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NF-E2-related Factor-2 Mediates Neuroprotection against Mitochondrial Complex I Inhibitors and Increased Concentrations of Intracellular Calcium in Primary Cortical Neurons*

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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)[1] 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), glutamate-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 NF-E2-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*-butylhydroquinone, which induces Nrf2 nuclear translocation resulting in coordinate up-regulation of ARE-driven genes, attenuates H_2O_2- and platelet-activating factor-induced cell death (11). Similarly, *t*-butylhydroquinone-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 Q_{10} have been reported to

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[1] The abbreviations used are: ARE, antioxidant-responsive element; NQO1, NAD(P)H:quinone oxidoreductase-1; GST, glutathione *S*-transferase; Nrf2, NF-E2-related factor-2; PD, Parkinson's disease; MPP^+, 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine; DIV, days *in vitro*; GFAP, glial fibrillary acidic protein; MAP2, microtubule-associated protein-2; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium salt; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling; GCLC, glutamate-cysteine ligase catalytic subunit; GCLM, glutamate-cysteine ligase modifier subunit; RT, reverse transcription; Ad, adenovirus; GFP, green fluorescent protein; m.o.i., multiplicity of infection; dtBHQ, 2,5-di-(*t*-butyl)-1,4-hydroquinone; BAPTA-AM, 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid tetra(acetoxymethyl) ester.

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 overexpression of human ferritin in dopaminergic neurons or administration of an iron chelator prevents 1-methyl-4-phenyl-1,2,3,6-tetrapyridine-induced death of dopaminergic neurons *in vivo* (27). Furthermore, superoxide dismutase and glutathione 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 antioxidant genes such as glutamate-cysteine ligase, superoxide dismutase, glutathione peroxidase, and ferritin is dependent on Nrf2. Together, these observations suggest that coordinate upregulation of these programmed cell life genes through an Nrf2-ARE pathway may protect neurons from reactive oxygen species-induced neuronal cell death.

In this study, we raised and tested the hypothesis that decreased levels of detoxification enzymes and antioxidant proteins in $Nrf2^{-/-}$ neuronal cultures result in increased susceptibility to oxidative stress. To investigate this hypothesis, we used $Nrf2^{-/-}$ and $Nrf2^{+/+}$ neuronal cultures treated with 1-methyl-4-phenyl-1,2,5,6-tetrahydropyridine (MPP^+) or rotenone. Both of these compounds inhibit mitochondrial respiration (complex I) and are widely used in animal and/or cell culture models of PD (31–33). Furthermore, oligonucleotide microarray analysis was used to identify genes associated with this Nrf2-dependent differential sensitivity to inhibition of mitochondrial respiration.

EXPERIMENTAL PROCEDURES

Primary Neuronal Culture—$Nrf2^{+/-}$ mice were bred with $Nrf2^{+/-}$ mice, and primary neuronal cultures were prepared individually. Cerebral cortices from littermate embryos (gestation day 16) were removed, placed in ice-cold Hanks' balanced salt solution (3 ml/embryo; Invitrogen), centrifuged ($300 \times g$, 2 min), and digested individually (0.5 mg/ml trypsin (Invitrogen) in Hanks' balanced salt solution, 37 °C, 10 min). Tissues were washed twice with Hanks' balanced salt solution and resuspended in minimal essential medium with Earle's salt (Invitrogen) containing heat-inactivated (55 °C, 30 min) fetal bovine serum (10%; Atlanta Biologicals, Inc.) and horse serum (10%; Atlanta Biologicals, Inc.). Cell suspensions were sieved through cell strainers (70 μm; Falcon) and plated at a density of 1×10^6 cells/ml. After 45 min of initial plating, the medium was changed to neurobasal medium ($[Ca^{2+}] = 1.8$ mM) supplemented with B27 Minus AO (antioxidant) (Invitrogen). Cultures were maintained at 37 °C in a humidified tri-gas incubator (5% O_2, 90% N_2, and 5% CO_2; Forma Scientific, Inc.). The Nrf2 genotype of each culture was determined by a PCR-based method as described previously (11). Cultures were used for experiments between 2 and 7 days *in vitro* (DIV). Over 90% of the cells in the cultures (both $Nrf2^{-/-}$ and $Nrf2^{+/+}$) were neurons as determined by immunostaining of the astrocyte-specific marker glial fibrillary acidic protein (GFAP; Dako Corp.) and the neuron-specific markers microtubule-associated protein-2 (MAP2; Chemicon International, Inc.) and β_{III}-tubulin (Promega) (data not shown). In addition, there was no difference in the ratio of cell type (neurons *versus* astrocytes) between $Nrf2^{+/+}$ and $Nrf2^{-/-}$ cultures (data not shown).

NQO1 Activity—NQO1 enzymatic activity was determined by a colorimetric method for whole cell extracts (34) and by histochemistry for fixed cultures as described previously (35).

Cytotoxicity Assay—3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium salt (MTS; Promega) cytotoxicity assay and terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL; Roche Applied Science) staining were performed according to the manufacturers' protocols.

