# THE ROLE OF NRF2 IN EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYELITIS-A MOUSE MODEL OF MULTIPLE SCLEROSIS AND NEUROINFLAMMATION

By

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# DEDICATION

I DEDICATE THIS THESIS TO JEFF-MY LOVING HUSBAND AND BEST FRIEND

AND TO MY DAUGTERS TAYLOR AND EMMA CLAIRE-MY CHEERLEADERS!

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# ABBREVIATIONS AND SYMBOLS

| ABBREVIATIONS AND SYMBOLS | DEFINITION |
|---|---|
| AD | Alzheimer's disease |
| AD-GFP | adenovirus-green fluorescent protein |
| AD-Nrf2 | adenovirus-Nrf2 |
| ALI | acute lung injury |
| ALS | amyotrophic lateral sclerosis |
| AP-1 | activator protein-1 |
| ARE | antioxidant response element |
| BBB | blood brain barrier |
| BLC | B cell attractant chemokine |
| BTB | bric-a-brac, tamtrack, broad-complex domain |
| CAT | catalase |
| CBP | CREB binding protein |
| CD | cluster of differentiation |
| CDDO-Im | 1-2-cyano-3-12-dioxooleana-1,9(11)-dien-28–oyl]-imidazole |
| CFA | complete Freund's adjuvant |
| CMV | cytomegalovirus |
| CNC | cap-n-collar |
| CNS | central nervous system |
| COX-2 | cyclooxygenase-2 |
| CREB | cAMP response element-binding |
| CTL | cytotoxic T lymphocyte |
| DEM | diethyl maleate |
| DEP | diesel exhaust particles |
| DMF | dimethyl fumarate |
| E3 | E3 ubiquitin ligase |
| E14-16 | embryonic day 14-16 |
| EAE | experimental autoimmune encephalomyelitis |
| ER | endoplasmic reticulum |
| FBS | fetal bovine serum |
| GAPDH | glyceraldehyde 3-phosphate dehydrogenase |
| GCLC | glutamate-cysteine ligase-catalytic subunit |

| | |
|---|---|
| GCLM | glutamate-cysteine ligase-modulatory subunit |
| GFAP | glial fibrillary acidic protein |
| Gpx | glutathione peroxidase |
| GR | glutathione reductase |
| GSH | glutathione (reduced) |
| GST | glutathione S-transferase |
| GSSG | oxidized glutathione |
| hPAP | human placental alkaline phosphatase |
| HPLC | high performance liquid chromatography |
| HO-1 | heme oxygenase-1 |
| $H_2O_2$ | hydrogen peroxide |
| Iba1 | ionized calcium binding adaptor molecule 1 |
| IκB | Inhibiter protein of NF-κB |
| Iκκ | IκB kinase |
| IL | interleukin |
| iNOS | inducible nitric oxide synthase |
| INF | interferon |
| Keap1 | Kelch ECH associating protein |
| LFB | luxol fast blue |
| LPS | lipopolysaccharide |
| MAP-2 | microtubule associated protein-2 |
| MMP | matrix metalloprotease |
| MPTP | 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine |
| MS | multiple sclerosis |
| NADH | nicotinamide adenine dinucleotide |
| NADPH | nicotinamide adenine dinucleotide phosphate |
| Neh | Nrf2-ECH homologous domian |
| NO | nitric oxide |
| Nrf | nuclear factor erythroid 2-related factor |
| NF-E2 | nuclear factor erythroid 2 |
| Nrf2-KO | Nrf2-knock out |
| NQO1 | NAD(P)H quinone oxidoreductase 1 |
| Maf | musculoaponeurotic fibrosarcoma oncogene homolog |
| MBP | myelin basic protein |
| MIG | monokine induced by interferon-γ |
| MOG | myelin oligodendrocyte glycoprotein |

| | |
|---|---|
| MPO | myeloperoxidase |
| NF-κB | nuclear factor kappa-light chain-enhancer of activated B cells |
| $O_2^{\bullet-}$ | superoxide radical |
| $^{\bullet}OH$ | hydroxyl radical |
| $ONOO^-$ | peroxynitrite |
| P1-2 | postnatal day 1-2 |
| PD | Parkinson's disease |
| PGE2 | prostaglandin E2 |
| PLP | proteolipid protein |
| Prx | peroxiredoxin |
| qPCR | quantitative real time polymerase chain reaction |
| RANTES | Regulated upon Activation, Normal T-cell Expressed and Secreted |
| RLU | relative luminescent units |
| $ROO^{\bullet}$ | peroxyl radical |
| $RO^{\bullet}$ | alkoxyl radical |
| ROS | reactive oxygen species |
| SFN | sulforaphane |
| tBHQ | tert-butylhydroquinone |
| RNS | reactive nitrogen species |
| RRMS | relapsing remitting multiple sclerosis |
| SD | standard deviation |
| SPMS | secondary progressive multiple sclerosis |
| Th | T helper cell |
| TNF | tumor necrosis factor |
| TRE | TPA-response element |
| TRX | thioredoxin |
| TXNRD1 | thioredoxin reductase-1 |
| SEM | standard error of the mean |
| SOD | superoxide dismutase |

## ABSTRACT

The nuclear factor erythroid 2-related factor 2 (Nrf2)-antioxidant response element (ARE) signaling pathway plays an essential role in protection from oxidative, nitrosative and electrophilic insult. Activation of this pathway drives gene clusters that mediate cellular redox homeostasis and protective mechanisms important for cell survival. Many studies using deficient mice (Nrf2-KO) have demonstrated the significance of Nrf2 in the prevention of diseases associated with oxidative stress. In many cases, the Nrf2-KO mice had an enhanced inflammatory response.

