Journal of Neurochemistry, 2007, **101**, 109–119 doi:10.1111/j.1471-4159.2006.04345.x

Dopamine activates Nrf2-regulated neuroprotective pathways in astrocytes and meningeal cells

Andy Y. Shih,*,† Heidi Erb*,† and Timothy H. Murphy*,†,‡

Kinsmen Laboratory of Neurological Research, Department of Psychiatry, †Brain Research Centre, ‡Department of Physiology, University of British Columbia, Vancouver, British Columbia, Canada

Abstract

The transcription factor Nrf2 controls inducible expression of multiple antioxidant/detoxification genes. We previously found that $Nrf2^{-/-}$ mice have increased sensitivity to *in vivo* mitochondrial stress and ischemia. Although Nrf2 regulated these forms of neuronal toxicity, it was unclear which injury-triggered signal(s) led to Nrf2 activation *in vivo*. In this study, we use primary cultures to test the hypothesis that excessive dopamine release can act as an endogenous Nrf2-inducing signal. We cultured two cell types that show increased Nrf2 activity during ischemia *in vivo*, astrocytes and meningeal cells. Cultures were infected with an adenovirus reporter of Nrf2 transcriptional activity. Dopamine-induced Nrf2 activity in both cell types by generating oxidative stressors, H_2O_2 and dopamine-quinones. Nrf2 activation in meningeal cells was significantly higher than astrocytes. The effect of dopamine was blocked by antioxidants, and by over-expression of either dominant-negative Nrf2 or Keap1. Nrf2 induction was specific to oxidative stress caused by catecholaminergic neurotransmitters as epinephrine also induced Nrf2, but the monoamine serotonin had no significant effect. These *in vitro* results suggest Nrf2 activity in astrocytes and meningeal cells link the neurotoxic actions of dopamine to neuroprotective pathways that may potentially modulate ischemic injury and neurodegeneration.

Keywords: tertiary-butylhydroquinone, antioxidant response element, glutathione, ischemia, Phase 2 enzymes, Keap1.
J. Neurochem. (2007) **101**, 109–119.

The inducible transcription factor Nrf2 regulates multiple lines of cellular defense that limit oxidative stress during stroke and neurodegenerative disease (van Muiswinkel and Kuiperij 2005; Shih *et al.* 2005a). Nrf2 is normally sequestered in the cytoplasm and targeted for proteosomal degradation by its regulatory protein Keap1, but is released for nuclear translocation upon exposure to oxidative stress (Itoh *et al.* 1999; Motohashi and Yamamoto 2004). In the nucleus, Nrf2 binds the antioxidant response element (ARE) found in the promoters of numerous antioxidant/detoxification genes (Phase 2 genes) (Venugopal and Jaiswal 1996; Itoh *et al.* 1997). In astrocytes, this inducible mechanism coordinates expression of several cellular defense pathways including: detoxification of reactive oxygen/nitrogen species and xenobiotics, glutathione (GSH) synthesis, and generation of NADPH (Eftekharpour *et al.* 2000; Lee *et al.* 2003b; Shih *et al.* 2003; Kraft *et al.* 2004).

$Nrf2^{-/-}$ mice have increased sensitivity to focal ischemia and striatal neurodegeneration caused by the mitochondrial toxin 3-nitropropionic acid (3-NP) (Shih *et al.* 2005a,b). Conversely, increasing Nrf2 activity with viral over-expression of Nrf2 protein or small molecule electrophilic Nrf2 inducers confers neuroprotection *in vivo* (Calkins *et al.* 2005; Shih *et al.* 2005a,b; Satoh *et al.* 2006). Although, it is clear that Nrf2 serves a protective role in brain, the mechanisms leading to activation of endogenous Nrf2 during injury have been difficult to determine. *In vivo* stroke studies suggest that injury induces Nrf2 target genes most strongly in astrocytes and meningeal cells (Campagne *et al.* 2000; Laxton *et al.* 2001). However, measurement of gene expression is complicated in injured tissues as large reductions in protein synthesis may occur acutely and specific Nrf2-dependent induction of Phase 2 genes could be masked by chronic microglial activation, gliosis, and immune cell invasion (Kochanek and Hallenbeck 1992; Hossmann 1994). Thus, we have used relatively pure

Received June 22, 2006; revised manuscript received August 28, 2006; accepted September 20, 2006.

Address correspondence and reprint requests to Timothy H. Murphy, 4N1-2255 Wesbrook Mall, University of British Columbia, Vancouver, BC, Canada, V6T 1Z3. E-mail: thmurphy@interchange.ubc.ca

Abbreviations used: ARE, antioxidant response element; DA, dopamine; MOI, multiplicity of infection; PAP, placental alkaline phosphatase; LDH, lactate dehydrogenase.

astrocyte and meningeal cell culture preparations to more clearly examine potential endogenous triggers for Nrf2 activation.

Excessive nigrostriatal release of the catecholamine neurotransmitter, dopamine (DA), is a proposed mechanism for striatal injury during stroke and 3-NP toxicity (Globus *et al.* 1987, 1988a; Maragos *et al.* 1998; Reynolds *et al.* 1998; Johnson *et al.* 2000). DA causes neurotoxicity by generating hydrogen peroxide (H_2O_2) and DA-quinones during auto-oxidation or monoamine oxidase-dependent metabolism (Graham 1978; Smythies and Galzigna 1998). We previously demonstrated that pre-activation of Nrf2 using electrophilic agents protected cells from DA toxicity, attributable in part to enhanced H_2O_2 scavenging by the GSH system, and detoxification of reactive quinones by NAD(P)H : quinone oxidoreductase 1 (NQO1) (Duffy *et al.* 1998). In this study, we show that excessive extracellular DA itself can be an endogenous signal to activate Nrf2-dependent neuroprotective pathways.

Materials and methods

Reagents

All reagents were obtained from Sigma unless otherwise stated.

Cell culture and treatments

Astrocyte and meningeal cultures were prepared from 1–2-day-old postnatal rat pups. Cerebral cortical tissue for astrocyte cultures, and pial/arachnoid layers from the surface of the cerebral and cerebellar cortices for meningeal cultures, were dissected, minced, and papain dissociated (Shih *et al.* 2003). Cells were plated in minimal essential media (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (HyClone), 2 mmol/L glutamine, 5 mmol/L D-glucose, 100 U/mL penicillin, and 0.1 mg/mL streptomycin in un-coated 10 cm plates (Falcon, San Jose, CA, USA). After 1 day *in vitro* (DIV) the medium was replaced, and the primary cultures were grown to 7 DIV. Adherent neurons were removed from the astrocyte cultures by repeated pipetting of the media. All cultures were used within 10 DIV because older, quiescent cultures appeared to lack the Nrf2-induced antioxidant response. The conditions used to generate astrocyte cultures primarily resulted in a population of type I and II astrocytes, as assessed by anti-glial fibrillary acidic protein (GFAP) staining (Shih *et al.* 2003). A very small proportion of the total cells were positive for oligodendrocyte marker RIP (Fig. 1). We cannot exclude the possibility that a relatively small number of microglia may also exist in the astrocyte cultures, as microglial markers were not tested.

Transient transfection of astrocytes using Lipofectamine 2000 was performed as described previously (Shih *et al.* 2005a,b).

