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NRF2-MEDIATED PROTECTION AGAINST 6-HYDROXYDOPAMINE

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Abstract

Parkinson's disease (PD) is a neurodegenerative movement disorder characterized by cell loss in the substantia nigra resulting in striatal dopamine depletion. Although the cause of sporadic PD is unknown, oxidative stress is thought to contribute to disease pathogenesis. One mechanism by which cells defend themselves against oxidative stress is through the transcriptional upregulation of cytoprotective genes. Under oxidative stress conditions, the transcription factor NF-E2 related factor (Nrf2) binds to the antioxidant response element (ARE) to induce antioxidant and phase II detoxification enzymes. Here we show that loss of Nrf2-mediated transcription exacerbates vulnerability to the neurotoxin 6-hydroxydopamine (6-OHDA) both *in vitro* and *in vivo*. We further demonstrate that activation of the Nrf2-ARE pathway by the known chemical inducer *tert*-butylhydroquinone can protect against 6-OHDA *in vitro*. Induction of this pathway by transplantation of astrocytes overexpressing Nrf2 can protect against 6-OHDA-induced damage in the living mouse. This suggests that the Nrf2-ARE pathway is a promising target for therapeutics aimed at reducing or preventing cell death in PD.

1. INTRODUCTION

There is abundant evidence supporting a role for oxidative stress in neurodegenerative diseases such as Parkinson's disease (PD). Cells respond to oxidative stress by upregulating a number of antioxidant and phase II detoxification genes via the antioxidant response element (ARE), a *cis*-acting enhancer sequence found in the promoter of the genes it regulates [38]. Transcriptional regulation by the ARE is accomplished via binding of the transcription factor NF-E2 related factor (Nrf2) to the ARE [48]. Under basal conditions, Nrf2 is sequestered by Keap1 in the cytosol [12,20]. Upon exposure to ARE activators, including oxidative stress conditions, Nrf2 is released from Keap1 and translocates to the nucleus, whereby it can bind to the ARE and activate transcription of ARE-regulated genes such as heme oxygenase-1

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(HO-1) [35], NAD(P)H quinone oxidoreductase-1 (NQO1) [15,47], glutathione-S-transferases (GSTs) [39], and other glutathione synthesizing enzymes [16,17,31]. Indeed, the chemicals sulforaphane and *tert*-butylhydroquinone (tBHQ) have been shown to protect a variety of different cell types under toxic conditions via an Nrf2-ARE mechanism [18,21,23,34,42,45].

Nrf2, the protein responsible for inducible ARE activation, is a basic leucine zipper protein and a member of the cap'n'collar family of transcription factors [30]. Nrf2 knockout mice are normal in appearance; however, they do display abnormally grayish-white teeth [25,49]. A proportion of aged Nrf2^{-/-} mice demonstrate autoimmune hemolytic anemia, autoantibodies, and a lupus-like phenotype in a 3:1 (females:males) ratio [25,29,50]. Adult Nrf2 knockout mice have decreased basal activity of some ARE-regulated genes and normal expression of others, suggesting multiple mechanisms of transcriptional activation of ARE-regulated genes [24]. However, Nrf2 knockout mice do not display inducible ARE activity in response to changes in redox status and oxidative stress [24]. Consequently, Nrf2 null mice demonstrate increased vulnerability to a variety of stressors including carcinogens [36], acetaminophen [13], and acute pulmonary injury [6,8].

Parkinson's disease (PD) is characterized by loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) that project to the striatum. The resultant dopamine depletion manifests as tremor, akinesia, rigidity, and postural instability. Most PD cases are considered to be idiopathic, although a subset of PD patients display mutations in known genes. The cause of sporadic PD is unknown and likely multifactorial. Still, the mechanisms of disease pathogenesis are being revealed.