Immunohistochemistry—Cells on chamber slides were rinsed with phosphate-buffered saline; fixed in 4% paraformaldehyde for 20 min; and blocked with phosphate-buffered saline containing 1% goat serum, 1% bovine serum albumin, and 0.3% Triton X-100 for 1 h. Cells were incubated with primary antibodies (rabbit anti-MAP2 (1:200), mouse anti-β_{III}-tubulin (1:200), rabbit anti-GFAP (1:500), and rabbit anti-cleaved caspase-3 (1:200) (Cell Signaling); rabbit anti-Nrf2 (1:2000) and rabbit anti-GST Mu1 (1:1000) (Biotrin); and rabbit anti-GCLC (1:1000)) overnight at 4 °C, followed by incubation for 1 h with Texas Red- or fluorescein-conjugated secondary antibodies (1:200; Vector Laboratories, Inc.). Hoechst 33258 was used for nuclear counterstaining. Fluorescence microscope pictures were taken under the same conditions.

Western Blot Analysis and Total GSH Measurement—Western blot analysis for GST Mu1, GCLM, and GCLC were performed, and total GSH levels were measured as described previously (11).

Reverse Transcription (RT)-PCR—Total RNA was isolated by TRIzol reagent (Invitrogen), and cDNA was synthesized using a reverse transcription system (Promega) according to the manufacturers' protocols. Aliquots of cDNA were used for PCR amplification with *Taq* DNA polymerase (Promega). PCR primers specific to each gene are as follows: Nrf2 exons 1–3, 5′-AGTTCTCGCTGCTCGGACTA-3′ and 5′-AGGCATCTTGTTTGGGAATG-3′; Nrf2 exon 5, 5′-TCTCCTCGCTGGAAAAAGAA-3′ and 5′-AATGTGCTGGCTGTGCTTTA-3′; NQO1, 5′-CATTCTGAAAGGCTGGTTTGA-3′ and 5′-CTAGCTTTGATCTGGTTGTCAG-3′; GST Alpha4, 5′-GCCAAGTACCCTTGGTTGAA-3′ and 5′-CAATCCTGACCACCTCAACA-3′; GST Mu1, 5′-CTCCCGACTTTGACAGAAGC-3′ and 5′-CAGGAAGTCCCTCAGGTTTG-3′; GST Mu3, 5′-AATCTTGGCCTGGATTTTCC-3′ and 5′-GAGGAAGCGGCTACTCTTCA-3′; GCLM, 5′-ACCTGGCCTCCTGCTGTGTG-3′ and 5′-GGTCGGTGAGCTGTGGGTGT-3′; GCLC, 5′-ACAAGCACCCCCGCTTCGGT-3′ and 5′-CTCCAGGCCTCTCTCCTCCC-3′; visinin-like 1, 5′-AAGTCATGGAGGACCTGGTG-3′ and 5′-CAGGCCATCCTCATTCATTT-3′; calbindin-28K, 5′-TGTGGCACATTCTTTTCTGC-3′ and 5′-TGGCTACCTTCCCTTACCAA-3′; synaptotagmin, 5′-GACAAAAGTCCACCGGAAAA-3′ and 5′-CTCGAACGGAACTTCAAAGC-3′; hippocalcin, 5′-AGGAAGGGGCTAAGCAAGAC-3′ and 5′-CGTGAGTGCACGAGAGAGAG-3′; and β-actin, 5′-AGAGCATAGCCCTCGTAGAT-3′ and 5′-CCCAGAGCAAGAGAGGTATC-3′.

Oligonucleotide Microarray Analysis—Total RNA was obtained from $Nrf2^{-/-}$ and $Nrf2^{+/+}$ primary neuronal cultures. Biotinylated cRNA was prepared, and fragmented cRNA was hybridized to MG U74Av2 arrays (11, 19). Affymetrix Microarray Suite Version 5.0 was used to scan and analyze the relative abundance of each gene (analysis parameters: scaling target signal, 2500; Alpha1, 0.04; Alpha2, 0.06; Tau, 0.015; Gamma1L and Gamma1H, 0.025; Gamma2L and Gamma2H, 0.05; and pertubation, 1.1). Data were analyzed by rank analysis as previously described (11, 19). The reproducibility of comparisons was based on the coefficient of variation (cv; S.D./mean) for the -fold change of ranked genes. A distribution curve of the cv was used to determine a cv cutoff value. The cutoff values were cv < 1.0- and ≥ 1.3-fold for increased genes and cv > -1.0- and ≤ -1.3-fold for decreased genes. Gene categorization was based on the NetAffex Database.[2]

Adenoviral Infection—Recombinant adenoviral vectors (Ad-GFP, expressing GFP alone; and Ad-Nrf2, expressing GFP and Nrf2) were constructed using the Cre-lox system (Canadian Stroke Network Core Facility, University of Ottawa) (36). The Nrf2 cDNA was excised from pEF/Nrf2 (4) by *Not*I and *Hin*dIII restriction. Titers of all viruses were determined on HEK293 cells. $Nrf2^{-/-}$ primary neuronal cultures were infected with Ad-GFP or Ad-Nrf2 at a multiplicity of infection (m.o.i.) of 50 at 2 DIV for 48 h. Typically, 10–20% of the neurons and virtually 100% of the astrocytes were infected with viral vectors as determined by GFP fluorescence and MAP2 and GFAP staining.