Studies herein explored the role of Nrf2 in experimental autoimmune encephalomyelitis (EAE), an autoimmune inflammatory model of multiple sclerosis (MS). Nrf2-KO mice immunized with antigenic myelin oligodendrocytic glycoprotein (MOG) 35-55, demonstrated increased severity of disease compared to WT. Clinical scores, as well as peripheral immune cell infiltration, microgliosis and inflammatory markers in spinal tissue were all significantly increased in Nrf2-KO versus WT mice with EAE.

When activated, microglia secrete cytotoxic reactive oxygen and nitrogen species. Based on this, as well as greater reactive microgliosis in Nrf2-KO mice with EAE, we investigated the effects of increased or lack of expression of Nrf2 on activation of microglia/macrophages. Our studies demonstrated a gene dose dependent

-activation of Nrf2 in primary cortical microglia from Nrf2-KO and WT mice and from mice overexpressing Nrf2 specifically in microglia/macrophage. Several ARE-driven genes, including the glutathione synthesizing genes, *GCLC* and *GCLM,* resulted in dose dependent gene expression. In addition, glutathione levels positively correlated with *GCLC* and *GCLM* gene expression in these same cultures. LPS-activated microglia in transwell co-cultures with neurons, or in organotypic spinal cord cultures, were attenuated by tBHQ-activation or microglia specific overexpression of Nrf2, respectively. Strikingly, we observed Nrf2-dependent neuronal protection from LPS-activated microglia in the transwell co-cultures treated with tBHQ. EAE experiments using mice overexpressing Nrf2 in microglia/macrophages had significantly lower clinical scores of disease compared with WT.

The data in this thesis describe the Nrf2-dependent attenuation of EAE and microglial activation. Activation of the Nrf2-ARE signaling pathway in microglia may be an important therapeutic target for alleviation of the deleterious effects of inflammation in diseases such as PD, ALS and MS, and thus contribute to neuronal protection.

# CHAPTER 1

## Introduction

**1.1 Reactive Oxygen and Nitrogen Species**

Our cells are continuously exposed to endogenous and extrinsic forms and sources of reactive oxygen (ROS) and nitrogen species (RNS). ROS and RNS are highly reactive ionic molecules. The most abundant source of reactive oxygen species (ROS) is initiated by the accidental leakage of electrons from mitochondrial respiration, a requisite of aerobic metabolism. These electrons bombard triplet oxygen ($O_2$) creating the superoxide molecule, $O_2^{\bullet -}$. Table 1, depicts several of the prominent reactive species that are generated in aerobic biological systems (Gate et al., 1999; Winterbourn, 2008).

The most highly reactive oxygen species is the hydroxyl radical $^{\bullet}OH$ with second order rate constants of $10^9$ to $10^{10}$ $M^{-1}$ $sec^{-1}$. In addition, ROS and RNS are produced by NADPH oxidase and inducible nitric oxide synthase (iNOS) enzyme, respectively, in phagocytic cells as necessary intermediates for the destruction and defense against invading pathogens (Halliwell and Cross, 1994). Extrinsic exposure to environmental toxins or drugs can also generate ROS. For instance, the herbicide paraquat undergoes a one-electron reduction by NADPH cytochrome c reductase to form paraquat radicals. The latter radicals immediately transfer their radical electrons to $O_2$, generating $O_2^{\bullet -}$ (Winterbourn, 2008). Abnormal environmental conditions such as hypo- or hyperoxia can generate ROS as well (Bowen, 2003).