Adenovirus construction

Adenoviral vectors were of the E1-substituted type, generated using the Cre-lox system (Canadian Stroke Network core facility, University of Ottawa, Ottawa, Canada), as described previously (Hardy *et al.* 1997; Crocker *et al.* 2001; Shih *et al.* 2003). For the Ad-rQR51 adenovirus construct, a human heat-stable placental alkaline phosphatase gene driven by a promoter carrying the rat NAD(P)H : quinone oxidoreductase ARE was excised from the rQR51wt DNA plasmid (using Not I) and incorporated into an adenovirus construct (Wasserman and Fahl 1997; Ahlgren-Beckendorf *et al.* 1999; Murphy *et al.* 2001). An adenovirus carrying the placental alkaline phosphatase gene with a mutated ARE sequence (Ad-rQR51mut) was used as a control and background subtraction in all experiments. An additional adenovirus carrying the placental alkaline phosphatase gene with no promoter (Ad-TiPAP) was also useful as a negative control (data not shown). The Keap1 cDNA sequence used to make Ad-Keap1 was a generous gift from Dr Masayuki Yamamoto (University of Tsukuba) (Itoh *et al.* 1999). Construction and use of Ad-GFP, Ad-Nrf2, and Ad-Nrf2DN was described previously (Lee *et al.* 2003a; Shih *et al.* 2003; Kraft *et al.*

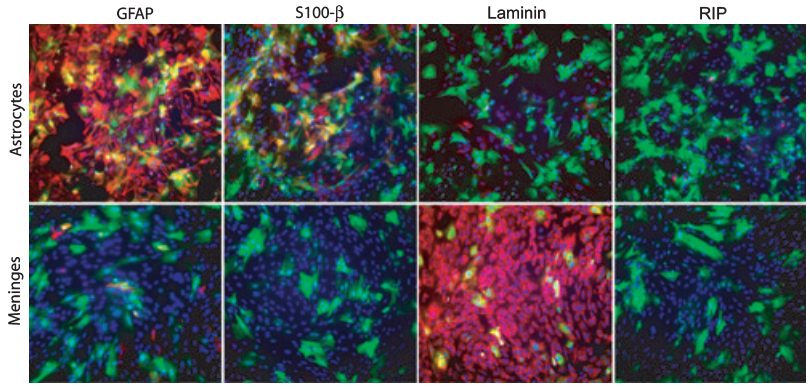

Fig. 1 Immuncytochemical characterization of astrocyte and meningeal cultures. Primary astrocyte and meningeal cultures (7 DIV) were infected with Ad-rQR51 reporter virus (green), fixed 24 h after infection, and immunostained (red) for GFAP, S100-β, laminin, or RIP. Astrocyte cultures were labeled only by GFAP and S100-β. Meningeal cultures were labeled only by laminin. The majority of Ad-rQR51-infected astrocytes and meningeal cells were co-labeled with GFAP or laminin, respectively (yellow). Images are representative of 3 separately prepared cultures. Scale bar = 80 μm.

2004). All adenoviral constructs, except for Ad-Keap1, expressed GFP through a separate CMV promotor for monitoring infection efficiency.

Adenovirus infection

7 DIV astrocyte and meningeal cells were split into 96-well culture plates (astrocytes: 3×10^3 cells/well, meningeal cells: 1×10^3 cells/well). Meningeal cells grew at a faster rate than astrocytes and were plated at a 1/3 lower density. Adenovirus infection was performed 24 h after cell splitting. To minimize possible adverse effects on Nrf2 activity due to viral toxicity, a lower multiplicity of infection (MOI) was used in this study (25 plaque forming units/cell), compared with our previous studies (200 plaque forming units/cell), (Shih *et al.* 2003, 2005a,b). When calculating MOI, we used the number of cells/well at the time of splitting. To start infection, viral stocks (usually on the order of 1×10^{11} plaque forming units/mL) were diluted in culture media and 30 μL was applied to each well. After a 2 h incubation period with occasional gentle swirling, fresh culture media was added to bring each well volume to 100 μL. Experimental treatments were applied in fresh media (100 μL/well) 24 h after infection.

Immunocytochemistry

Immunocytochemistry was performed as described previously (Shih *et al.* 2003). Antibodies used in this study include anti-GFAP (rabbit, Sigma, Oakville, ON, Canada), anti-S100β (mouse, Sigma), anti-laminin (rabbit, Sigma), anti-RIP (mouse, Chemicon, Temecula, CA, USA). After immunostaining, cultures were incubated with Hoechst nuclear counterstain (10 μg/mL) for 10 min and washed prior to mounting with Fluoromount G. Fluorescent images were taken using Northern Eclipse Software and a wide-field fluorescence microscope (Zeiss Axiophot, Oberkochen, Germany) equipped with a Retiga Exi CCD camera (QImaging, Burnaby, BC, Canada).

Placental alkaline phosphatase assay and stain

The placental alkaline phosphatase activity assay previously described (Shih *et al.* 2005a,b), was adapted for 96-well plates in this study. Infected and treated cells in 96 well culture plates were lysed with a buffer composed of 10 mmol/L Tris–HCl (pH 8), 1 mmol/L $MgCl_2$, and 0.1% Triton X-100 (50 μL/well). Plates were shaken at 960 rpm for 10 min to ensure complete cell lysis. For normalization of cell plating density, 10 μL of the sample was used to determine protein concentration with a BCA protein assay kit (Pierce, Rockford, IL, USA). For normalization of infection efficiency, fluorescence of viral-expressed GFP was measured in the remaining sample in each well using a Fluoroskan Ascent multiplate fluorescence reader (Thermo LabSystems, Beverly, MA, USA). The wells were then sealed with masking tape and the plates were heated to 65°C in a shallow water bath for 30 min to destroy endogenous phosphatase activity. Plates were cooled on ice for 10 min and samples were transferred to a new 96-well plate. To assay placental alkaline phosphatase activity, each well received 80 μL of reaction buffer (0.73 mol/L diethanolamine with 0.36 mmol/L $MgCl_2$, pH 9.8), followed by 20 μL of phosphatase substrate (13.6 mmol/L *p*-nitrophenyl phosphate) to initiate the reaction. The reaction was measured at 405 nm every 1 min over a 30 min period using a Multiskan Ascent multiplate absorbance reader (Thermo LabSystems). During data analysis, the average activity from Ad-rQR51mut-infected wells (included in triplicate for each experiment) was subtracted as ARE-independent background activity from all experimental groups. Then, both GFP and protein concentration measurements were used to normalize data values between experimental groups.

Histochemical PAP staining was performed as described previously (Murphy *et al.* 2001). Images of stained cells were taken with a microscope (Zeiss Axiophot) equipped with a Retiga Exi CCD camera (QImaging) or scanned with an Epson scanner (Long Beach, CA, USA).

Total intracellular GSH assay

The GSH assay was performed as described previously, except that cells were lysed in a detergent-containing buffer (10 mmol/L phosphate buffer with 0.1% Triton X-100, pH 7.4) to facilitate harvesting from 96-well plates (Shih *et al.* 2003, 2005a). Total intracellular GSH was quantified by the method of Tietze (Tietze 1969).

NQO1 enzymatic activity assays

The NQO1 assay was performed as described previously, except cells were lysed in the same detergent-containing buffer as described for the GSH assay (Shih *et al.* 2003, 2005a). Although significant NQO1 induction can be detected using this buffer, changes are not as robust as when cells are harvested in PBS (Shih *et al.* 2005a).

Semi-quantitative reverse-transcriptase PCR of rat brain tissues

Total RNA was isolated from fresh brain tissue samples using TRIzol Reagent (Invitrogen). One μg of RNA was reverse transcribed using random hexamer primers and Thermoscript reverse transcriptase (Invitrogen). The resulting cDNA was then amplified by PCR using various primer sets (Lee *et al.* 2003a; Shih *et al.* 2003): mouse Nrf2, 5′-TCTCCTCGCTGGAAAAAGAA-3′ and 5′-AATGTGCTGGCTGTGCTTTA-3′; NQO1, 5′-CATTCTGAAAGGCTGGTTTGA-3′ and 5′-CTAGCTTTGATCTGGTTGTCAG-3′; xCT, 5′-TTGCAAGCTCACAGCAATTC-3′ and 5′-CGTCAGAGGATGCAAAACAA-3′; GCLC, 5′-ACAAGCACCCCCGCTTCGGT-3′ and 5′-CTCCAGGCCTCTCTCCTCCC-3′; Actin, 5′-AGAGCATAGCCCTCGTAGAT-3′ and 5′-CCCAGAGCAAGAGAGGTATC-3′.