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. 1*A*). Similarly, the NQO1 activity of $Nrf2^{+/+}$ neuronal cultures was significantly higher than that of $Nrf2^{-/-}$ neuronal cultures (Fig. 1*B*). This correlated with a complete lack of NQO1 histochemical staining in the $Nrf2^{-/-}$ neuronal cultures (Fig. 1*C*) and 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.

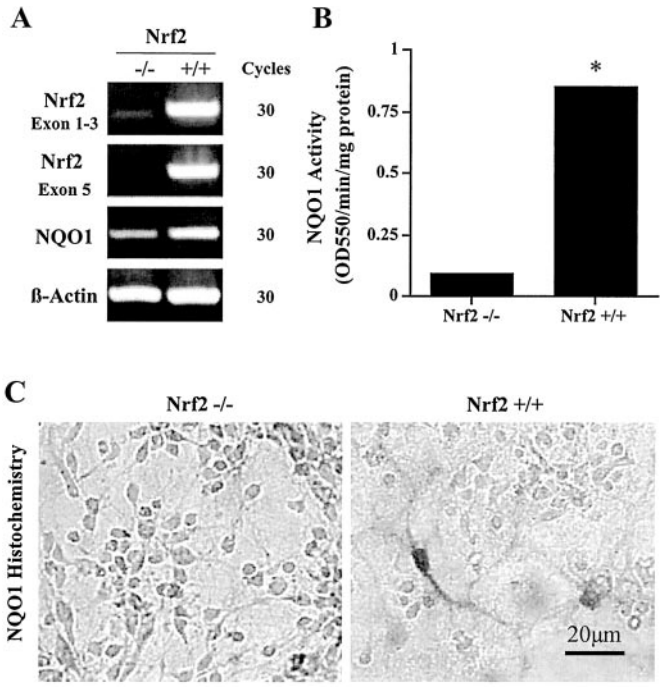

FIG. 1. **Nrf2-dependent NQO1 gene expression in primary neuronal cultures.** Total RNA was isolated from primary neuronal cultures, and cDNA was synthesized for PCR amplification of Nrf2, NQO1, and β-actin (all 30 cycles) (*A*). Two specific primer sets were used for selective amplification of the transactivation domain (exons 1–3) and basic leucine zipper domain (exon 5) of Nrf2 as indicated. NQO1 activity was determined by a colorimetric method (*B*) or by histochemistry (*C*) as described under "Experimental Procedures." *Data bars* represent means ± S.E. ($n = 6$). *, significantly different from $Nrf2^{-/-}$ neuronal cultures by Student's *t* test ($p < 0.05$).

gate whether this loss of Nrf2-dependent gene expression confers increased sensitivity of neurons to oxidative stress, we treated primary neuronal cultures with the well known mitochondrial toxins MPP^+ (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 $[Ca^{2+}]_i$. As shown in Fig. 2*A*, $Nrf2^{-/-}$ neuronal cultures were much more sensitive to MPP^+- or rotenone-induced cytotoxicity. Rotenone (0.1 μM) and MPP^+ (50 μM) induced 85% cell death (equivalent to 100% neuronal cell death) in $Nrf2^{-/-}$ neuronal cultures (Fig. 2*A*). The remaining MTS activity is presumably due to the surviving astrocytes. Strikingly, the same concentrations of MPP^+ and rotenone induced little or no cell death in $Nrf2^{+/+}$ neuronal cultures, respectively (Fig. 2*A*). This is reflected in the numbers of TUNEL-positive cells in MPP^+- or rotenone-treated $Nrf2^{-/-}$ neuronal cultures (Fig. 2*B*). There were far greater numbers of TUNEL-positive cells in the $Nrf2^{-/-}$ neuronal cultures than in the $Nrf2^{+/+}$ neuronal cultures (Fig. 2*B*). Double labeling with selective astrocyte or neuronal markers indicated that these apoptotic cells were neurons, not astrocytes (data not shown). MPP^+ and rotenone also preferentially activated the caspase-3 pathway in $Nrf2^{-/-}$ 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 $Nrf2^{+/+}$ and $Nrf2^{-/-}$ primary neuronal cultures. A total of 142 increased and 175 decreased genes were identified using rank analysis ($R \geq 4$, $R \leq -4$), 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