There is evidence for oxidative stress in PD [22]. Post mortem brain tissue from PD patients has revealed increased oxidized proteins, lipids, and nucleic acids such as protein carbonyls, 4-hydroxy-2-nonenol, and 8-hydroxy-2-deoxyguanosine [2,3,5,9–11]. There is also evidence for increased antioxidant activity such as superoxide dismutase-1 [40] and utilization of reduced glutathione [44]. Furthermore, experimental interventions aimed reducing oxidative stress, such as overexpression of glutathione peroxidase [37], N-acetylcysteine [32], and vitamin E [4] have shown to be neuroprotective in animal models. Taken together, the data suggest that oxidative stress may be a factor in the pathogenesis of PD.

There is increasing evidence of a relationship between the Nrf2-ARE pathway and neurodegenerative disease. ARE-driven genes, such as NQO1 and HO-1, are found to be upregulated in post mortem brain tissue from PD patients [41,46,51]. Recently, we have shown that 6-hydroxydopamine (6-OHDA), a reactive oxidative stressor and complex I inhibitor used to model PD, activates the ARE through a combination of oxidative, excitotoxic, and likely structural factors, both *in vitro* and *in vivo* [21]. This activation *in vivo* follows a similar time course as the loss of tyrosine hydroxylase (TH) immunoreactivity, the rate-limiting enzyme in dopamine synthesis. This suggests that ARE activation induced by 6-OHDA and resultant transcription of cytoprotective genes may reflect an attempt to protect against the loss of synaptic terminals. Our lab has also shown that Nrf2^{-/-} neurons are more vulnerable to two complex I inhibitors, rotenone and MPP+ *in vitro* [28].

The current study tested the hypothesis that Nrf2 is critical in mediating the toxicity of 6-OHDA both *in vitro* and *in vivo*. More specifically, we examined whether the absence of Nrf2 protein exacerbates cell loss due to 6-OHDA both *in vitro* and *in vivo*. We also observed whether further induction of the Nrf2-ARE pathway by either chemical or genetic means could protect against 6-OHDA toxicity. Here we show that Nrf2 plays a crucial role in controlling damage due to complex I inhibitors. Moreover, we show that further ARE activity can protect both *in vitro* and *in vivo*.

2. RESULTS

TBHQ ACTIVATES THE ARE

tBHQ is a known ARE activator via stabilization of Nrf2. In ARE-hPAP+ cortical cells, tBHQ (10 μ M) can cause an over 30-fold induction in ARE activity [21]. The dopaminergic N27 cell line has never been assessed for ARE-responsiveness and activity. Therefore, in order to determine if this line responds similar to primary cortical cells, N27 cells were transfected with an ARE-luciferase (ARE-luc) construct with β -galactosidase (β -gal) to control for number of cells per well. Cells were then exposed to tBHQ overnight and assayed for activity. N27 cells demonstrate a dose-dependent increase in ARE-luc activity, with maximal activation at 10 μ M ($p < 0.05$; FIG. 1A). At doses higher than 30 μ M, ARE activation falls below control values suggesting toxicity due to tBHQ (FIG. 1A).

ARE activation was further supported by RT-PCR analysis of N27 cells treated with tBHQ for 24 hours. Cells were harvested for total RNA; using RT-PCR, the relative amounts of some ARE-regulated genes were qualitatively revealed (FIG. 1B). In comparison to cells exposed to vehicle, tBHQ treatment increased the relative expression levels of GSTmu3, NQO1, and HO-1, all ARE-regulated genes, suggesting that the N27 line demonstrates ARE induction by tBHQ.

TBHQ PROTECTS AGAINST 6-OHDA IN N27 CELLS

To assess if tBHQ can protect in a dopaminergic cell line, N27 cells were plated in 96-well plates and treated with tBHQ or vehicle for 24 hours. The tBHQ was removed and cells were treated with varying doses of 6-OHDA and assayed for cell viability using the MTS assay. 6-OHDA caused a dose-dependent toxic effect in N27 cells ($p < 0.05$). Pretreatment with tBHQ significantly protected against cell death due to 6-OHDA as compared to vehicle control (37.5–150 μ M; $p < 0.05$; FIG. 1C).