**Table 1.1 Examples of Reactive Oxygen and Nitrogen Species**

| Reactive Oxygen or Nitrogen Species | Sources of ROS/RNS | Stability and Reactivity |
|---|---|---|
| **Super Oxide** $O_2^{\bullet -}$ | •Mitochondrial electron transport leakage<br>•NADPH Oxidase enzymes-respiratory burst<br>•Microsomal cytochrome P450 and corresponding electron transporting enzymes<br>•Ionizing radiation | •Unstable<br>•Spontaneously dismutases: $O_2^{\bullet -} + H \rightarrow 0.5H_2O_2 + 0.5O_2$<br>•pKa=4.8 thus In equilibrium with $HO_2^{\bullet}$ (very unstable-excellent oxidant) |
| **Hydrogen Peroxide** $H_2O_2$ | •Superoxide dismutase (SOD) enzyme reaction: $2O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2$<br>•Other enzymes: urate oxidase, L-amino acid oxidase and glycolate oxidase | •Stable<br>•Good oxidant<br>•Low steady state cellular concentrations are not toxic<br>•Proposed as a second messenger in redox-regulated signaling pathways |
| **Hydroxyl Radical** $^{\bullet}OH$ | •Fenton reaction: $(Fe^{2+}/Fe^{3+})$ $H_2O_2 \rightarrow {}^{\bullet}OH + OH^-$<br>•Haber Weiss reaction: $(Fe^{2+}/Fe^{3+})$ $H_2O_2 + O_2^{\bullet -} \rightarrow {}^{\bullet}OH + OH^- + O_2$ | •Very unstable<br>•The most highly reactive oxygen radical known-excellent oxidant |
| **Nitric Oxide** NO | •Inducible nitric oxide synthase (iNOS) reaction: $Arg + O_2 + NADPH \rightarrow NO + Citrulline + NADP^+$ | •Stable<br>•Poor oxidant |
| **ONOO$^-$** | $O_2^{\bullet -} + NO \rightarrow ONOO^- \xrightarrow{CO_2} {}^{\bullet}NO_2 + {}^{\bullet}CO_3$ | •Powerful oxidant (itself)<br>•Quickly reacts with cellular $CO_2$<br>•$^{\bullet}NO_2 + {}^{\bullet}CO_3$ believed to be toxic radicals |
| **Peroxyl, Alkoxyl Radical** $ROO^{\bullet}$, $RO^{\bullet}$ | •Lipid peroxidation: $RH + {}^{\bullet}OH \rightarrow R^{\bullet} + O_2 \rightarrow ROO^{\bullet} + H_2O$<br>$ROO^{\bullet} + RH \rightarrow ROOH + R^{\bullet}$<br>$ROOH \rightarrow RO^{\bullet} \rightarrow$ Malondialdehyde | •Both unstable<br>•Both powerful oxidants |

Representative references:
Halliwell and Cross 1994; Gate, Paul et al. 1999; Bowen 2003; Olson 2007; Winterbourn 2008.

### 1.2 Cellular Mechanisms of Protection Against ROS/RNS

Reactive intermediates are short lived, unstable and highly reactive with surrounding macromolecules. ROS and RNS are capable of reacting with and damaging DNA and protein, and can initiate lipid peroxidation reactions. Together these result in cellular toxicity and possibly cell death. Fortunately, our biological systems have evolved to counteract and protect against these potential deleterious effects (Gate et al., 1999; Winterbourn, 2008).

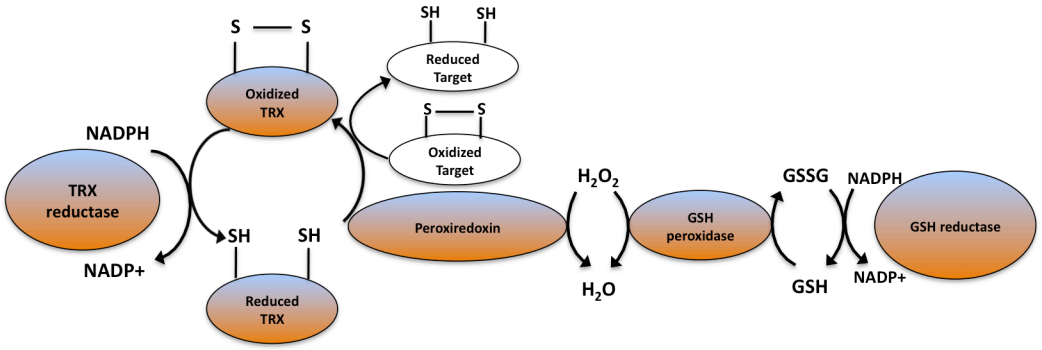
#### 1.2.1 Antioxidants and Antioxidant Enzymes

Cellular enzymatic systems and antioxidants convert reactive species to non-toxic molecules and serve to maintain cellular homeostasis. Glutathione (GSH) is the most abundant cellular antioxidant. It is a small three amino acid peptide, γ-glutamate-cysteine-glycine, with an active nucleophilic sulfhydryl group on the cysteine. GSH is thus an important mediator in the prevention of oxidative and nitrosative stress in cells. GSH is capable of directly reacting with reactive species and also can convert $H_2O_2$ to water and reduce lipid peroxides with the aid of glutathione peroxidases. The oxidized GSSG is reduced back to GSH by GSH reductase. One other important cysteine rich protein found in the cytoplasm is thioredoxin (TRX). This protein reduces oxidized targeted species such as cellular proteins as well as the enzyme peroxiredoxin (another enzyme important in the reduction of ROS and

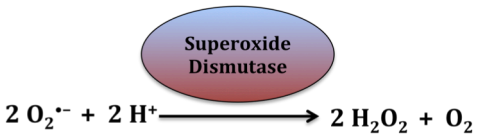
RNS) (Fig. 1.1A) (Gate et al., 1999; Halliwell and Cross, 1994). TRX reductase reduces the oxidized TRX to regenerate reduced TRX.