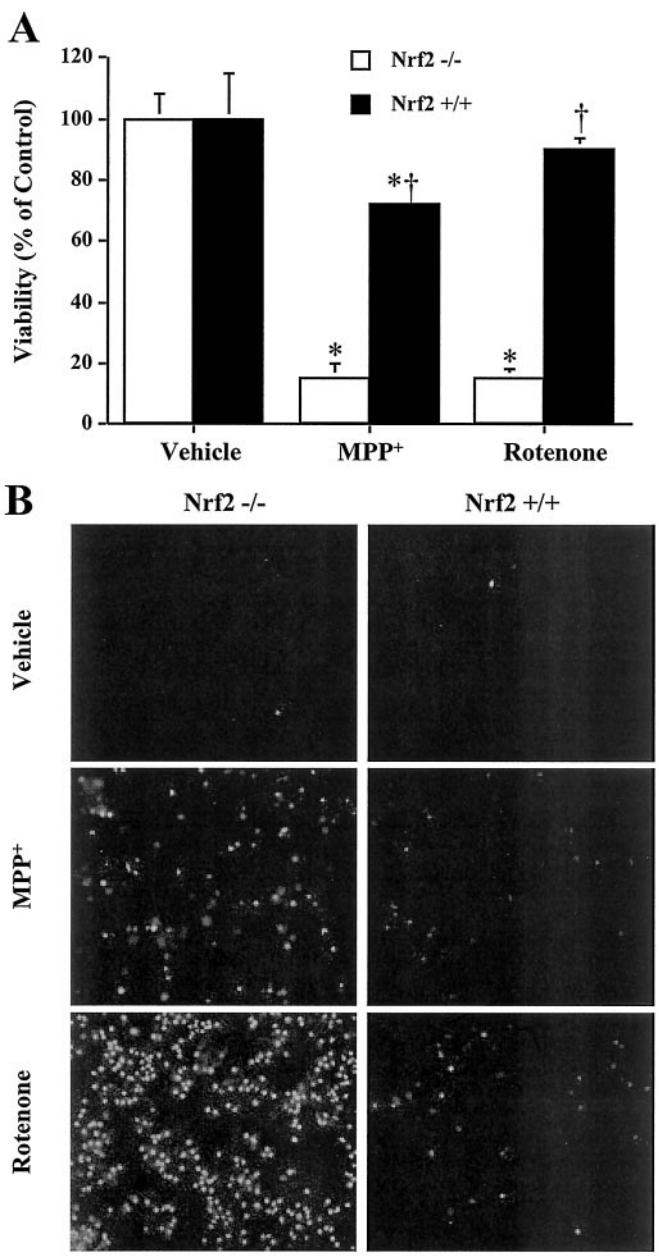

FIG. 2. **Differential sensitivity of neurons to mitochondrial toxins.** Primary neuronal cultures were treated with vehicle (0.01% Me_2SO), MPP^+ (50 μM), or rotenone (0.1 μM). After 24 h, MTS cytotoxicity assay (*A*) and TUNEL staining (*B*) were performed as described under "Experimental Procedures." *Data bars* represent means ± S.E. ($n = 6$). *, 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 ($p < 0.05$). Magnification is ×200 in *B*.

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).[3] 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/

[3] A complete list of all increased and decreased genes, including genes of unknown function and expressed sequence tags, is available upon request.

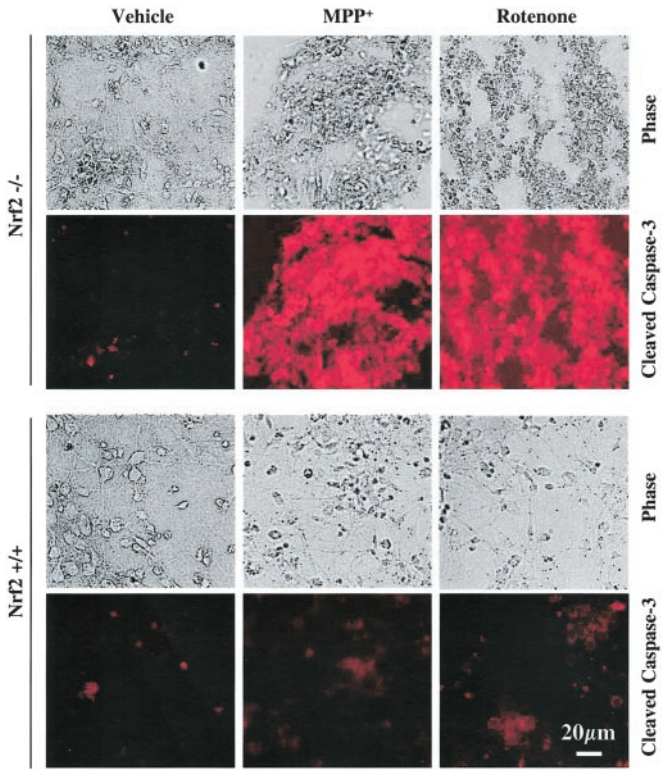

FIG. 3. **Preferential activation of caspase-3 in Nrf2$^{-/-}$ neuronal cultures by mitochondrial toxins.** Primary neuronal cultures were treated with vehicle (0.01% Me_2SO), MPP^+ (50 μM), or rotenone (0.1 μM). After 24 h, cells were stained for cleaved caspase-3 as described under "Experimental Procedures."

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. 4*A*) correlated well with the microarray data (Fig. 4*B*). In addition, Western blot analysis showed that the Nrf2$^{+/+}$ neuronal cultures had higher GST Mu1 and GCLC protein levels (Fig. 4*C*), and the total GSH level in Nrf2$^{+/+}$ neuronal cultures was significantly higher than that in Nrf2$^{-/-}$ neuronal cultures (Fig. 4*D*).

Important Role of Nrf2 in Calcium Homeostasis—Our microarray (Fig. 5*B* and Table I) and RT-PCR (Fig. 5*A*) 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. 5 (*A* and *B*) and Table I). 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 $[Ca^{2+}]_i$ 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. 5*C*). 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. 6*B*) and GFAP (astrocyte-specific marker) staining (Fig. 6*C*). 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. 6*C*). Infection of Nrf2$^{-/-}$ neuronal cultures with Ad-Nrf2 increased Nrf2 immunostaining (Fig. 6*D*) and its downstream targets such as GST Mu1 (Fig. 7*A*), GCLC (Fig. 7*B*), and NQO1 (Fig. 7*C*). 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. 7*D*). 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 μM rotenone or 1 μM 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 μM), 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 recovers 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,

TABLE I

Identification of Nrf2 target genes in primary neuronal cultures

The genes changed by Nrf2 were functionally categorized. R, rank; CV, coefficient of variation of fold; GEF, guanine nucleotide exchange factor; GABA, α-aminobutyric acid; LH-RH, luteinizing hormone-releasing hormone.