INCREASED VULNERABILITY OF NRF2^{-/-} CELLS TO 6-OHDA *IN VITRO*

The role of Nrf2 was examined in Nrf2 knockout and wildtype cultures. Nrf2 heterozygote mice were mated and E16-18 pups were individually cultured for cortical neurons and later genotyped. Cortical neurons were used in order to get a quantity of cells sufficient for the assays. Cells plated in chamber slides were exposed to 50 μ M 6-OHDA for 24 hours (FIG. 2). Slides were then fixed and stained for apoptotic cells using the TUNEL assay (FIG. 2A). The proportion of TUNEL+ cells was determined as a fraction of Hoechst-stained nuclei for each embryo. In the presence of 6-OHDA, both genotypes demonstrated significantly more toxicity as compared to vehicle alone (FIG. 2B; $p < 0.05$). However, Nrf2^{-/-} cells demonstrated significantly more TUNEL+ cells (45.13 $\pm$ 6.7%) as compared to wildtype cells (20.38 $\pm$ 3.8%) in the presence of 6-OHDA ($p < 0.05$; FIG. 2B).

Individual Nrf2^{+/+}, ^{+/-}, and ^{-/-} embryos were cultured in 96-well plates and exposed to various concentrations of 6-OHDA (FIG. 2C). Nrf2 null cells demonstrated significantly more cell death as compared to wildtype cells, as measured by the MTS assay at 25 μ M, 50 μ M, and 75 μ M 6-OHDA, ($p < 0.05$; FIG. 2C). Nrf2 heterozygote cells were not significantly more vulnerable than wildtype cells at any concentration tested ($p > 0.05$; FIG. 2C).

INCREASED VULNERABILITY IN NRF2^{-/-} MICE *IN VIVO*

To assess if Nrf2 knockout mice would also display increased vulnerability to 6-OHDA similar to cultured cells, animals were stereotactically administered 6-OHDA into the right striatum. After a week, animals were sacrificed and tissue was processed and stained for TH immunoreactivity (THir; FIG. 3).

Nrf2 knockout mice demonstrated increased loss of striatal THir as compared to wildtype animals receiving the same dose of 6-OHDA (FIG. 3A). Lesion volume was quantified and Nrf2 null animals demonstrated lesions that were approximately twice as large as lesions in wildtype animals ($Nrf2^{+/+}$, $1.317 \pm 0.345 \text{ mm}^3$; $Nrf2^{-/-}$, $2.498 \pm 0.385 \text{ mm}^3$; $p < 0.05$; FIG. 3B). Loss of TH⁺ cells in the SNpc was also more prominent in the knockout mice as compared to the wildtype mice (FIG. 3C). Contralateral striata were injected with saline; however, both wildtype and knockout mice did not show lesions due to the vehicle control (FIG. 3A and C).

NRF2-EXPRESSING ASTROCYTES PROTECT AGAINST 6-OHDA *IN VIVO*

Currently, there are no compounds known to activate the ARE that cross the blood brain barrier to a significant degree. Consequently, to assess whether Nrf2 expression could protect against 6-OHDA *in vivo* we used a transplantation approach. Cultured astrocytes were infected with either adeno-Nrf2 or adeno-GFP and transplanted into the right striata of wildtype mice with a contralateral vehicle control. Five weeks later, the animals were subjected to bilateral intrastriatal 6-OHDA injections. One week following the 6-OHDA lesions, animals were euthanized and tissue was collected for lesion volume analysis and assessment of transplant viability. After the transplant surgeries, the remaining untransplanted cells were plated and assessed for infection (FIG. 4A). Approximately 75% of cells express the GFP construct after 24 hours at 200MOI (data not shown). Cells were also stained for GFAP immunoreactivity to demonstrate that they were primarily astrocytes (FIG. 4A). Greater than 80% of the cells demonstrated GFAP-immunoreactivity at 8 DIV (data not shown).