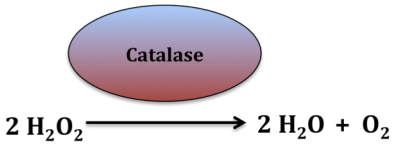
In addition to the systems described above, the enzymes superoxide dismutase (SOD) and catalase also detoxify the superoxide anion $O_2^{\bullet -}$ and $H_2O_2$, respectively. SOD converts $O_2^{\bullet -}$ to $H_2O_2$ and $O_2$, whilst catalase subsequently converts $H_2O_2$ to $H_2O$ and $O_2$ (Fig. 1.1B).

**Figure 1.1 Cellular Mechanisms for the Detoxification of Reactive Oxygen and Nitrogen Species.**
*(A)* $H_2O_2$ and lipid hydroperoxides can be reduced by peroxiredoxins (Prx). In turn, Prxs are reduced by thioredoxins (TRX). TRXs are reduced by TRX reductase. In addition, GSH can reduce $H_2O_2$ and lipid hydroperoxides via GSH peroxidase. The oxidized state of glutathione, GSSG, can be reduced back to GSH by GSH reductase. Lastly, oxidized protein targets are reduced by TRXs. *(B)* Superoxide Dismutase (SOD) converts superoxides to $H_2O_2$ and $O_2$; catalase converts $H_2O_2$ to $H_2O$ and $O_2$. *(C)* A two electron reduction of a quinone to hydroquinone by NAD(P)H quinone oxidoreducatase (NQO1) prevents the redox cycling of the quinone and generation of reactive oxygen species. Quinones can also be reduced by GSH to a GS-conjugated hydroquinone. Note-Shaded balloons are genes expressed by the Nrf2-ARE signaling pathway.

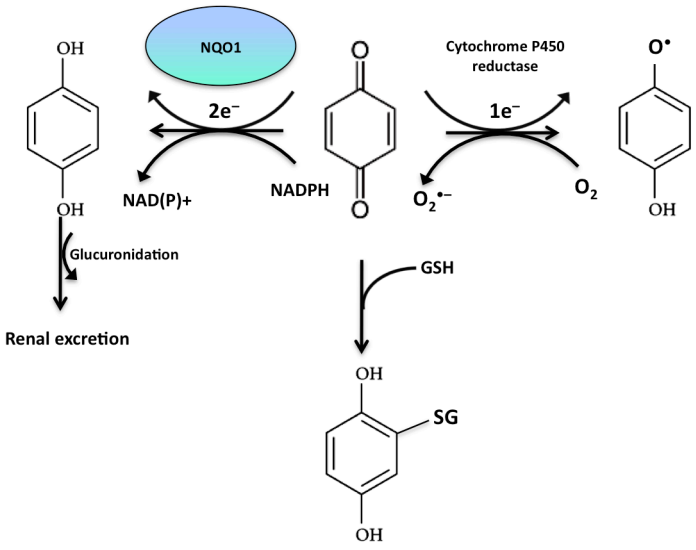

S — S
SH SH
Reduced Target
Oxidized TRX
S — S
Oxidized Target
NADPH
TRX reductase
NADP+
SH SH
Reduced TRX
Peroxiredoxin
$H_2O_2$
$H_2O$
GSH peroxidase
GSSG
NADPH
GSH
NADP+
GSH reductase

B


Superoxide Dismutase
$2\ O_2^{\bullet-} + 2\ H^+ \rightarrow 2\ H_2O_2 + O_2$


Catalase
$2\ H_2O_2 \rightarrow 2\ H_2O + O_2$

C


OH
NQO1
2e⁻
NAD(P)+
NADPH
O
O
Cytochrome P450 reductase
1e⁻
$O_2^{\bullet-}$
$O_2$
O•
OH
OH
Glucuronidation
Renal excretion
GSH
OH
SG
OH

## 1.3 ARE-Nrf2 Signaling Mechanisms Mediate Cellular Homeostasis and Protection

### 1.3.1 The Antioxidant Response Element

The ARE is a *cis*-acting gene enhancer sequence located in the promoter site of a battery of genes whose functions are to promote cellular homeostasis and survival (Li and Jaiswal, 1992; Rushmore et al., 1990). ROS, RNS and thiol reactive (electrophilic) compounds activate the ARE and induce gene transcription. Through DNA microarray analysis, ARE-driven genes were identified and clustered into the following categories all of which are important for maintenance of cellular homeostasis and protection: i) metabolize and conjugate xenobiotics or electrophiles such as the Phase II enzymes NAD(P)H-quinone oxidoreductase (NQO1), multiple glutathione S-transferases (GSTs) and UDP- glycosyltransferase 1A6 (UGT); and ii) boost a cells antioxidant potential including glutamate-cysteine ligase (GCL; glutathione (GSH) production), heme oxygenase-1 (HO-1), thioredoxin reductase-1 (TXNRD1), TRX, and ferritin (converts $Fe^{2+}$ to $Fe^{3+}$ and sequesters it, thereby preventing $Fe^{2+}$ from participating in the Fenton reaction) (Kraft et al., 2004b; Lee et al., 2003a; Li et al., 2002; Shih et al., 2003b).