For tissue collection, brains were extracted from three young Wistar rats (~1 month of age), and placed in ice-cold PBS. Pial/arachnoid layers (meninges) were first collected from the surface of the cerebral and cerebellar cortices. The forebrain was then sliced into 1 mm thick coronal sections. Cortex (parietal) and striatum (caudate/putamen) was excised from the section corresponding to bregma 0.0 mm. All tissues were immediately homogenized in TRIzol after dissection.

Statistical analysis

Results are presented as the mean ± SEM. Statistical analysis of raw data was performed with GraphPad Prism 2.0. Experimental groups were compared by one-way ANOVA or Student's *t*-test. $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

Results

High Nrf2 expression and activity in meningeal cells

In vivo, both astrocytes and meningeal cells exhibit robust induction of ARE-mediated gene expression following stroke (Campagne *et al.* 2000). To simplify the examination of mechanisms underlying endogenous Nrf2 activation, primary astrocytes and meningeal cells were cultured from P0-2 postnatal rat pups. Astrocyte cultures were positive for the conventional astrocyte-specific markers GFAP and S100β, but negative for laminin (basement membrane marker) and RIP (oligodendrocyte marker) (Fig. 1, upper panels). In contrast, meningeal cultures were laminin-positive, but negative for GFAP, S100β, and RIP (Fig. 1, lower panels). Previous studies have also used laminin to identify the pial basement membrane of the meninges (Halfter *et al.* 2002).

To measure Nrf2 activity, cultures were infected with an adenovirus (Ad-rQR51) expressing human heat-stable placental alkaline phosphatase (PAP) under the control of an ARE derived from the rat NQO1 gene promoter (Wasserman and Fahl 1997; Ahlgren-Beckendorf *et al.* 1999; Moehlenkamp and Johnson 1999; Murphy *et al.* 2001). Ad-rQR51 also expressed GFP through a separate CMV promoter to aid identification of infected cells. Ad-rQR51 exhibited similar tropism for both GFAP-positive astrocytes and laminin-positive meningeal cells (data not shown). To minimize viral toxicity and its effects on Nrf2 activity, we infected cultures with a relatively low concentration of Ad-rQR51 (25 plaque forming units/cell) compared with our previous studies (Shih *et al.* 2003). Confirming that Ad-rQR51 was reporting Nrf2 activity, co-infection with an Nrf2 over-expressing adenovirus (Ad-Nrf2) led to a ~12-fold and ~24-fold induction in PAP expression in astrocyte and meningeal cultures, respectively (Fig. 2a), while the Ad-GFP control produced no significant change. Conversely, Ad-Nrf2DN (a dominant-negative that blocks endogenous Nrf2 activity by competing for the ARE binding site), and Ad-Keap1 (Keap1 over-expressing virus) both suppressed basal PAP expression (Fig. 2b) (Alam *et al.* 1999; Itoh *et al.* 1999).

Inducible activity of endogenous Nrf2 was examined in both cell types using the electrophilic agent, *tert*-butylhydroquinone (tBHQ). Astrocyte Nrf2 activity was induced ~4-fold over basal levels after tBHQ treatment for 24 h (Fig. 2c) (Lee *et al.* 2003b; Shih *et al.* 2005b). Although astrocytes and meningeal cells were similar in basal Nrf2 activity, meningeal cells showed a significantly larger ~8-fold induction by tBHQ (Fig. 2c). The induction of ARE-mediated gene expression could also be demonstrated as an *in situ* PAP stain in 96-well plates, which confirmed that the majority of infected astrocyte or meningeal cells were responding, not a sub-population (Fig. 2d). Importantly, negative control viruses Ad-rQR51mut (mutated ARE sequence) and Ad-TiPAP (no promoter) did not report basal or inducible Nrf2 activity (data not shown) (Murphy *et al.* 2001).

Consistent with results from the Ad-rQR51 reporter virus, tBHQ treatment produced larger increases in GSH content and NQO1 enzymatic activity in meningeal cells than astrocytes (Fig. 2e). To relate our findings with the *in vivo* situation, we compared mRNA levels of Nrf2 and its gene targets amongst various freshly dissected brain tissues using semi-quantitative RT-PCR. As expected from the cell culture data, meningeal tissue expressed the highest levels of Nrf2 and Phase 2 gene mRNAs including NQO1 and the cystine/glutamate exchanger (xCT) (Fig. 2f)(Sun *et al.* Society for Neuroscience online abstracts 2005, #221.14). Remarkably, meningeal tissue expressed a similar level of Nrf2 mRNA as liver tissue, which is among the most highly expressing regions in the body (Chan *et al.* 1996). Surprisingly, the rate-limiting enzyme for GSH synthesis γ-glutamylcysteine synthetase (GCLC) which is also under Nrf2 regulation, was highest within the striatum and not the meninges (Wild and Mulcahy 2000).

Dopamine-mediated Nrf2 induction is dependent on oxidative stress

DA release and degradation (auto-oxidation and/or metabolism) is one possible mechanism by which ischemia and 3-NP promote neurodegeneration (Globus *et al.* 1987, 1988a; Maragos *et al.* 1998; Reynolds *et al.* 1998; Johnson *et al.* 2000). We hypothesized that H_2O_2 and DA-quinone generation by DA could act as a signal for Nrf2 activation *in vitro* (Graham 1984; Stokes *et al.* 1999). Using Ad-rQR51 infected cultures, we found that DA exposure (30–100 μmol/L for 24 h) robustly increased Nrf2 activity in both astrocytes and meningeal cells, with a significantly higher response in meningeal cells (Figs 3a and b). Short-term exposure of DA (1 h) was not sufficient to induce Nrf2. DA exposure also increased cellular GSH content and NQO1 enzymatic activity without affecting lactate dehydrogenase (LDH) activity, a house-keeping gene not regulated by Nrf2 (Fig. 3c). Consistent with a role for Nrf2, both Ad-Nrf2DN and Ad-Keap1 blocked the effect of DA in meningeal cultures (Fig. 3d). Interestingly, the relatively strong Nrf2-inducing effect of DA in astrocyte cultures was blocked by Ad-Keap1, but not completely by Ad-Nrf2DN (~60% reduction in stimulated activity), which may be due to the lower virus titer used here when compared with our previous studies (25 vs. 200 plaque forming units/cell) (Shih *et al.* 2003, 2005a,b). Reporter activity induced by DA could also be clearly resolved using the histochemical PAP stain (Fig. 3e).

DA-mediated Nrf2 induction was significantly reduced by co-application of antioxidants (500 μmol/L GSH and 200 μmol/L α-tocopherol for 24 h) (Fig. 3e and Fig. 4a,b), consistent with DA oxidation and generation the oxidative stressors H_2O_2 and DA-quinones. Direct exposure to H_2O_2 activated Nrf2 at nearly equimolar concentrations as DA (100–200 μmol/L for 24 h) (Fig. 4c). However, the H_2O_2