Six weeks after the initial transplant surgery, and one week after bilateral 6-OHDA lesions, animals were euthanized and serial sections were mounted on glass slides and coverslipped. Astrocytes transduced with adeno-GFP survived equally well in the mouse parenchyma as cells infected with adeno-Nrf2 (FIG. 4B), as demonstrated by fluorescent microscopy. GFP expression from the transplants was strong enough to be detected without immunocytochemical enhancement.

Floating brain sections were stained for THir to demonstrate 6-OHDA-induced lesions (FIG. 5). The lesion areas of both transplanted and control striata were assessed and lesion volumes across the rostral-caudal extent were calculated (FIG. 5). Due to the experimental design employed by this study, the effect of the transplanted astrocytes upon lesion volume was statistically compared to the contralateral striata in a paired comparison. Adeno-GFP astrocyte transplants showed a trend toward protection; however, this was not statistically significant when compared with the vehicle pre-treated contralateral hemisphere (FIG. 5B). However, animals transplanted with adeno-Nrf2 infected astrocytes demonstrated significantly reduced lesion volumes as compared to the contralateral control hemisphere ($p < 0.05$; FIG. 5B).

3. DISCUSSION

The ARE is an enhancer element found in the promoter of many genes such as GSTs, NQO1, and HO-1 that can detoxify and defend against changes in redox status. Under conditions of oxidative stress, the inducible transcription factor Nrf2 may bind to the ARE to activate the transcription of these protective genes. In the current study, we have shown the importance of the Nrf2-ARE pathway both *in vitro* and *in vivo* in a model of PD.

There is ample evidence to support a role for oxidative stress in the pathogenesis of PD [22]. Oxidative stress may be a consequence of mitochondrial complex I inhibition, excitotoxicity, or inflammation. Due to the prominent role of oxidative stress in PD pathogenesis and evidence for increased ARE-regulated genes in post mortem PD brains [41,46], we hypothesized that the Nrf2-ARE pathway may play a role in PD.

Previously, we have shown that 6-OHDA activates the ARE both *in vivo* and *in vitro* [21]. Furthermore, the complex I inhibitors rotenone and MPP⁺ were both shown to be more toxic in cultured Nrf2^{-/-} neurons [28]. This suggests that the Nrf2-ARE pathway may play a role in limiting complex I-mediated cellular dysfunction. However, this had never been shown *in vivo*. Here we show that both in culture and in the living animal, endogenous Nrf2 expression is critical to control the extent of cellular damage due to the neurotoxin 6-OHDA. Cultured neurons from Nrf2 null embryos exposed to 6-OHDA show increased apoptotic cells as well as attenuated cellular viability as compared to cells that can engage an Nrf2-ARE response (FIG. 2). This *in vitro* data was further supported by 6-OHDA injections into the striata of Nrf2^{-/-} mice. Animals lacking Nrf2 demonstrate lesions almost twice the size of wildtype animals (FIG. 3).

Cell death still occurs in animals with intact Nrf2-ARE transcriptional pathways. This may be due to several factors: the system may not be working at maximal capacity, the toxin may kill cells before a transcriptional defense can be mounted, or the genes controlled by the ARE are insufficient to prevent toxicity due to 6-OHDA. Likely, it is some combination of these factors. Here, we tested the ability of further induction of the ARE to protect against 6-OHDA in culture and *in vivo*.

The compound tBHQ is a known ARE activator at doses that do not cause oxidative stress [27]. In comparison to the maximal induction caused by 6-OHDA, tBHQ at a non-toxic dose causes a five-fold induction in primary cells, which is approximately 30-fold induction above vehicle alone [21]. When 6-OHDA is added to tBHQ, the ARE is not significantly activated above induction due to tBHQ alone [21]. This suggests that the dose of tBHQ used in this study maximally induces the ARE pathway.