Initial studies presumed that members of the AP-1 transcription factor family regulated the ARE, since there were similarities between the ARE core sequence, 5'-TGACnnnGC-3' and the TPA-response element (TRE) core sequence 5'-TGACTCA-3'. AP-1 binds to the TRE and is activated by numerous signals including growth factors,

UV-radiation and oxidative stress (Lee et al., 1987). However, later studies using mutational analysis, determined the ARE has different signaling pathways than the TRE. Table 2 describes numerous genes that contain an ARE as well TRE-like elements required for full the activation of the gene (Nguyen et al., 1994; Nguyen et al., 2003).

**Table 1.2 Anti-oxidant genes carrying the ARE core sequence TGACnnnGC.**
ARE sequences are bolded and underlined in black. TRE-like sequences are underlined in red. -Note that full basal gene expression requires the TRE-like sequences.

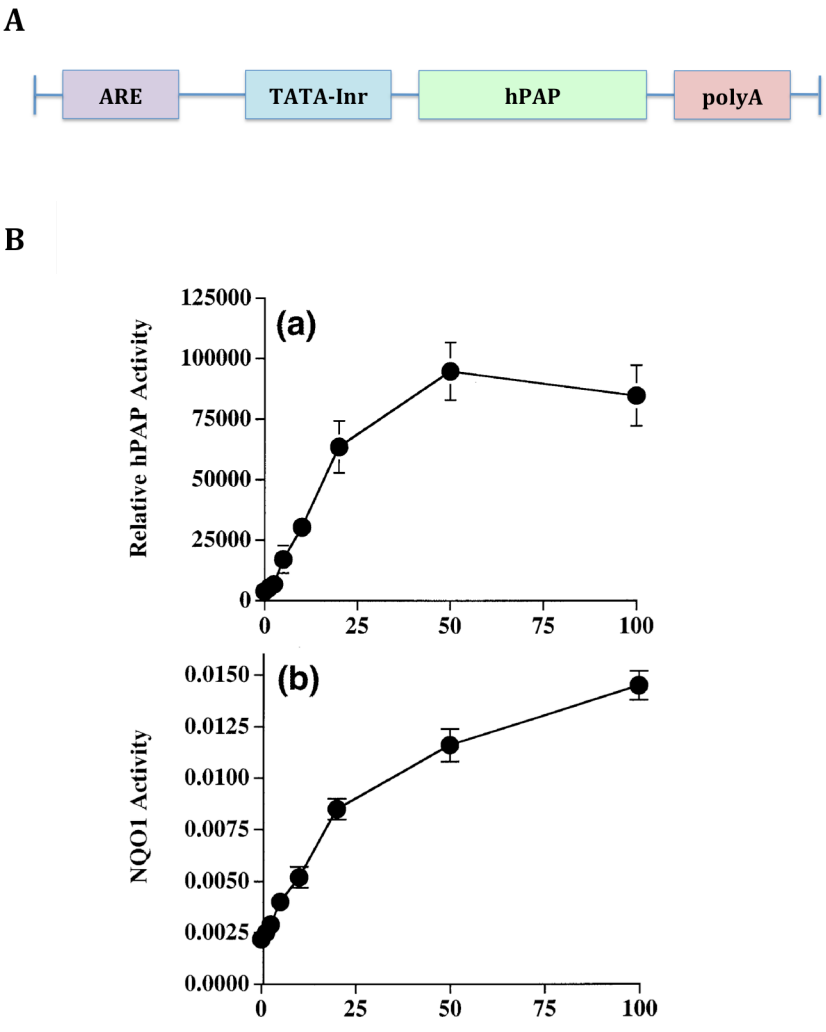
| Gene | Sequence of ARE |
|---|---|
| *GSTA2 (rat)* | 5′ -AAATGGCATTGCTAATGG **TGACAAAGC** AACT-3′ |
| *Gsta1 (mouse)* | 5′ -AAATGACATTGCTAATGG **TGACAAAGC** AACT-3′ |
| *GSTP (rat)* | 5′ -AGTAGTCAGTCACTA **TGATTCAGC** AACA-3′ |
| *NQO1 (rat)* | 5′ -AGTCTAGAGTCACAG **TGACTTGGC** AAAA-3′ |
| *NQO1 (human)* | 5′ -AAATCGCAGTCACAG **TGACTCAGC** AGAA-3′ |
| *GCLC (human)* | 5′ -AGC **GCTGAGTCA** CGGGG- . . . - CCCGCGCAGTCAC-3′ |
| *GCLM (human)* | 5′ -GGATGAGTAACGG- . . . –TTCT **GCTTAGTCA** TTG-**3′** |
| *Ho-1 (mouse)* | 5′ -TCT **GCTGAGTCA** AGGTCCG-3′ |
| NF-E2 (consensus) | 5′ -TGT **GCTGAGTCA** CTG-3 |
| TRE (consensus) | 5′ -GCA **TGAGTCA** GAC-3′ |

Adapted from *Regulatory Mechanisms Controlling Gene Expression Mediated by the Antioxidant Response Element* (Nguyen, Sherratt and Pickett; 2003)