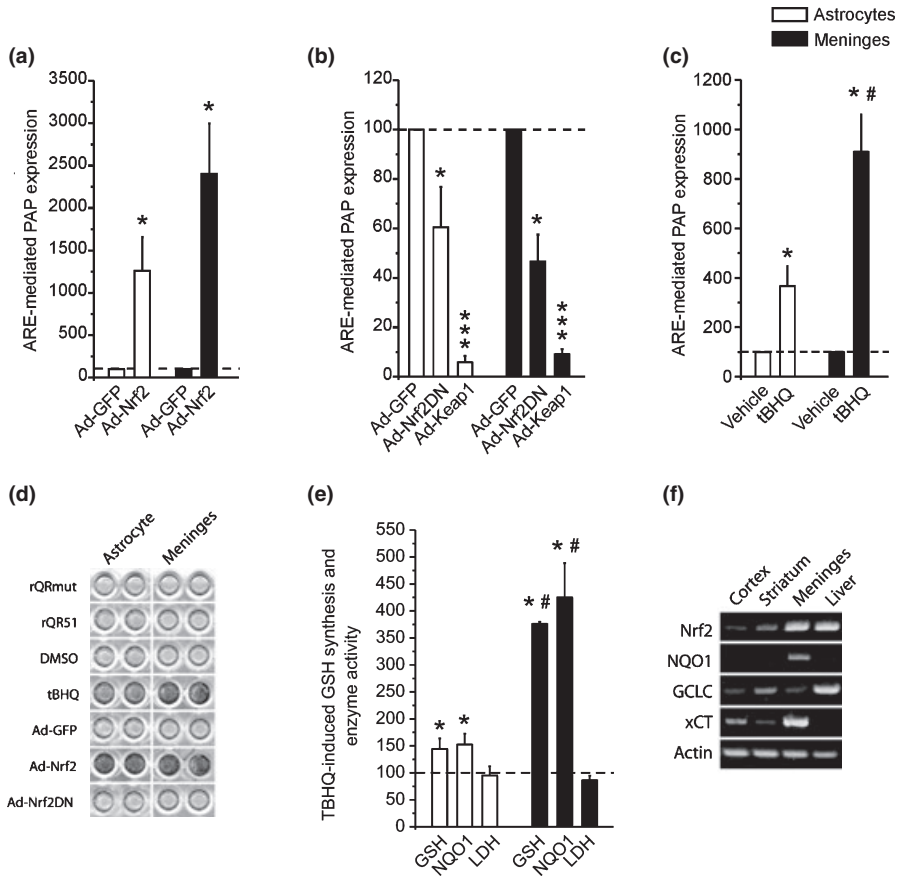

Fig. 2 Ad-rQR51 validation and characterization of Nrf2 activity in astrocyte and meningeal cultures. (a) Astrocyte and meningeal cultures were co-infected with Ad-rQR51 and Ad-Nrf2 (Nrf2 overexpressing virus) or Ad-GFP control. Ad-Nrf2 co-infection led to a robust increase in ARE-dependent PAP expression in both culture types. (b) Ad-Nrf2DN (dominant-negative Nrf2) and Ad-Keap1 (cytoplamic regulatory protein) suppressed basal ARE-dependent gene expression, confirming specific measurement of Nrf2 activity. $^{*}p < 0.05$, $^{***}p < 0.001$ compared with Ad-GFP control. (c) Treatment with the electrophilic agent, tBHQ (20 μmol/L), increased Nrf2 activity more robustly in meningeal cultures than astrocytes. $^{*}p < 0.05$ compared with vehicle control, $^{\#}p < 0.05$ compared with tBHQ-treated astrocytes. (d) Histochemical PAP stain in 96 well plates. All wells except first row (Ad-rQR51mut) were infected with Ad-rQR51. Both tBHQ treatment and Ad-Nrf2 co-infection led to robust purple staining of Ad-rQR51-infected cells. (e) In response to tBHQ treatment meningeal cultures exhibited a larger induction of GSH synthesis and NQO1 activity. $^{*}p < 0.05$ compared with vehicle control, $^{\#}p < 0.05$ compared with tBHQ-treated astrocytes. LDH was not Nrf2-regulated and thus showed no change. For cell culture experiments, data represent the mean ± SEM collected from at least three independent experiments on separately prepared cultures. (f) Fresh tissue was collected from various brain regions for RT-PCR. Meningeal tissue expressed higher levels of Nrf2, NQO1, and xCT mRNA than cortex (parietal) and striatum (caudate/putamen). The RT-PCR gel is representative of three independent experiments using separate Wistar rats (~1 month of age).

scavenging enzyme catalase (100 U/mL) only partially blocked DA-mediated Nrf2 activation (Fig. 4d). The blocking effect of catalase was saturated at 100 U/mL and higher concentrations did not further reduce Nrf2 activity (data not shown). Thus, it is likely that simultaneous production of DA-quinones and H_2O_2 underlies DA-mediated Nrf2 induction. Although we could not test DA-quinones directly due to their unstable nature (Graham 1978), we demonstrate that many quinone-based compounds (menadione, hydroquinone, and tBHQ) induce Nrf2, at least in part, by increasing oxidative stress (Fig. 4e). Nrf2 activation by menadione could be completely blocked by addition of antioxidants, whereas hydroquinone and tBHQ could be only partially blocked.

To determine whether Nrf2 induction by DA was relatively specific, we also examined other neurotransmitters that auto-oxidize and are implicated in neurotoxicity (Rosenberg 1988; Fu *et al.* 1998; Wrona and Dryhurst 1998). Another catecholamine neurotransmitter, epinephrine, also induced Nrf2 at a similar concentration range as DA, albeit in a less potent manner. The effect of epinephrine could be blocked by co-application of antioxidants (Fig. 5a). In contrast, the

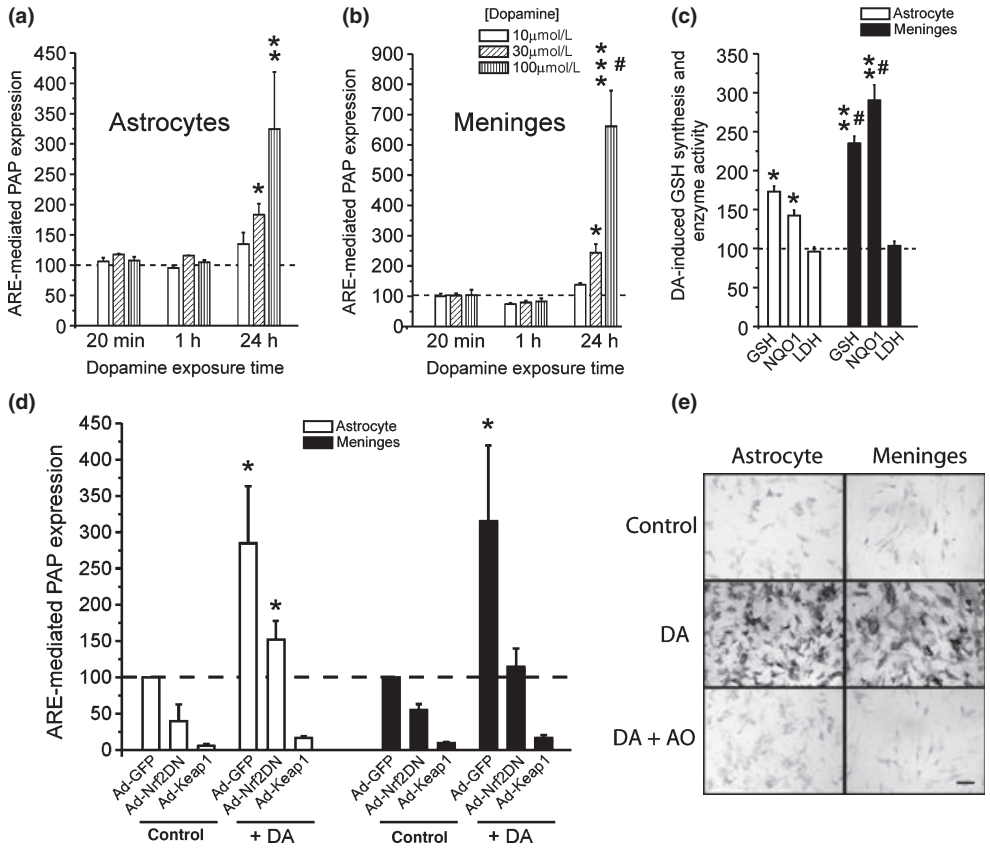

Fig. 3 Dopamine (DA) induces Nrf2 activity in both astrocyte and meningeal cultures. (a and b) DA significantly increased Nrf2 activity in both cell types, but meningeal cells responded more robustly than astrocytes. The 100 μM concentration produced the largest response and was used for subsequent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared with vehicle control, $^{\#}p < 0.05$ compared with same DA concentration for astrocytes. (c) DA treatment enhanced GSH synthesis and NQO1 activity in astrocytes and meningeal cells. $^{*}p < 0.05$ compared with vehicle control, $^{\#}p < 0.05$ compared with DA-treated astrocytes. LDH activity was unchanged with DA treatment. (d) Ad-Nrf2DN and Ad-Keap1 largely blocked DA-mediated increase in Nrf2 activity. $^{*}p < 0.05$ compared with vehicle control with same virus infection. (e) Histochemical PAP staining showed that DA-mediated Nrf2 induction is blocked by co-application of antioxidants (AO) (500 μmol/L GSH and 200 μmol/L α-tocopherol) (Scale bar = 80 μm). Data represent the mean ± SEM over at least three independent experiments on separately prepared cultures.

monoamine neurotransmitter serotonin failed to induce Nrf2 (Fig. 5b).