In order to assess the effects of further ARE induction in a dopaminergic cell line, we first demonstrated that tBHQ also induces the transcription of ARE-regulated genes in these cells (FIG. 1A and B). The responsiveness of the N27 cells to tBHQ was not as dramatic as that seen in cortical cells. This may be due to a variety of reasons. ARE activation was measured by two different mechanisms, transfection versus transgenic reporter gene [21]. There may be differences in basal ARE activity due to the immortalization process in the N27 cell line. Lastly, in the previously published experiments involving primary cells, tBHQ was applied for 48 hours as compared to 24 hours in the N27 cells. This modification was taken due to the high rate of cell division in the N27 line, as the cells double in less than 24 hours and must be seeded at extremely low cell numbers to prevent overgrowth of the wells (unpublished observations). Even when plated at low concentrations, a 48-hour exposure followed by another 24 hours of 6-OHDA treatment led to concentrations of cells that were no longer vulnerable to 6-OHDA due to cellular overgrowth (data not shown). Still, as in primary cells, tBHQ also significantly protects against 6-OHDA in N27 cells (FIG. 1C).

Currently, there is a lack of known ARE activators that enter the brain at concentrations high enough to cause measurable ARE activation. Consequently, to demonstrate that Nrf2-ARE activation can protect against 6-OHDA *in vivo*, Nrf2- or GFP-expressing astrocytes were transplanted into one hemisphere of wildtype animals (FIG. 4–5). *Ex vivo* gene manipulation with adenovirus was used to circumvent the immune reaction that can occur when adenovirus is administered into the *in vivo* brain. Transduced astrocytes survived six weeks with no difference between adeno-GFP or adeno-Nrf2 cells. Mice that received adeno-GFP astrocytes demonstrated mild protection from 6-OHDA, which did not reach statistical significance. However, astrocytes expressing Nrf2 provided significant protection against 6-OHDA (FIG. 5). This shows that additional Nrf2 can have a protective effect against 6-OHDA *in vivo*.

At present, there are no neuroprotective drugs for neurodegenerative disease such as PD. Clinical trials have explored the use of antioxidants, energy supplementation, anti-excitotoxic, and growth factors to no avail [14,19,43]. Consequently, it is critical that researchers find new drug targets for neuroprotection. The data shown here strongly suggest that modulating the ARE may be a promising therapeutic target in PD.

4. EXPERIMENTAL PROCEDURES

ANIMALS

All mice used in these studies were housed in the vivarium at the University of Wisconsin School of Pharmacy and treated according to the regulations set by the university's IACUC as well as the laboratory's approved animal protocol. All animals were maintained under standard laboratory conditions, with a 12:12 hour light:dark schedule, and food and water available *ad libitum*. Wild-type C57Bl6/SJL mice were obtained directly from Jackson labs. The transgenic Nrf2^{-/-} line was created through a targeted disruption of the Nrf2 allele [7] and kindly provided to us by Dr. Y.W. Kan of the University of California-San Francisco Comprehensive Cancer Center. The presence of a transgene was confirmed by PCR amplification of a region of DNA bridging the insertion site.

CHEMICALS AND REAGENTS

All chemicals and reagents used were obtained from Sigma unless specifically noted. 6-Hydroxydopamine was dissolved in 0.15% ascorbate in 0.9% sterile saline. *tert*-butylhydroquinone (tBHQ, Acros) was dissolved in 0.1% DMSO, with appropriate DMSO vehicle controls. All other compounds used *in vitro* were dissolved in sterile media specific to the culture type, unless otherwise noted.

PRIMARY CULTURE

Primary cortical neuronal cultures were generated from individual E16-18 embryos derived from crossing Nrf2^{+/-} male and Nrf2^{+/-} female mice. Briefly, embryonic tails were removed for genotyping analysis. Individual cortices were dissected and dissociated with trypsin, and plated on individual poly-D-lysine coated 96-well plates or on CC2-treated chambers slides (LabTek) at a density of 100,000 cells or 400,000 cells, respectively. Cells were initially plated in "CMEM" media containing modified eagle media (MEM), fetal bovine serum (FBS), horse serum, L-glutamine, and penicillin/streptomycin/fungicide (PSF). After one hour, the media was replaced with neurobasal (Gibco BRL) containing B27 (without antioxidants), PSF, and L-glutamine for the duration of the experiment. Two days after plating, cells were exposed to 6-OHDA for 24 hours. All data points were collected in triplicate, with each embryo serving as an individual N and are reported as averages $\pm$ standard error or measurement (SEM). Data were analyzed with unpaired, two-tailed student t-tests.