Gene	Accession no.	R.	Signal of Nrf2$^{-/-}$	−Fold ± S.E.	CV
Detoxification/antioxidant/reducing potential					
NQO1	U12961	4	120.8	4.9 ± 2.24	0.91
GST Alpha4	L06047	8	440.0	4.7 ± 2.06	0.88
GST Mu1	J03952	8	22,336.8	2.2 ± 0.11	0.10
GST Mu3	J03953	8	5050.8	1.7 ± 0.03	0.03
Malic enzyme, supernatant	J02652	6	4830.8	1.6 ± 0.19	0.25
GCLC	U85414	4	1256.1	1.3 ± 0.13	0.19
Catalase-1	M29394	7	1486.0	1.3 ± 0.04	0.06
Calcium Homeostasis					
Visinin-like 1	D21165	6	10,455.3	1.8 ± 0.44	0.48
Calbindin-28K	D26352	5	1265.1	1.8 ± 0.38	0.42
Synaptotagmin-1	D37792	8	5245.5	1.6 ± 0.16	0.20
Hippocalcin	AB015200	6	2892.9	1.5 ± 0.24	0.32
Synaptotagmin-5	AB026806	4	8830.2	1.4 ± 0.19	0.27
Nucleobindin-2	AJ222586	4	453.0	1.4 ± 0.09	0.13
Calmodulin III	AI842328	6	27,383.9	1.4 ± 0.16	0.24
Ryanodine receptor-3	X83934	4	1485.9	1.3 ± 0.06	0.09
Growth factors					
Nerve growth factor-γ	AV043739	4	38.1	2.7 ± 0.46	0.34
Fibroblast growth factor-13	AF020737	4	1481.6	1.3 ± 0.16	0.24
Brain-derived neurotrophic factor	X55573	4	1610.6	1.3 ± 0.15	0.23
Signaling					
Regulator of G-protein signaling 11	AF061934	4	1677.3	2.4 ± 0.75	0.63
Rho GEF-3	A1853706	4	453.8	1.6 ± 0.27	0.34
Protein-tyrosine phosphatase receptor type D	D13903	4	2446.4	1.4 ± 0.20	0.29
Neuronal GEF	AW050346	6	4796.3	1.4 ± 0.14	0.19
Protein kinase inhibitor-α	AW125442	4	21,774.3	1.4 ± 0.17	0.25
GABA-A receptor-α2	M62374	8	3896.4	1.4 ± 0.11	0.16
Megakaryocyte-associated tyrosine kinase	D45243	6	2644.9	1.4 ± 0.09	0.13
Phosphatidylinositol-4-phosphate 5-kinase type 1-γ	AB006916	4	543.1	1.3 ± 0.15	0.22
Mitogen-activated protein kinase-10	L35236	4	7146.5	1.3 ± 0.13	0.20
Protein-tyrosine phosphatase receptor type O	U37465	5	6840.5	1.3 ± 0.13	0.20
Protein kinase C-β	X53532	4	1782.6	1.3 ± 0.04	0.07
G-protein-γ3	AF069953	4	35,274.8	1.3 ± 0.13	0.20
GABA-A receptor-1	U14418	4	6611.1	1.3 ± 0.16	0.23
GABA-B receptor-1	AL078630	6	13,361.3	1.3 ± 0.13	0.21
Adenylate cyclase-activating polypeptide-1	AB010149	4	955.8	1.3 ± 0.16	0.26
Inosine 5′-phosphate dehydrogenase-1	U00978	5	2082.8	1.3 ± 0.11	0.17
Receptor/channel/carrier protein					
Potassium voltage-gated channel shaker-related β1	AF033003	4	444.9	2.5 ± 0.75	0.60
Potassium large conductance Ca^{2+}-activated channel Mα1	U09383	6	3434.3	1.4 ± 0.14	0.21
Solute carrier family 7 (y^+ system), member 8	AW122706	6	563.7	1.4 ± 0.10	0.15
Solute carrier family 3, member 1	AW121154	6	9570.4	1.3 ± 0.08	0.11
Chloride channel 4–2	Z49916	4	4720.2	1.3 ± 0.16	0.24
Potassium voltage-gated channel shaker-related β2	U65592	4	1033.0	1.3 ± 0.11	0.18
Solute carrier family 1, member 1	U73521	4	1276.6	1.3 ± 0.17	0.26
Solute carrier family 30 (zinc transporter), member 4	AF004100	6	2080.8	1.3 ± 0.02	0.04
Neuron-specific proteins					
Kinesin family member C2	U92949	4	78.0	4.1 ± 1.24	0.60
Tubby	U54643	4	184.1	3.1 ± 0.56	0.37
Nasal embryonic LH-RH factor	AI849565	4	1258.8	2.1 ± 0.29	0.28
Myocyte enhancer factor-2C	L13171	8	4204.2	2.0 ± 0.31	0.31
Neurogenic differentiation-1	U28068	4	543.7	1.6 ± 0.29	0.35
L1 cell adhesion molecule	X12875	6	2279.1	1.6 ± 0.28	0.34
Reelin	U24703	8	8198.4	1.6 ± 0.26	0.31
Secretogranin II	X68837	6	2516.5	1.5 ± 0.20	0.27
Chromogranin B	X51429	6	7396.8	1.4 ± 0.16	0.22
Synaptosome-associated protein, 25 kDa	M22012	6	11,907.0	1.4 ± 0.18	0.26
Vesicular membrane protein p24	D83206	6	1555.5	1.4 ± 0.08	0.12
Synaptophysin	X95818	4	2938.4	1.4 ± 0.11	0.17
Synapsin II	AF096867	4	4310.7	1.4 ± 0.11	0.17
Prolyl endopeptidase	AB022053	4	799.2	1.4 ± 0.11	0.16
Amyloid-β (A4) precursor protein binding A2	L34676	4	2672.2	1.3 ± 0.14	0.20
Dynactin-3	AF098508	6	12,963.5	1.3 ± 0.07	0.10
Defense/immune/inflammation					
Small inducible cytokine B subfamily, member 5	U27267	6	350.7	2.3 ± 0.63	0.56
Platelet-activating factor acerylhydrolase	U34277	6	1532.7	1.6 ± 0.29	0.37
Interferon-γ receptor-2	U69599	4	2847.5	1.3 ± 0.10	0.16
Interferon-related developmental regulator-1	V00756	4	1727.6	1.3 ± 0.12	0.19