Discussion

Ad-rQR51-infected cultures: an assay system for quantifying Nrf2 activity

To measure Nrf2 activity, we produced an efficient and specific assay system using the Ad-rQR51 adenoviral reporter construct. Ad-rQR51-mediated PAP expression was strongly enhanced by tBHQ treatment or over-expression of Nrf2, and repressed by over-expression of Nrf2DN or Keap1. Further, our previous studies using the DNA plasmid-based version of rQR51 showed that basal and inducible ARE-dependent PAP expression were significantly reduced in $Nrf2^{-/-}$ mice (Shih *et al.* 2005a). In addition to astrocytes and meningeal cells, Ad-rQR51 would be useful for studying Nrf2 activity in a variety of cell-types that are difficult to transfect with plasmid DNA *in vitro*. However, the adenovirus has poor tropism for mature cortical neurons, but can be used to infect immature cortical neurons (Shih *et al.* 2003). Ad-rQR51-infected cultures prepared in multi-well plates can be used as a relatively high-throughput system for screening potential Nrf2-inducing compounds, complementing existing NQO1-based screening assays (Prochaska *et al.* 1992). Meningeal cells would be an excellent culture system for screening purposes, given their high level of Nrf2 expression/inducibility and rapid growth in culture. Ad-rQR51 may also be useful for studying treatments that induce Nrf2 activity *in vivo*, complementing existing methods with transgenic reporter mice (Calkins *et al.* 2005; Jakel *et al.* 2005).

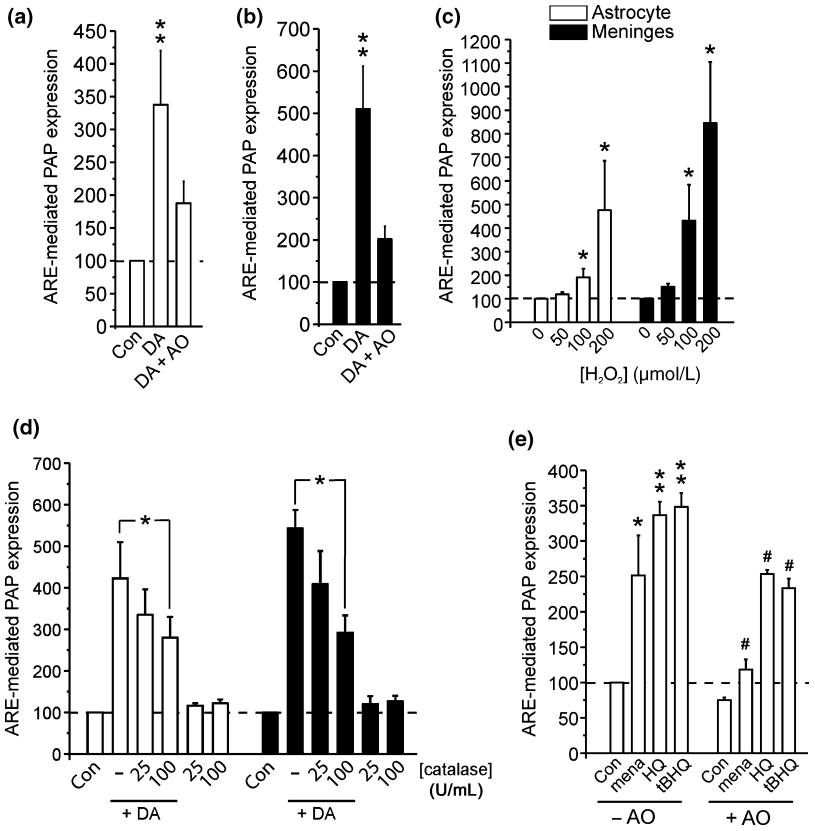

Fig. 4 Dopamine induces Nrf2 activity by increasing oxidative stress. (a and b) Assayed Nrf2 activity was increased by DA exposure and blocked by co-application of AO. $^{**}p < 0.01$ compared with vehicle control. (c) Treatment with H_2O_2 for 24 h led to a dose-dependent increase in Nrf2 activity. $^{*}p < 0.05$ compared with vehicle control. (d) DA-mediated Nrf2 induction was only partially blocked by co-application of catalase at 100 U/mL (saturating concentration). $^{*}p < 0.05$ for comparison. (e) Various quinone-based molecules induced Nrf2 (mena = menadione, HQ = hydroquinone). $^{*}p < 0.05$ compared with DMSO vehicle control, $^{\#}p < 0.05$ compared with same quinone with no AO. Data represent the mean ± SEM collected from at least three independent experiments on separately prepared cultures.

Nrf2 induction is primarily observed in non-neuronal cells: focus on meninges

Cortical neurons express particularly low levels of Nrf2 protein and exhibit reduced basal and inducible ARE-mediated gene expression (Ahlgren-Beckendorf *et al.* 1999; Eftekharpour *et al.* 2000; Murphy *et al.* 2001; Shih *et al.* 2003). In a mixed neuron-astrocyte culture system, Nrf2 activation by electrophilic agents was restricted to the astrocyte population (Murphy *et al.* 2001). Similarly, Han *et al.* found that L-dopa only up-regulated GSH synthesis in mixed neuron-astrocyte cultures, but not pure neuronal cultures, consistent with the idea that neurons exhibit little or no Nrf2 activation in response to DA toxicity (Han *et al.* 1996). In contrast, we show here that meningeal cells respond to electrophilic Nrf2 inducers and DA even more robustly than astrocytes, and have Nrf2 and Phase 2 gene expression levels in excess of that expressed by liver. Indeed, meningeal cells are confined to the brain surface and may not necessarily respond to DA released from distant brain regions such as the substantia nigra. However, our results give proof of principle evidence that meningeal cells are uniquely responsive to Nrf2-inducing agents and that this characteristic is preserved in culture allowing convenient study. For these reasons, we have examined the effect of DA toxicity on meningeal cells as an important comparison/contrast to the classical neuroprotective role of astrocytes.

Based on several findings from our lab and others, we postulate that meningeal cells serve a neuroprotective function *in vivo*, particularly during brain injury. First, ARE-mediated gene expression is strongly and rapidly induced in meningeal cells during *in vivo* focal ischemia (Campagne *et al.* 2000). Second, meningeal tissue is enriched in Nrf2-regulated enzymes including NQO1, glutathione S-transferase, metallothionein, and xCT that may be further upregulated in response to injury (Senjo *et al.* 1986; Murphy *et al.* 1998; van Lookeren Campagne *et al.* 1999; Laxton *et al.* 2001; Sato *et al.* 2002). Third, our previous work has shown that meningeal cells synthesize high levels of GSH *in vivo*, even when compared with cortical astrocytes (Sun *et al.* 2006). Since GSH efflux is dependent on intracellular GSH concentration, the meninges may support neuronal viability by releasing GSH to distant sites within the brain (Sagara *et al.* 1996). Finally, cultured meningeal cells are able to confer wide-spread protection of immature neurons during oxidative stress in a co-culture system (Sun *et al.* 2005; Shih *et al.* 2006).