N27 CELL LINE

The N27 cell line was kindly provided by Dr. Curt Freed of the University of Colorado Health Sciences Center. This dopaminergic cell line was previously created by transfecting 12 DIV rat fetal mesencephalic cells with the large T cell antigen of the SV 40 virus [33]. This cell line produces DA at levels higher than undifferentiated or differentiated murine neuroblastoma cells [1]. Cells were maintained in media containing RMPI 1640 with glutamine, 10% FBS, and 1% PSF and passaged by conventional trypsinization.

For experiments, cells were plated at a density of approximately 5000 cells per well of a 96-well plate. This concentration was predetermined to prevent cells from growing to confluency prior to the end of the experiment rendering them insensitive to toxins (data not shown). The

next day, cells were treated with 10 μ M tBHQ or vehicle. Twenty-four hours later, the media was changed and cells were exposed to varying concentrations of 6-OHDA. The next day, plates were assayed for cell death via the MTS assay described as follows.

STEREOTAXIC 6-OHDA INJECTIONS

A total of 12 mice (7 Nrf2 wildtype and 5 Nrf2 knockout), aged 7–15 months were anesthetized with isoflurane, and injected with 6-OHDA in ascorbate (4 μ g in 1 μ l) into the right striatum with vehicle control injected into the left at the coordinates: + 0.5mm anterior to bregma; $\pm$ 2.0mm lateral to midline; –3.1mm ventral to dura using a stereotactic frame. The solutions were administered in a Hamilton syringe, which was inserted into the proper coordinates and allowed to equilibrate for two minutes prior to injection over two minutes. Following another two-minute equilibration period, the syringe was withdrawn over three minutes.

PRIMARY ASTROCYTE CULTURES AND TRANSPLANTS

Primary astrocyte cultures were prepared from P1-2 mouse pups as previously described [24]. After 7 DIV, when cultures were approximately 70% confluent, astrocytes were infected with recombinant adenoviral-GFP, or adenoviral-Nrf2/GFP at 200MOI as previously published [23]. Twenty-four hours following the astrocyte infections, a total of 8 wildtype C57Bl6/SJL mice, aged 16 weeks, were stereotactically injected with saline into the left striatum. Into the right striatum, animals were either administered astrocytes infected with adeno-GFP or adeno-Nrf2/GFP. Animals received approximately 100,000 cells in 1 μ l using the following coordinates: 0.4mm anterior of bregma, 2.2mm lateral to midline, and 3.8mm ventral to the dura. The astrocytes were kept on ice during the entirety of the surgeries. The remaining cells were plated and stained for GFAP immunoreactivity. Following surgery, animals were allowed to recover and were returned to their home cage. After five weeks of recovery, all mice received bilateral 6-OHDA, prepared in ascorbate, lesions. At six weeks from the commencement of the experiment, the mice were sacrificed for histological analysis.

TISSUE COLLECTION AND HISTOLOGY

All animals were euthanized with CO₂. Mice were then perfused through the left ventricle with PBS followed by 4% paraformaldehyde. Brains were collected for histology, post-fixed overnight in paraformaldehyde, and cryoprotected in 30% sucrose in PBS. Brains were frozen in OCT and sectioned on a cryostat (Leica, Deerfield, IL). Serial sections were taken as free-floating in PBS containing azide and stored at 4°C until analysis.

IMMUNOCYTOCHEMISTRY

Brain sections were stained for anti-tyrosine hydroxylase (TH; Chemicon, 1:800). Cells were stained for anti-GFAP (Dako, 1:400) immunoreactivity as previously described [21]. Cells were also counterstained with with Hoescht 33258 to visualize nuclei.

