the corresponding Nrf2$^{-/-}$ neuronal cultures by Student's t test (p < 0.05).
play an important role in protecting neurons from oxidative stress or disturbance of calcium homeostasis. Although the list of Nrf2 target genes varies depending on cell type, these findings suggest that Nrf2 is critical to the cellular defense mechanism in many tissues, including brain.

Multiple studies have demonstrated that astrocytes confer neuroprotection. Astrocytes generate and release GSH, metabolize the excitotoxin glutamate, and secrete neurotrophic factors (43, 44). Recently, we reported that Nrf2 regulates GSH synthesis and release in astrocytes, increasing neuronal cell survival (14). In the neuron-enriched culture system used here, astrocytes account for ~10% of the total cell number. We speculate that Nrf2 target genes in a small number of astrocytes contribute to part of the observed neuroprotection. Astrocyte-conferred neuroprotection might be contributed by antioxidants such as glutamate-cysteine ligase, heme oxygenase-1,
thioredoxin reductase-1, ferritin, and peroxiredoxin, as previously identified by microarray analysis (11, 14). In addition to astrocyte-conferred neuroprotection, this study showed that overexpression of Nrf2 attenuated rotenone- and ionomycin-induced cytotoxicity in Nrf2−/− neurons. Furthermore, many surviving Nrf2−/− neurons after treatment with higher concentrations of ionomycin were GFP-positive, suggesting that Nrf2-overexpressing neurons are more resistant to neurotoxins without assistance from astrocytes. Together, these observations raise the possibility that the neuroprotective effect of Nrf2 is contributed by the genetic remodeling (i.e., ARE-driven gene expression) of both astrocytes and neurons and that Nrf2 is a major player in the neuroprotective process.

Oxidative stress has been implicated in the neuronal cell death of many progressive neurodegenerative diseases such as Alzheimer’s disease and Parkinson’s disease. Replacing the dying or dead neurons in these diseases has met with limited success (45). Therefore, approaches to prevent cell death and/or to attenuate the progression of these neurodegenerative diseases may have significant clinical utility in combating neurodegeneration. Here, we have reported one very efficient way to protect neurons from oxidative stress through the coordinate up-regulation of ARE-driven genes that is referred to as programmed cell life (19).

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Fig. 8. Neuroprotection by Nrf2. Primary Nrf2−/− neuronal cultures were infected with Ad-GFP (m.o.i. = 50) or Ad-Nrf2 (m.o.i. = 50) at 2 DIV (48 h). A and B, infected neuronal cultures were treated with rotenone or ionomycin (24 h), respectively, for MTS cytotoxicity assay. Data bars represent means ± S.E. (n = 6). C, infected neuronal cultures were treated with vehicle (0.01% MeSO₄) or ionomycin (10 μM) for 24 h and stained for MAP2. D, GFP and MAP2 staining showed that most of the surviving Ad-Nrf2-infected Nrf2−/− neurons (MAP2-positive) after ionomycin treatment (10 μM) were also GFP-positive, suggesting that Nrf2-expressing Nrf2−/− neurons are more resistant to ionomycin-induced cytotoxicity. Arrowheads indicate neurons.