### 1.3.2 The ARE is activated in Mouse Primary Cortical Cultures

Our laboratory created a transgenic reporter mouse that enables us to study activation of the ARE. Briefly, a reporter construct was derived consisting of 51 base pairs of the promoter region from the rat *NQO1* gene that contains an ARE and was coupled to the human placental alkaline phosphatase (hPAP) gene. Mouse blastocysts were injected with linearized DNA and implanted into donor females. Offspring founder mice were identified by PCR for positive insertion of the gene (Fig. 1.2A) (Johnson et al., 2002b). Our laboratory studies the prevention of neurodegenerative disorders such as Alzheimer's disease (AD), Parkinson's disease (PD) and Amyotrophic lateral sclerosis (ALS). More specifically, we study how overexpression of antioxidant genes can protect against the oxidative and nitrosative stress associated with these diseases. Hence, studies using the ARE-hPAP reporter mice have been fruitful in understanding how activation of the ARE has been neuroprotective. For instance, primary cortical mixed cultures (neurons and astrocytes) derived from these mice and treated with tert-butylhydroquinone (tBHQ), a known activator of the Nrf2-ARE, showed a dose dependent increase in hPAP activity representing Nrf2 activation. This increase is also reflected by an increase in NQO1 (known to be increased by tBHQ in a Nrf2-dependent manner) activity (Fig 1.2B) (Johnson et al., 2002b). Subsequent studies indicated that tBHQ selectively activated Nrf2 in the astrocytes and that this astrocytic activation significantly increased the resistance of neurons to oxidative stress-induced cytotoxicity (Kraft et al., 2004).

**Figure 1.2 The Antioxidant Response Element Reporter Mice.** *(A)* Transgenic reporter mice were developed by genomic insertion of 51 bp from the *NQO1* promoter region coupled to the *human placental alkaline phosphatase* (hPAP) gene. Transcription and detection of hPAP indicates activation of the ARE. *(B)* Activation of the ARE as measured by a luminescent substrate for phosphatase activity. Primary cortical cultures were treated with increasing doses of tBHQ for 24 hours. Data are presented as mean relative phosphatase activity ± SEM (n=5). To confirm ARE-activation, NQO1 activity was measured in the same cultures. Data are represented as mean NQO1 activity ± SEM (n=5).



### 1.3.3 Nrf2 is the Master Regulator of the Antioxidant Response Element

Nrf2 was first identified as a protein capable of binding to the NF-E2/AP-1 motif of the DNase I hypersensitive site-2 in the ß-globin locus control gene (Itoh et al., 1995; Kensler et al., 2007; Moi et al., 1994). Nrf2 is a basic leucine zipper transcription factor with a conserved *cap 'n' collar* (CNC) domain. The CNC domain was first identified in *Drosophila* and appropriately named, as it is required for development of the labral segments and mandible. There are six CNC family members in mammalian cells. Four of which are closely related, p45-NFE2, Nrf1, Nrf2 and Nrf3, and two that are distantly related, Bach1 and Bach2. All activate transcription except Bach 1 and 2, which function as transcriptional repressors (Kensler et al., 2007; Sykiotis and Bohmann, 2010).

Jaiswal and colleagues identified similarities of the DNA binding motifs of Nrf1 and Nrf2 to that of the ARE found in the human *NQO1* promoter region. In fact, when they transfected Nrf1 or Nrf2 plasmid in HepG2 cells, they observed increased ARE-reporter gene activity, suggesting the binding of these transcription factors to the ARE (Venugopal and Jaiswal, 1996). Although Nrf1 and Nrf3 respond to stress and can stimulate transcription of ARE-containing genes, Nrf2 is a more potent transcriptional activator (Kobayashi et al., 1999; Sykiotis and Bohmann, 2010). Both Nrf1 and Nrf3 are integral membrane proteins associated with the endoplasmic reticulum (ER). Upon ER stress, Nrf3 is activated however Nrf1 is

suppressed. How these two transcription factors translocate to the nucleus is not well understood (Zhang et al., 2006; Zhang et al., 2009).

Nrf2 is a cytoplasmic protein sequestered by the actin-bound protein Keap1 (Kelch ECH associating protein) (Itoh et al., 1999; Zipper and Mulcahy, 2002). Keap1 targets Nrf2 to Cullin3-based E3 ligase complexes where Nrf2 is poly-ubiquinated for subsequent proteasomal degradation (Itoh et al., 2003; McMahon et al., 2003). Oxidative stress or exposure to electrophilic agents react with Keap1 and through proposed mechanisms described below, Nrf2 is released and subsequently translocates to the nucleus. Nrf2 dimerizes with small Maf proteins and binds to the ARE transcriptionally driving expression of several detoxifying and antioxidant genes including *GSTs, HO-1, NQO1, TRX, Prx, GCLM, GCLC* (Fig. 1.3A) (Kensler et al., 2007; Sykiotis and Bohmann, 2010).