CYTOTOXICITY MEASUREMENTS

The MTS [3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2(4-sulfophenyl)-2H-tetrazolium salt] assay (Promega) was used according to the manufacturer's instructions to assess cell viability. To assess apoptotic cell death, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL; Roche) staining was used as per instructions. To visualize the total nuclei per field, cells were counterstained with Hoescht 33258. Five fields from each embryo were quantified for number of TUNEL+ cells and Hoescht+ cells by an observer who was blinded to the genotype of the cultures.

TRANSFECTION OF N27 CELLS

N27 cells were plated at a density of 10^4 cells/well. Twenty-four hours later, transfections were performed using Lipofectamine™ 2000 (Invitrogen) according to the supplier's instructions. Briefly, 0.5 µg ARE-luc and 0.5 µg CMV-β-gal were diluted and combined with the Lipofectamine™ 2000 and placed on cells in fresh media with antibiotics for 6 hours prior to replenishment with fresh media. All treatments were administered at the final media change and incubated for 24 hours until luciferase and β-gal assays were performed [26]. Transfection efficiency was estimated to be approximately 50% using the pSUPER GFP-containing reporter plasmid (OligoEngine).

REVERSE-TRANSCRIPTASE PCR (RT-PCR)

N27 cultures assessed for total mRNA were exposed to the designated treatment for 24 hours followed by total RNA isolation with TRIzol (Invitrogen) as per manufacturers directions. RNA was then analyzed by a spectrophotometer and a Bioanalyzer (Agilent) to assess concentration and quality. One µg of total RNA was converted to cDNA using the Reverse Transcription System (Promega). PCR for rat beta-actin, HO-1, GSTmu3, and NQO1 was completed using DNA 2X Master Mix (Promega) using the following primers: beta-actin (5'-CAG TGA GGC CAG GAT AGA GC-3'; 5'-CTG GGT ATG GAA TCC TGT GC-3') 248bp, HO-1 (5'-TGC TCG CAT GAA CAC TCT G-3'; 5'-TCC TCT GTC AGC AGT GCC T-3') 123bp, GSTmu3 (5'-GTT GGG AGC CAA CTA TCC AA-3'; 5'-TAT TTG GGG CAG CAA GTA GG-3') 303 bp, and NQO1 (5'-CTG GAA GGG TGG AAG AAG CGT C-3'; 5'-TCT GGT CGG CTG GAA TGG-3') 196bp.

LESION VOLUME QUANTIFICATION

Lesion volume was assessed on images of coronal sections immunohistochemically labeled for TH, taken by a brightfield camera (Zeiss) at 1.25X magnification. Using the Zeiss AxioVision 4.0 Interactive Measurement program, blinded observers circumscribed the striatal regions lacking TH-immunoreactivity, and the area was tabulated. Every third section of 50µm was analyzed, and each area measurement was multiplied by 150 (the distance in µm between sections) and summed to obtain the total estimated lesion area.

STATISTICAL ANALYSIS

All data are reported as means ± SEM, using $p < 0.05$ as the cutoff for statistical significance. For primary culture data, all data points were collected in triplicate and analyzed with unpaired student t-tests. *In vivo* knockout vs. wildtype data was acquired as described above. Mean lesion volume was analyzed by unpaired student t-tests. Transplant experiments were analyzed with paired student t-tests. Briefly, the transplanted side was compared to the contralateral vehicle-treated hemisphere as a paired control following bilateral administration of 6-OHDA.