Activation of Nrf2 by DA toxicity: implications for ischemic injury and neurodegeneration

The endogenous signal(s) leading to Nrf2 activation during stroke and neurodegeneration are poorly understood. Using the Ad-rQR51 assay system, we show that excessive

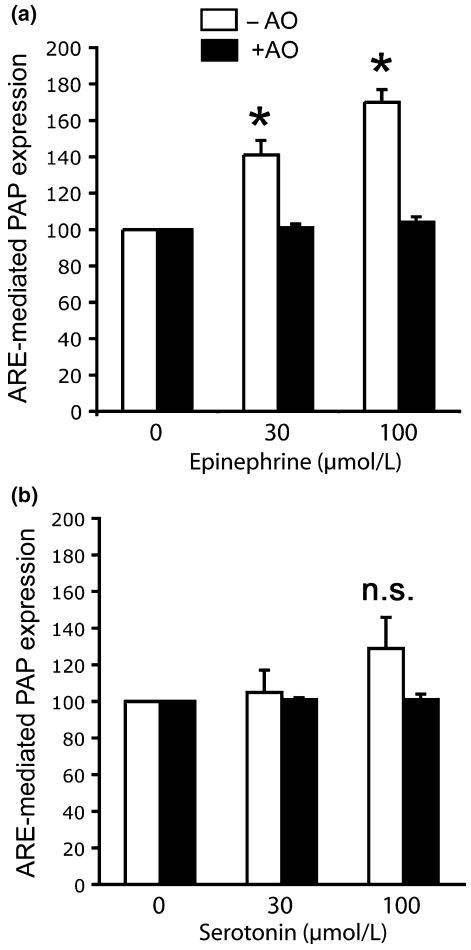

Fig. 5 Epinephrine induces Nrf2 activity, but not serotonin. (a) Nrf2 was induced by another catecholamine neurotransmitter epinephrine. The effect of epinephrine was blocked by co-application of AO. *$p < 0.05$ compared with vehicle control. (b) The monoamine serotonin had no significant effect on Nrf2 activity at the same concentrations (n.s. = not significant compared with no serotonin control). Data represent the mean ± SEM collected from three independent experiments on separately prepared cultures.

extracellular DA can trigger Nrf2 activation *in vitro* by generating oxidative stressors during auto-oxidation/metabolism. In agreement with these results, previous studies have shown that the DA precursor, L-dopa, up-regulates GSH content and NQO1 expression in cultured astrocytes (Han *et al.* 1996; van Muiswinkel *et al.* 2000). We believe that DA toxicity may activate Nrf2 in astrocytes and meningeal cells as a neuroprotective mechanism during injury (van Muiswinkel *et al.* 2004). *In vivo*, astrocytes are ideally positioned to support neuronal survival by inducing antioxidant and Phase 2 enzymes. In theory, the Phase 2 response could scavenge superoxide anion and H_2O_2 (superoxide dismutase, catalase, GSH peroxidase, peroxiredoxins, and thioredoxin) (Motohashi and Yamamoto 2004), detoxify DA-quinones (NQO1) (Cadenas 1995), and sequester iron to reduce catalysis of DA oxidation and production of the hydroxyl radical via the Fenton reaction (ferritin) (Halliwell and Gutteridge 1999; Pietsch *et al.* 2003). In addition, Nrf2 up-regulates all enzymes necessary for GSH synthesis (xCT, GCLC, GSH synthase) and release (multi-drug resistance protein 1), that could compensate for the GSH depleting effects of DA toxicity (Hirrlinger *et al.* 2002; Shih *et al.* 2003). We previously showed that Nrf2-mediated induction of GSH synthesis/release in astrocytes was sufficient to protect neighboring neurons from oxidative stress caused by GSH depletion and H_2O_2 exposure (Shih *et al.* 2003).

Consistent with Nrf2 responding to DA toxicity, the concentrations of DA capable of activating Nrf2 (30 and 100 μmol/L) have been commonly employed for *in vitro* neurotoxicity models (Rosenberg 1988; Hoyt *et al.* 1997; Fu *et al.* 1998). Neurotoxic potency of the neurotransmitter appears important for Nrf2 activation since previous studies have shown that both DA and epinephrine at 100 μmol/L cause significant death of cultured neurons, whereas up to 200 μmol/L serotonin remains relatively non-toxic (Fu *et al.* 1998). Accordingly, our results show that DA and epinephrine are Nrf2 inducing neurotransmitters, while serotonin is not.

Under normal conditions *in vivo*, the concentration of extracellular DA in the brain is likely too low to induce Nrf2 (nmol/L range in striatum, with even lower levels are detected in the cortex) (Moghaddam *et al.* 1990). However, DA toxicity is one important mechanism underlying striatal neurodegeneration caused by both focal ischemia and mitochondrial toxins in rodents (Buisson *et al.* 1991; Maragos *et al.* 1998, 1999). During ischemia, for example, extracellular DA concentrations in the striatum can rise dramatically reaching 120 μmol/L, due to loss of DA re-uptake and enzymatic degradation (Globus *et al.* 1988a). This concentration may be sufficient to activate Nrf2 during stroke based on our results. Interestingly, extracellular DA levels rapidly return to baseline upon restoration of blood flow (Globus *et al.* 1988b), suggesting that prolonged DA exposure necessary for Nrf2 induction might be achieved primarily during permanent ischemia or near the damaged stroke core after transient ischemia. DA released excessively from nerve terminals within the stroke core could emanate to the stroke border-zone where perfusion remains adequate to generate ATP for Phase 2 expression (Hossmann 1994). Consistent with this idea, cortical infarcts caused by transient distal middle cerebral artery occlusion are delineated by astrocytes and meningeal cells undergoing robust ARE-mediated gene expression (Campagne *et al.* 2000). A similar effect has also been observed in the border-zones surrounding striatal lesions induced by malonate and 6-hydroxydopamine (Calkins *et al.* 2005; Jakel *et al.* 2005).

Importantly, Van Muiswinkel and colleagues have detected NQO1 up-regulation in astrocytes and endothelial cells of the substantia nigra pars compacta of patients with Parkinson's

disease (van Muiswinkel *et al.* 2004), suggesting that Nrf2 activation could exist as a protective mechanism against DA toxicity in humans. Pharmacological pre-activation of Nrf2 in the human brain may therefore help attenuate DA toxicity and other forms of oxidative injury during stroke and neurodegeneration. As many Nrf2 inducers exist naturally in fruits and vegetables, prophylactic dietary-based strategies may be practical and effective (Dinkova-Kostova and Talalay 1999; Fahey and Talalay 1999; Kensler *et al.* 2000; Chen and Kong 2004; Chen *et al.* 2005).

Acknowledgements

We thank Lei Jiang for excellent technical assistance, Steve Callaghan and Dominique Vaillant at the Adenovirus core facility (University of Ottawa, Ottawa) for adenovirus production, and the staff at the UBC Animal Research Unit. AYS was supported by studentships from the Canadian Institute of Health Research (CIHR) and the Michael Smith Foundation for Health Research (MSFHR). THM is a CIHR investigator and MSFHR senior scholar. THM is also supported by grants from the Heart and Stroke Foundation of British Columbia and Yukon and the Canadian Stroke Network.

References

Ahlgren-Beckendorf J. A., Reising A. M., Schander M. A., Herdler J. W. and Johnson J. A. (1999) Coordinate regulation of NAD(P)H:quinone oxidoreductase and glutathione- S-transferases in primary cultures of rat neurons and glia: role of the antioxidant/electrophile responsive element. *Glia* **25**, 131–142.

Alam J., Stewart D., Touchard C., Boinapally S., Choi A. M. and Cook J. L. (1999) Nrf2, a Cap'n'Collar transcription factor, regulates induction of the heme oxygenase-1 gene. *J. Biol. Chem.* **274**, 26 071–26 078.

Buisson A., Plotkine M. and Boulu R. (1991) Lesioning the substantia nigra reduces striatal infarct volume following focal ischemia in rats. *Fundam. Clin. Pharmacol.* **5**, 645–647.

Cadenas E. (1995) Antioxidant and prooxidant functions of DT-diaphorase in quinone metabolism. *Biochem. Pharmacol.* **49**, 127–140.