inhibit mitochondria complex I, resulting in increased reactive oxygen species production, increased $[Ca^{2+}]_i$, 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 $[Ca^{2+}]_i$ induced by

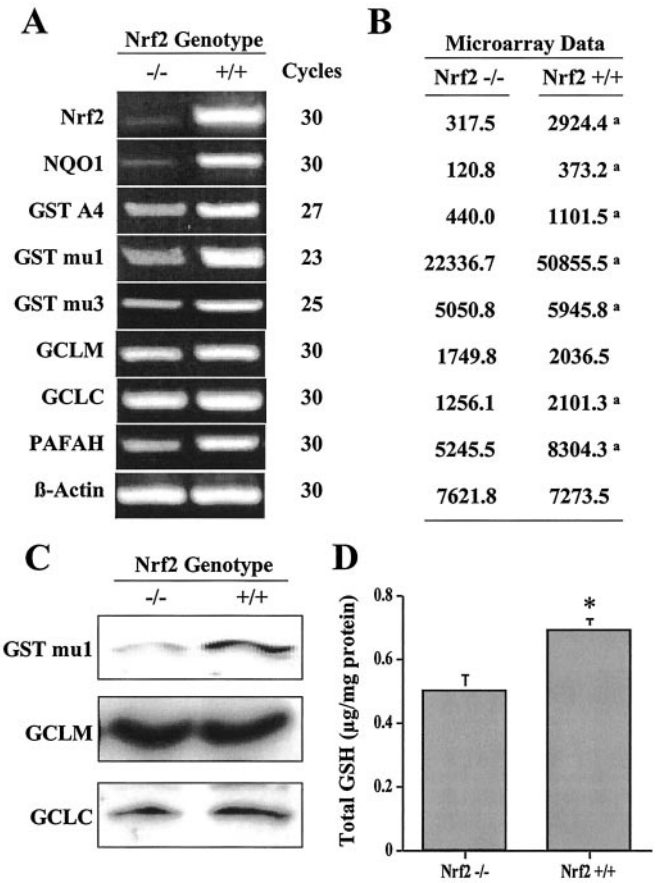

FIG. 4. **Verification of microarray data.** *A*, total RNA was isolated from primary neuronal cultures, and cDNA was synthesized for PCR amplification. *GST A4*, GST Alpha4; *PAFAH*, platelet-activating factor acetylhydrolase. *B*, the corresponding averages of the signal values from the microarray 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. ($n = 6$). [a], genes called "increased" in the microarray analysis; *, significantly different from $Nrf2^{-/-}$ neuronal cultures by Student's *t* test ($p < 0.05$).