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FIGURE 1. tBHQ induces ARE regulated genes in N27 cells and protects against 6-OHDA

N27 cells were transfected with ARE-luc/b-gal construct and exposed to tBHQ. As compared to vehicle control, tBHQ causes ARE activation (1 μM, $p=0.007$; 3 μM, $p=1E-5$; 10 μM, $p=1E-6$; 30 μM, $p=4E-14$; unpaired, 2-tailed t-tests; A). Note that maximal ARE activation occurs at approximately 10 μM (A). N27 cells were exposed to tBHQ for 24 hours and harvested for RNA (B). RT-PCR reveals that treatment with tBHQ increases the amount of GSTmu3, NQO1, and HO-1 mRNA. β actin was used as a control for amount of RNA assayed. tBHQ protects against 6-OHDA *in vitro* (C). 6-OHDA causes a dose-dependent increase in cell death in N27 cells (as compared to 0 μM 6-OHDA: 37.5 μM, $p=0.002$; 75 μM, $p=0.0001$; 150 μM, $p=0.00001$). Pretreatment of N27 cells with 10 μM tBHQ as compared to vehicle prior to exposure to 6-OHDA significantly attenuates cell death as measured by the MTS assay (37.5 μM, $p=0.0002$; 75 μM, $p=0.003$; 150 μM, $p=0.041$).

* significantly different from corresponding vehicle-treated control sample ($p<0.05$).

significantly different from corresponding sample in the absence of tBHQ ($p<0.05$).

FIGURE 2. Increased vulnerability of Nrf2^{-/-} cells to 6-OHDA *in vitro*

Nrf2^{+/+} and ^{-/-} cells were exposed to 50μM 6-OHDA and assessed for TUNEL staining (A). The proportion of TUNEL+ cells per total cells was assessed for both genotypes (A and B). As compared to vehicle treatment, 6-OHDA increased cell death (Nrf2^{+/+}, p=0.02; Nrf2^{-/-}, p=0.00002; B). However, in the presence of 6-OHDA, there was significantly more apoptosis in Nrf2^{-/-} cultures as compared to controls (p=0.003; B). A 6-OHDA dose-response curve also demonstrated increased vulnerability in null cultures as compared to wildtype cells (25μM, p=0.0041; 50μM, p=0.0212; 75μM, p=0.0395; C). Heterozygote cells were not significantly different than wildtype control cells (p>0.05; C).

* significantly different from corresponding vehicle-treated control sample (p<0.05).

significantly different from corresponding sample in the absence of tBHQ (p<0.05).

FIGURE 3. Increased vulnerability of Nrf2^{-/-} mice to 6-OHDA *in vivo*

6-OHDA was stereotactically injected into the striatum of wildtype and knockout Nrf2 mice with contralateral vehicle controls. After one week, mice were sacrificed and tissue was stained for THir (A). Nrf2 null mice demonstrated significantly larger lesions as revealed by THir and quantified by lesion volume analysis (A and B; $p=0.0278$). The 6-OHDA-induced reduction of THir in the SNpc was also more pronounced in the null mice as compared to the wildtypes (C).

* significantly different as compared to Nrf2 wildtype animals ($p<0.05$).

FIGURE 4. Transplanted astrocytes

During the transplant surgeries, harvested astrocytes were kept on ice. Remaining cells (A) were plated on chamber slides overnight and stained for nuclei with Hoescht (blue), or anti-GFP for astrocytes (red). Infection with adenovirus expressing GFP can be seen (green, A). Unstained striatal sections shown above are from either Adeno-GFP or Adeno-Nrf2 transplanted animals and imaged for GFP fluorescence indicating surviving GFP-expressing astrocytes (B). Upon visual inspection, astrocytes survived equally well in both groups. Scale bar = 100 μ M.

FIGURE 5. Astrocytes expressing Adeno-Nrf2 significantly protect against 6-OHDA

Brain tissue collected from transplanted mice was stained for THir (A and B). Every third section was assessed for lesion area. Images taken at 1.25X magnification; scale bar = 1mm. Lesion volume was calculated for both hemispheres (C). Transplantation of Adeno-GFP treated astrocytes resulted in a trend toward mild protection that did not reach statistical significance ($p > 0.05$). Animals receiving astrocytes infected with Adeno-Nrf2 demonstrated significantly reduced lesion volume as compared to the contralateral striata ($p = 0.0326$).

* significantly different from contralateral vehicle-pretreated hemisphere ($p < 0.05$).