REFERENCES
1. Bushmore, T. H., King, R. G., Paulson, K. E., and Pickett, C. B. (Proc. Natl. Acad. Sci. U. S. A. 87, 3826–3833, 1990
2. Jaiswal, A. K. (1991) Biochemistry 26, 15647–16553
3. Mulcahy, R. T., Wartman, M. A., Bailey, H. H., and Gipp, J. J. (1997) J. Biol. Chem. 272, 7445–7454
4. Alam, J., Stewart, D., Touchard, C., Boinapally, S., Choi, A. M., and Cook, J. L. (1999) J. Biol. Chem. 274, 26071–26078
5. Huang, H. C., Nguyen, T., and Pickett, C. B. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 12475–12480
6. Huang, H. C., Nguyen, T., and Pickett, C. B. (2002) J. Biol. Chem. 277, 42769–42774
7. Alam, J., Wicks, C., Stewart, D., Gong, P., Touchard, C., Otterbein, S., Choi, A. M., Burrow, M. E., and Tou, J. (2000) J. Biol. Chem. 275, 27694–27702
8. Wild, A. C., Moinova, H. R., and Mulcahy, R. T. (1999) J. Biol. Chem. 274, 33627–33636
9. Lee, J.-M., Hanson, J. M., Chu, W. A., and Johnson, J. A. (2001) J. Biol. Chem. 276, 20011–20016
10. Lee, J.-M., Moehlenkamp, J. D., Hanson, J. M., and Johnson, J. A. (2001) Biochem. Biophys. Res. Commun. 286, 296–299
11. Lee, J. M., Calkins, M. J., Chan, K., Kan, Y. W., and Johnson, J. A. (2003) J. Biol. Chem. 278, 12029–12038
12. Mui, P., Chan, K., Anzulis, I., Cao, A., and Kan, Y. W. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 9926–9930
13. Thimmulappa, R. K., Mai, K. H., Srisuma, S., Kensler, T. W., Yamamoto, M., and Biswal, S. (2002) Cancer Res. 62, 5186–5203
14. Shih, A. Y., Johnson, D. A., Wong, G., Kraft, A. D., Jiang, L., Erb, H., Johnson, J. A., and Murphy, T. H. (2003) J. Neurosci. 23, 3394–3406
15. Chan, K., Han, X. D., and Kan, Y. W. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 4611–4616
16. Chan, K., and Kan, Y. W. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 12731–12736
17. Cho, H. Y., Jedicke, A. E., Reddy, S. P., Kensler, T. W., Yamamoto, M., Zhang, L. Y., and Kleeberger, S. R. (2002) Am. J. Respir. Cell Mol. Biol. 26, 175–182
18. Duffy, S., So, A., and Murphy, T. H. (1998) J. Neurochem. 71, 69–77
19. Li, J., Lee, J.-M., and Johnson, J. A. (2002) J. Biol. Chem. 277, 388–394
20. Adams, J. D., Jr., Chang, M. L., and Raisman, L. (2001) Curr. Med. Chem. 8, 809–814
21. Nakamura, K., Wang, W., and Kang, U. J. (1997) J. Neurochem. 69, 1850–1858
22. Beal, M. F., Matthews, R. T., Tieleman, A., and Shults, C. W. (1998) Brain Res. 783, 109–114
23. Shults, C. W., Oakes, D., Kieburtz, K., Beal, M. F., Haas, R., Plumb, S., Juncos, J. L., Nutt, J., Shoulson, I., Carter, J., Kompoliti, K., Perlmuter, J. S., Reich, S., Stern, M., Watts, R. L., Kurian, K., Molho, E., Harrison, M., Lew, M., and the Parkinson Study Group. (2002) Arch. Neurol. 59, 1541–1550
24. Bharath, S., Hsu, M., Kaur, D., Rajagopalan, S., and Andersen, J. K. (2002) Biochem. Pharmacol. 64, 1037–1046
25. Dexter, D. T., Carayon, A., Vidailhet, M., Ruberg, M., Agid, F., Agid, Y., Lees, A. J., Wells, F. R., Jenner, P., and Marsden, C. D. (1990) J. Neurochem. 55, 16–20
26. Mann, V. M., Cooper, J. M., Daniel, S. E., Srai, K., Jenner, P., Marsden, C. D., and Schapira, A. H. (1994) Ann. Neurol. 36, 876–881
27. Kaur, D., Yantiri, F., Rajagopalan, S., Kumar, J., Mo, J. Q., Boonplueang, R., Viswanath, V., Jacobs, R., Yang, L., Beal, M. F., DiMonte, D., Volitaskis, I., Ellerby, L., Cherny, R. A., Bush, A. I., and Andersen, J. K. (2003) Neuron 37, 899–909
28. Przedborski, S., Kostic, V., Jackson-Lewis, V., Naini, A. B., Simonetti, S., Fahn, S., Carlson, E., Epstein, C. J., and Cadet, J. L. (1992) J. Neurosci. 12, 1658–1667
29. Zhang, J., Graham, D. G., Montine, T. J., and Ho, Y. S. (2000) J. Neuropathol. Exp. Neurol. 59, 53–61
30. Klivenyi, P., Andreassen, O. A., Ferrante, R. J., Dedeglu, A., Mueller, G., Lancelot, E., Bogdanov, M., Andersen, J. K., Jiang, D., and Beal, M. F. (2000) J. Neurosci. 20, 1–7
31. Offen, D., Beart, P. M., Cheung, N. S., Passon, C. J., Hochman, A., Gorodin, S., Melamed, E., Bernard, R., and Bernard, O. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 5789–5794
32. Betarbet, R., Sherer, T. B., MacKenzie, G., Garcia-Osuna, M., Panov, A. V., and Greenamyre, J. T. (2000) Nat. Neurosci. 3, 1301–1306
33. Sherer, T. B., Betarbet, R., Stout, A. K., Lund, S., Baptista, M., Panov, A. V., Cookson, M. R., and Greenamyre, J. T. (2002) J. Neurosci. 22, 7006–7015
34. Prechaska, H. J., and Santamaria, A. B. (1988) Anal. Biochem. 169, 328–336
35. Johnson, D. A., Andrews, G. K., Xu, W., and Johnson, J. A. (2002) J. Neurochem. 81, 1233–1241
36. Hardy, S., Kitamura, M., Harris-Stanfill, T., Dai, Y., and Phipps, M. L. (1997) J. Virol. 71, 1842–1849
37. Mercer, E. A., Korhonen, L., Skogloisa, Y., Olsson, P. A., Kukkonen, J. P., and Lindholm, D. (2000) EMBO J. 19, 3597–3607
38. Figuereiro-Gardenes, G., Harris, C. L., Anderson, K. D., and Reiner, A. (1998) Exp. Neurol. 149, 356–372
39. Putney, J. W., Jr., and Bird, G. S. (1995) Cell 75, 199–201
40. Kwak, M. K., Wakabayashi, N., Itoh, K., Motshachi, H., Yamamoto, M., and Kessler, T. W. (2005) J. Biol. Chem. 278, 8135–8145
41. Ramos-Gomez, M., Kwak, M. K., Dolan, P. M., Itoh, K., Yamamoto, M., Talalay, P., and Kessler, T. W. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 3410–3415
42. Fahey, J. W., Haristoy, X., Dolan, P. M., Kessler, T. W., Scholtus, I., Stephenson, K. K., Talalay, P., and Lozniewski, A. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 7610–7615
43. Aschner, M. (2000) Neurotoxicology 21, 1101–1107
44. Mount, H. T., Dean, D. O., Alberch, J., Dreyfus, C. F., and Black, I. B. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 9692–9696
45. Freed, C. R., Greene, P. E., Breeze, R. E., Tsai, W. Y., DuMouchel, W., Kao, R., Dillen, S., Winfield, H., Culver, S., Trejanowski, J. Q., Edelberg, D., and Fahn, S. (2001) N. Engl. J. Med. 344, 710–719