Nrf2 contains six well-conserved Nrf2-ECH homologous (Neh) domains that support molecular function. The CNC and DNA binding regions are located in Neh1 domain as is the Maf dimerization site. Neh4 and Neh5 are necessary for recruitment of transcription factor and other canonical proteins required for gene expression (Fig. 1.3B) (Zhang, 2006). Molecular studies determined that the Neh2 domain was required for the cytoplasmic localization of Nrf2 (without Neh2 Nrf2 continually translocated to the nucleus). Yeast two-hydridization screening using the Neh2 domain from Nrf2 as bait identified Keap1 as an Nrf2 binding protein. Eighty

percent of the independently isolated clones screened were Keap1 positive suggesting specificity of the Keap1-Nrf2 interaction (Itoh et al., 1999). Keap1 has two canonical domains, the Kelch domain and the bric-a-brac, tramtrack, broad-complex (BTB) domain. The Kelch domain binds actin and thus tethers the Keap1-Nrf2 complex to the cytoskeleton. The BTB domain is important for protein dimerization of Keap1 molecules. There are many cysteine residues in the Keap1 protein that potentially function as sensors of oxidants and electrophiles; humans have 27 and rat and mouse have 25 (Dinkova-Kostova et al., 2002; Kensler et al., 2007). Two mechanistic theories exist that describe the Keap1-Nrf2 interaction. The "hinge and latch" model proposes that two Keap1 molecules bind Nrf2 at high and low affinity sites located in the Neh2 domain (Tong et al., 2006b). The hinge domain, EGTE, supports high affinity and the latch domain, DLG, low affinity. When Keap1 senses oxidative or electrophilic stress, the low affinity domain binding Nrf2 is abolished and proteosomal degradation of Nrf2 is disrupted (Tong et al., 2006a). The "quaternary complex" theory is based on cell culture studies that found a 2:2 and not 2:1 stoichiometry of Keap1 to Nrf2 molecules (Eggler et al., 2009; Lo et al., 2006; Small et al., 2010). This model proposes that two Keap1 molecules dimerize at BTB domains; each binding to an Nrf2 molecule via the high affinity EGTE domain (Sykiotis and Bohmann, 2010).

Microarray analysis of tissues and cell cultures from WT and Nrf2-KO mice as well as primary cortical astrocyte and mixed neuronal cultures transformed with

adenoviral vectors containing mouse cDNA encoding Nrf2, depicted several genes that were basally expressed or induced by Nrf2. Many gene clusters were identified and include antioxidant and reducing agents, detoxification enzymes, signal transduction proteins, anti-inflammatory response genes, metabolic enzymes. All clusters are important for cellular redox homeostasis and protective mechanisms important for cell survival (Table 1.3) (Lee et al., 2003a; Li et al., 2002; Reddy et al., 2007; Shih et al., 2003b).

**Figure 1.3 The Keap1-Nrf2-ARE Pathway.** *(A)* The Nrf2 transcription factor is sequestered in the cytoplasm by Keap1. ROS, RNS and electrophiles react with cysteine residues in Keap1 liberating Nrf2. Nrf2 quickly translocates the nucleus and with small Maf proteins, binds to the ARE located in promoter regions of many antioxidative genes. If Keap1 is not stimulated by ROS, RNS or electrophiles, the E3-like ligase activity associated with Keap1 polyubquinates Nrf2 promoting its proteosomal degradation. *(B)* The Nrf2 gene contains six conserved Nrf2-ECH homologous (Neh) domains that support molecular function. Neh2 is the negative regulatory domain required for inhibiting trans-activity of Nrf2 and is the site where Nrf2 is bound to Keap1.