Calkins M. J., Jakel R. J., Johnson D. A., Chan K., Kan Y. W. and Johnson J. A. (2005) Protection from mitochondrial complex II inhibition in vitro and in vivo by Nrf2-mediated transcription. *Proc. Natl Acad. Sci. USA* **102**, 244–249.

Campagne M. V., Thibodeaux H., van Bruggen N., Cairns B. and Lowe D. G. (2000) Increased binding activity at an antioxidant-responsive element in the metallothionein-1 promoter and rapid induction of metallothionein-1 and -2 in response to cerebral ischemia and reperfusion. *J. Neurosci.* **20**, 5200–5207.

Chan K., Lu R., Chang J. C. and Kan Y. W. (1996) NRF2, a member of the NFE2 family of transcription factors, is not essential for murine erythropoiesis, growth, and development. *Proc. Natl Acad. Sci. USA* **93**, 13 943–13 948.

Chen C. and Kong A. N. (2004) Dietary chemopreventive compounds and ARE/EpRE signaling. *Free Radic. Biol. Med.* **36**, 1505–1516.

Chen C. Y., Jang J. H., Li M. H. and Surh Y. J. (2005) Resveratrol upregulates heme oxygenase-1 expression via activation of NF-E2-related factor 2 in PC12 cells. *Biochem. Biophys. Res. Commun.* **331**, 993–1000.

Crocker S. J., Lamba W. R., Smith P. D., Callaghan S. M., Slack R. S., Anisman H. and Park D. S. (2001) c-Jun mediates axotomy-induced dopamine neuron death in vivo. *Proc. Natl Acad. Sci. USA* **98**, 13 385–13 390.

Dinkova-Kostova A. T. and Talalay P. (1999) Relation of structure of curcumin analogs to their potencies as inducers of Phase 2 detoxification enzymes. *Carcinogenesis* **20**, 911–914.

Duffy S., So A. and Murphy T. H. (1998) Activation of endogenous antioxidant defenses in neuronal cells prevents free radical-mediated damage. *J. Neurochem.* **71**, 69–77.

Eftekharpour E., Holmgren A. and Juurlink B. H. (2000) Thioredoxin reductase and glutathione synthesis is upregulated by t- butylhydroquinone in cortical astrocytes but not in cortical neurons. *Glia* **31**, 241–248.

Fahey J. W. and Talalay P. (1999) Antioxidant functions of sulforaphane: a potent inducer of Phase II detoxication enzymes. *Food Chem. Toxicol.* **37**, 973–979.

Fu W., Luo H., Parthasarathy S. and Mattson M. P. (1998) Catecholamines potentiate amyloid beta-peptide neurotoxicity: involvement of oxidative stress, mitochondrial dysfunction, and perturbed calcium homeostasis. *Neurobiol. Dis.* **5**, 229–243.

Globus M. Y., Ginsberg M. D., Dietrich W. D., Busto R. and Scheinberg P. (1987) Substantia nigra lesion protects against ischemic damage in the striatum. *Neurosci. Lett.* **80**, 251–256.

Globus M. Y., Busto R., Dietrich W. D., Martinez E., Valdes I. and Ginsberg M. D. (1988a) Intra-ischemic extracellular release of dopamine and glutamate is associated with striatal vulnerability to ischemia. *Neurosci. Lett.* **91**, 36–40.

Globus M. Y., Busto R., Dietrich W. D., Martinez E., Valdes I. and Ginsberg M. D. (1988b) Effect of ischemia on the *in vivo* release of striatal dopamine, glutamate, and gamma-aminobutyric acid studied by intracerebral microdialysis. *J. Neurochem.* **51**, 1455–1464.

Graham D. G. (1978) Oxidative pathways for catecholamines in the genesis of neuromelanin and cytotoxic quinones. *Mol. Pharmacol.* **14**, 633–643.

Graham D. G. (1984) Catecholamine toxicity: a proposal for the molecular pathogenesis of manganese neurotoxicity and Parkinson's disease. *Neurotoxicology* **5**, 83–95.

Halfter W., Dong S., Yip Y. P., Willem M. and Mayer U. (2002) A critical function of the pial basement membrane in cortical histogenesis. *J. Neurosci.* **22**, 6029–6040.

Halliwell B. and Gutteridge J. M. C. (1999) *Free Radicals in Biology and Medicine*, 3rd edn. Pp. xxxi. 936, [919] of col. plates. Oxford University Press, Oxford, England.

Han S. K., Mytilineou C. and Cohen G. (1996) L-DOPA up-regulates glutathione and protects mesencephalic cultures against oxidative stress. *J. Neurochem.* **66**, 501–510.

Hardy S., Kitamura M., Harris-Stansil T., Dai Y. and Phipps M. L. (1997) Construction of adenovirus vectors through Cre-lox recombination. *J. Virol.* **71**, 1842–1849.

Hirrlinger J., Schulz J. B. and Dringen R. (2002) Effects of dopamine on the glutathione metabolism of cultured astroglial cells: implications for Parkinson's disease. *J. Neurochem.* **82**, 458–467.

Hossmann K. A. (1994) Viability thresholds and the penumbra of focal ischemia. *Ann. Neurol.* **36**, 557–565.

Hoyt K. R., Reynolds I. J. and Hastings T. G. (1997) Mechanisms of dopamine-induced cell death in cultured rat forebrain neurons: interactions with and differences from glutamate-induced cell death. *Exp. Neurol.* **143**, 269–281.

Itoh K., Chiba T., Takahashi S. *et al.* (1997) An Nrf2/small Maf heterodimer mediates the induction of phase II detoxifying enzyme genes through antioxidant response elements. *Biochem. Biophys. Res. Commun.* **236**, 313–322.

Itoh K., Wakabayashi N., Katoh Y., Ishii T., Igarashi K., Engel J. D. and Yamamoto M. (1999) Keap1 represses nuclear activation of anti-

oxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain. *Genes Dev.* **13**, 76–86.

Jakel R. J., Kern J. T., Johnson D. A. and Johnson J. A. (2005) Induction of the protective antioxidant response element pathway by 6-hydroxydopamine *in vivo* and *in vitro*. *Toxicol. Sci.* **87**, 176–186.

Johnson J. R., Robinson B. L., Ali S. F. and Binienda Z. (2000) Dopamine toxicity following long term exposure to low doses of 3-nitropropionic acid (3-NPA) in rats. *Toxicol. Lett.* **116**, 113–118.

Kensler T. W., Curphey T. J., Maxiutenko Y. and Roebuck B. D. (2000) Chemoprotection by organosulfur inducers of phase 2 enzymes: dithiolethiones and dithiins. *Drug Metab. Drug Interact.* **17**, 3–22.

Kochanek P. M. and Hallenbeck J. M. (1992) Polymorphonuclear leukocytes and monocytes/macrophages in the pathogenesis of cerebral ischemia and stroke. *Stroke* **23**, 1367–1379.

Kraft A. D., Johnson D. A. and Johnson J. A. (2004) Nuclear factor E2-related factor 2-dependent antioxidant response element activation by tert-butylhydroquinone and sulforaphane occurring preferentially in astrocytes conditions neurons against oxidative insult. *J. Neurosci.* **24**, 1101–1112.

Laxton A. W., Sun M. C., Shen H., Murphy T. H. and Honey C. R. (2001) The antioxidant enzyme quinone reductase is up-regulated in vivo following cerebral ischemia. *Neuroreport* **12**, 1045–1048.

Lee J. M., Shih A. Y., Murphy T. H. and Johnson J. A. (2003a) NF-E2-related factor-2 mediates neuroprotection against mitochondrial complex I inhibitors and increased concentrations of intracellular calcium in primary cortical neurons. *J. Biol. Chem.* **278**, 37 948–37 956.