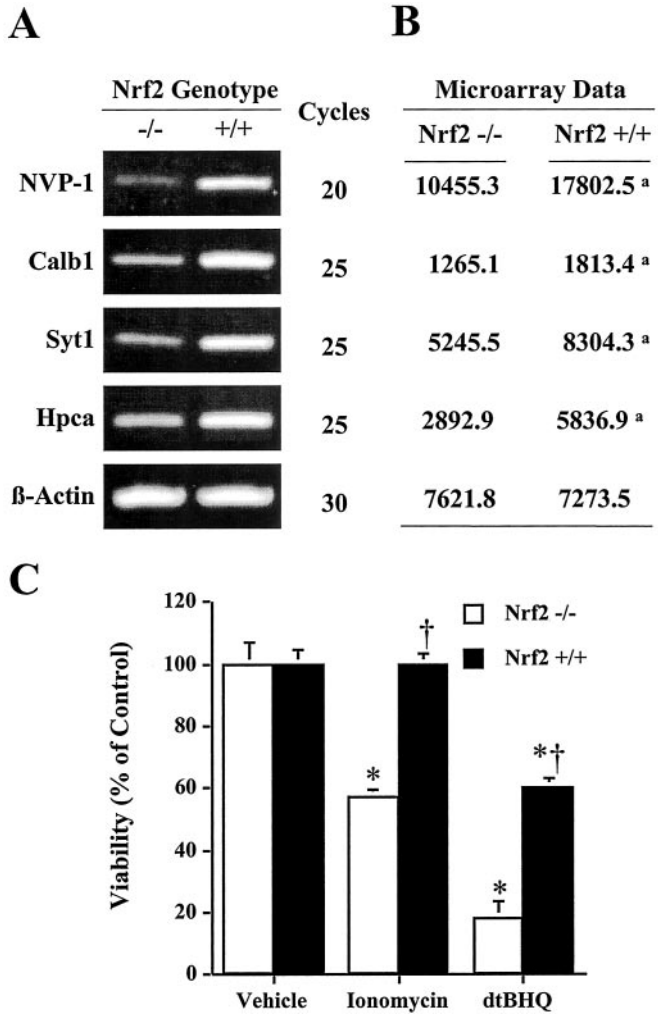

FIG. 5. **Important role of Nrf2 in Ca^{2+} homeostasis.** *A*, total RNA was isolated from primary neuronal cultures, and cDNA was synthesized for PCR amplification. *NVP-1*, visinin-like 1; *Calb1*, calbindin-28K; *Syt1*, synaptotagmin-1; *Hpca*, hippocalcin. *B*, the corresponding averages of the signal values from the microarray data are listed. *C*, primary neuronal cultures were treated with vehicle (0.01% Me_2SO), 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. ($n = 4$). [a], 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 ($p < 0.05$).

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 $[Ca^{2+}]_i$. 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_2O_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. Outside of these five genes, 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 very 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

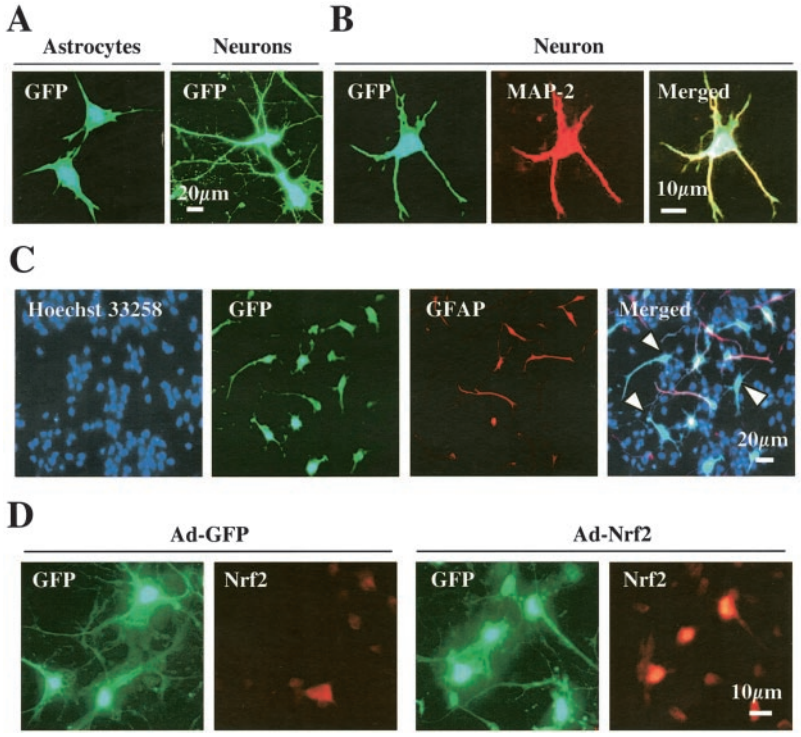

FIG. 6. **Adenoviral vector-mediated Nrf2 expression.** Primary Nrf2$^{-/-}$ neuronal cultures were infected with Ad-GFP (m.o.i. = 50) or Ad-Nrf2 (m.o.i. = 50) at 2 DIV (for 48 h). *A*, GFP fluorescence was observed in both astrocytes and neurons. *B*, neuronal infection was determined by GFP fluorescence and MAP2 staining as described under "Experimental Procedures." *C*, astrocytes were preferentially infected by adenoviral vector as determined by GFAP staining. *Arrowheads* indicate neurons. *D*, Ad-Nrf2-mediated Nrf2 expression was confirmed by GFP fluorescence and Nrf2 staining. Fluorescence microscope pictures were taken under the same conditions.