Lee J. M., Calkins M. J., Chan K., Kan Y. W. and Johnson J. A. (2003b) Identification of the NF-E2-related factor-2-dependent genes conferring protection against oxidative stress in primary cortical astrocytes using oligonucleotide microarray analysis. *J. Biol. Chem.* **278**, 12 029–12 038.

van Lookeren Campagne M., Thibodeaux H., van Bruggen N., Cairns B., Gerlai R., Palmer J. T., Williams S. P. and Lowe D. G. (1999) Evidence for a protective role of metallothionein-1 in focal cerebral ischemia. *Proc. Natl Acad. Sci. USA* **96**, 12 870–12 875.

Maragos W. F., Jakel R. J., Pang Z. and Geddes J. W. (1998) 6-Hydroxydopamine injections into the nigrostriatal pathway attenuate striatal malonate and 3-nitropropionic acid lesions. *Exp. Neurol.* **154**, 637–644.

Maragos W. F., Jakel R. J., Chesnut M. D., Geddes J. W. and Dwoskin L. P. (1999) Does dopamine contribute to striatal damage caused by impaired mitochondrial function? *Ann. NY Acad. Sci.* **893**, 345–349.

Moehlenkamp J. D. and Johnson J. A. (1999) Activation of antioxidant/electrophile-responsive elements in IMR-32 human neuroblastoma cells. *Arch. Biochem. Biophys.* **363**, 98–106.

Moghaddam B., Roth R. H. and Bunney B. S. (1990) Characterization of dopamine release in the rat medial prefrontal cortex as assessed by *in vivo* microdialysis: comparison to the striatum. *Neuroscience* **36**, 669–676.

Motohashi H. and Yamamoto M. (2004) Nrf2-Keap1 defines a physiologically important stress response mechanism. *Trends Mol. Med.* **10**, 549–557.

van Muiswinkel F. L. and Kuiperij H. B. (2005) The Nrf2-ARE Signalling pathway: promising drug target to combat oxidative stress in neurodegenerative disorders. *Curr. Drug Targets CNS Neurol. Disord.* **4**, 267–281.

van Muiswinkel F. L., Riemers F. M., Peters G. J., LaFleur M. V., Siegel D., Jongenelen C. A. and Drukarch B. (2000) L-Dopa stimulates expression of the antioxidant enzyme NAD(P)H:quinone oxidoreductase (NQO) in cultured astroglial cells. *Free Radic. Biol. Med.* **29**, 442–453.

van Muiswinkel F. L., de Vos R. A., Bol J. G., Andringa G., Jansen Steur E. N., Ross D., Siegel D. and Drukarch B. (2004) Expression of NAD(P)H:quinone oxidoreductase in the normal and Parkinsonian substantia nigra. *Neurobiol. Aging* **25**, 1253–1262.

Murphy T. H., So A. P. and Vincent S. R. (1998) Histochemical detection of quinone reductase activity in situ using LY 83583 reduction and oxidation. *J. Neurochem.* **70**, 2156–2164.

Murphy T. H., Yu J., Ng R., Johnson D. A., Shen H., Honey C. R. and Johnson J. A. (2001) Preferential expression of antioxidant response element mediated gene expression in astrocytes. *J. Neurochem.* **76**, 1670–1678.

Pietsch E. C., Chan J. Y., Torti F. M. and Torti S. V. (2003) Nrf2 mediates the induction of ferritin H in response to xenobiotics and cancer chemopreventive dithiolethiones. *J. Biol. Chem.* **278**, 2361–2369.

Prochaska H. J., Santamaria A. B. and Talalay P. (1992) Rapid detection of inducers of enzymes that protect against carcinogens. *Proc. Natl Acad. Sci. USA* **89**, 2394–2398.

Reynolds D. S., Carter R. J. and Morton A. J. (1998) Dopamine modulates the susceptibility of striatal neurons to 3-nitropropionic acid in the rat model of Huntington's disease. *J. Neurosci.* **18**, 10 116–10 127.

Rosenberg P. A. (1988) Catecholamine toxicity in cerebral cortex in dissociated cell culture. *J. Neurosci.* **8**, 2887–2894.

Sagara J., Makino N. and Bannai S. (1996) Glutathione efflux from cultured astrocytes. *J. Neurochem.* **66**, 1876–1881.

Sato H., Tamba M., Okuno S., Sato K., Keino-Masu K., Masu M. and Bannai S. (2002) Distribution of Cystine/Glutamate Exchange Transporter, System xc-, in the Mouse Brain. *J. Neurosci.* **22**, 8028–8033.

Satoh T., Okamoto S. I., Cui J., Watanabe Y., Furuta K., Suzuki M., Tohyama K. and Lipton S. A. (2006) Activation of the Keap1/Nrf2 pathway for neuroprotection by electrophillic phase II inducers. *Proc. Natl Acad. Sci. USA* **103**, 768–773.

Senjo M., Ishibashi T., Terashima T. and Inoue Y. (1986) Successive appearance of glutathione S-transferase-positive cells in developing rat brain: choroid plexus, pia mater, ventricular zone and astrocytes. *Neurosci. Lett.* **66**, 131–134.

Shih A. Y., Johnson D. A., Wong G., Kraft A. D., Jiang L., Erb H., Johnson J. A. and Murphy T. H. (2003) Coordinate regulation of glutathione biosynthesis and release by Nrf2-expressing glia potently protects neurons from oxidative stress. *J. Neurosci.* **23**, 3394–3406.

Shih A. Y., Li P. and Murphy T. H. (2005a) A small-molecule-inducible Nrf2-mediated antioxidant response provides effective prophylaxis against cerebral ischemia in vivo. *J. Neurosci.* **25**, 10 321–10 335.

Shih A. Y., Imbeault S., Barakauskas V., Erb H., Jiang L., Li P. and Murphy T. H. (2005b) Induction of the Nrf2-driven Antioxidant Response Confers Neuroprotection during Mitochondrial Stress in Vivo. *J. Biol. Chem.* **280**, 22 925–22 936.

Shih A. Y., Erb H., Sun X., Toda S., Kalivas P. W. and Murphy T. H. (2006) Cystine/glutamate exchange modulates glutathione supply for neuroprotection from oxidative stress and cell proliferation. *J. Neurosci.* **26**, 10 514–10 523.

Smythies J. and Galzigna L. (1998) The oxidative metabolism of catecholamines in the brain: a review. *Biochim. Biophys. Acta* **1380**, 159–162.

Stokes A. H., Hastings T. G. and Vrana K. E. (1999) Cytotoxic and genotoxic potential of dopamine. *J. Neurosci. Res.* **55**, 659–665.

Sun X., Shih A. Y., Erb H., Jiang L., Li P. and Murphy T. H. (2005) Meningeal cells efficiently protect neurons against oxidative stress by GSH-dependent mechanisms. Program No. 221.14. *2005 Abstract Viewer and Itinerary Planner*, Society for Neuroscience, Washington, DC. http://sfn.scholarone.com/itin2005

Sun X., Shih A. Y., Johannssen H. C., Erb H., Li P. and Murphy T. H. (2006) Two-photon imaging of glutathione levels in intact brain indicates enhanced redox buffering in developing neurons and cells at the cerebrospinal fluid and blood-brain interface. *J. Biol. Chem.* **281**, 17 420–17 431.

Tietze F. (1969) Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. *Anal. Biochem.* **27**, 502–522.

Venugopal R. and Jaiswal A. K. (1996) Nrf1 and Nrf2 positively and c-Fos and Fra1 negatively regulate the human antioxidant response element-mediated expression of NAD(P)H:quinone oxidoreductase1 gene. *Proc. Natl Acad. Sci. USA* **93**, 14 960–14 965.

Wasserman W. W. and Fahl W. E. (1997) Functional antioxidant responsive elements. *Proc. Natl Acad. Sci. USA* **94**, 5361–5366.

Wild A. C. and Mulcahy R. T. (2000) Regulation of gamma-glutamylcysteine synthetase subunit gene expression: insights into transcriptional control of antioxidant defenses. *Free Radic. Res.* **32**, 281–301.

Wrona M. Z. and Dryhurst G. (1998) Oxidation of serotonin by superoxide radical: implications to neurodegenerative brain disorders. *Chem. Res. Toxicol.* **11**, 639–650.