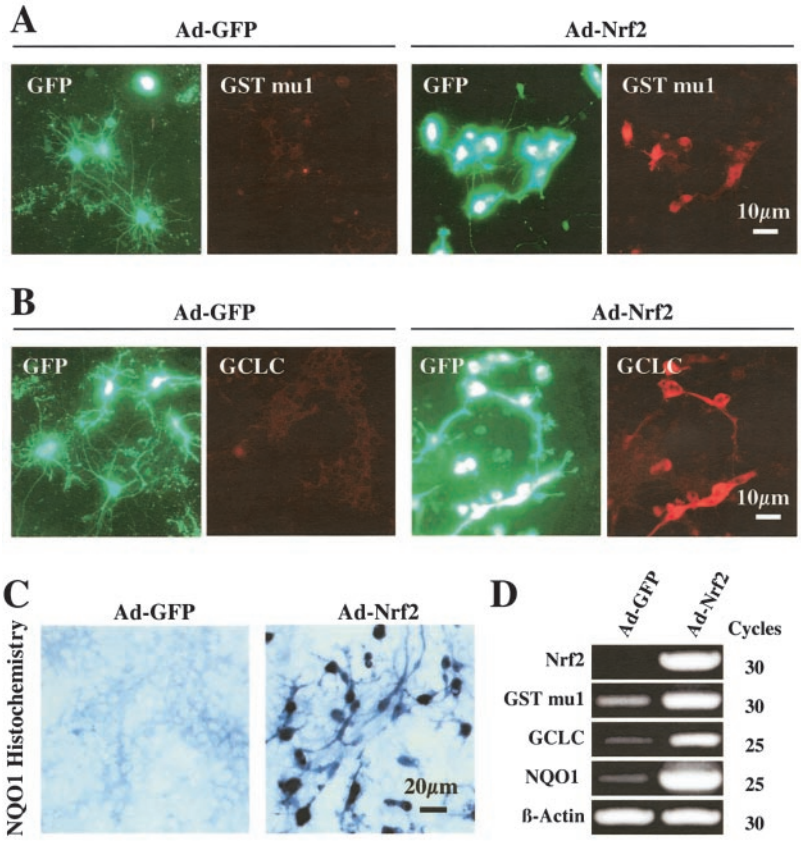

FIG. 7. **Nrf2-dependent ARE-driven gene expression.** Primary Nrf2$^{-/-}$ neuronal cultures were infected with Ad-GFP (m.o.i. = 50) or Ad-Nrf2 (m.o.i. = 50) at 2 DIV (for 48 h). Immunostaining (GST Mu1 (*A*) and GCLC (*B*)), histochemistry (NQO1 (*C*)), and RT-PCR (*D*) were performed as described under "Experimental Procedures." Fluorescence microscope pictures were taken under the same conditions.

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 survivability (14). In the neuron-enriched culture system used here, astrocytes account for ~10% the of 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,

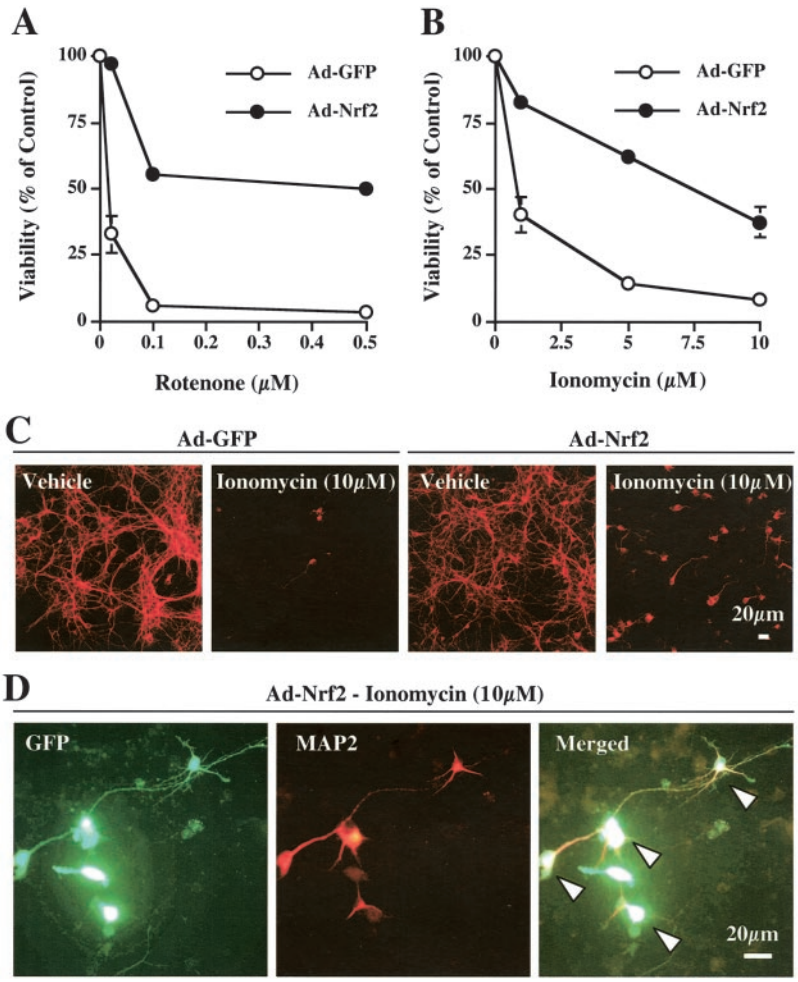

FIG. 8. **Neuroprotection by Nrf2.** Primary Nrf2$^{-/-}$ neuronal cultures were infected with Ad-GFP (m.o.i. = 50) or Ad-Nrf2 (m.o.i. = 50) at 2 DIV (for 48 h). *A* and *B*, infected neuronal cultures were treated with rotenone or ionomycin (24 h), respectively, for MTS cytotoxicity assay. *Data bars* represent means ± S.E. ($n = 6$). *C*, infected neuronal cultures were treated with vehicle (0.01% Me_2SO) 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.

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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