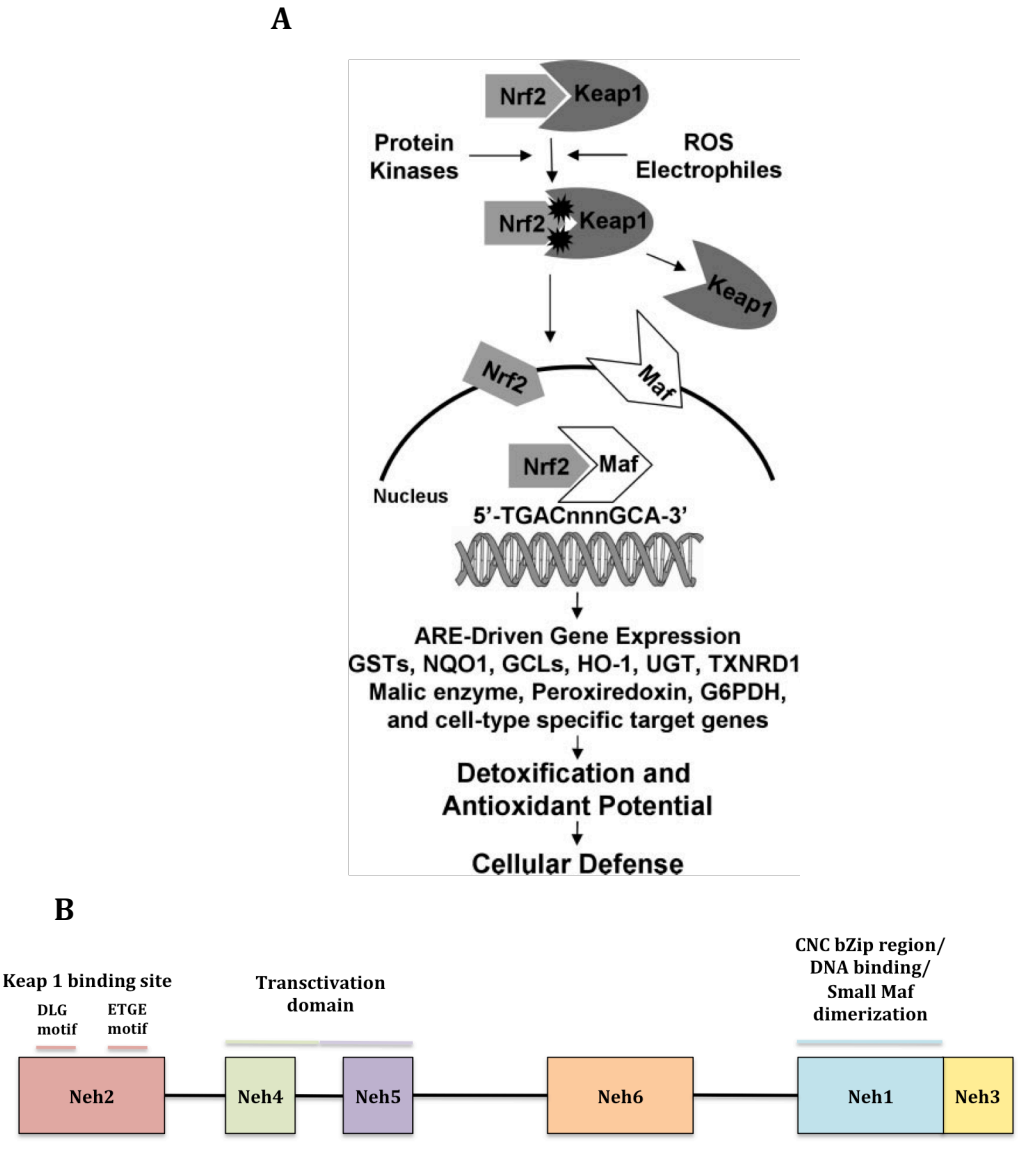



Figure 1.3A was used with permission by Lee *et. al.* (Gate et al., 1999; Lee et al., 2005)

**Table 1.3 Common Nrf2 Upregulated Genes in Astrocytes and Mixed Neuronal/Astrocyte Cortical Cultures Transformed with AD-GFP or AD-Nrf2.**

| GenBank accession number | Gene | Glial-enriched | | | | Neuron/glial mixed | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | R | Fold | CV | BS | R | Fold | CV | BS |
| | Detoxification | | | | | | | | |
| M58495 | NAD(P)H:quinone oxidoreductase | 8 | 4.3 | 0.24 | 2621 | 18 | 5.4 | 0.56 | 141 |
| K00136 | Glutathione *S*-transferase A1 or 2 | 6 | 17.6 | 0.41 | 24 | 12 | 8.8 | 0.80 | 100 |
| K01932 | Glutathione *S*-transferase A3 | 8 | 12.3 | 0.77 | 4657 | 18 | 19.8 | 0.56 | 223 |
| X62660 | Glutathione *S*-transferase A4 | 8 | 21.7 | 0.56 | 443 | 18 | 4.1 | 0.42 | 769 |
| X02904 | Glutathione *S*-transferase P2 | 8 | 3.4 | 0.38 | 22952 | 18 | 2.4 | 0.25 | 7230 |
| J02722 | Heme oxygenase 1 | 8 | 2.8 | 0.18 | 24137 | 18 | 21.3 | 1.2 | 358 |
| M11670 | Catalase | 8 | 4.1 | 0.34 | 1381 | 16 | 1.7 | 0.22 | 593 |
| M11794 | Metallothionein 1 or 2 | 4 | 3.7 | 0.56 | 15750 | 16 | 2.83 | 0.35 | 1274 |
| AI010083 | Peroxiredoxin 1 | 8 | 2.9 | 0.06 | 32993 | 18 | 2.5 | 0.25 | 6609 |
| AF014009 | Peroxiredoxin 6 (1-cys peroxiredoxin) | 8 | 3.3 | 0.12 | 6153 | 14 | 1.8 | 0.20 | 1772 |
| M21060 | Cu/Zn superoxide dismutase | 8 | 1.5 | 0.09 | 28627 | 16 | 1.9 | 0.27 | 14495 |
| AF106563 | *p*-glycoprotein/multi-drug resistance protein (MDR2) | 6 | 1.9 | 0.23 | 1459 | 10 | 5.9 | 0.71 | 2671 |
| X90642 | Multi-drug resistance protein (MRPI) | 8 | 2.3 | 0.33 | 3432 | 14 | 1.7 | 0.13 | 1317 |
| | Signal transduction | | | | | | | | |
| U09583 | Src-related tyrosine kinase | 8 | 22.6 | 0.90 | 89 | 18 | 25.6 | 0.16 | 56 |
| AI231354 | Stress-activated protein kinase, $\alpha$ | 8 | 3.1 | 0.10 | 1097 | 14 | 1.65 | 0.26 | 880 |
| D78610 | Protein tyrosine phosphatase receptor E | 7 | 6.7 | 0.27 | 83 | 14 | 0.0 | 0.00 | 858 |
| M15427 | c-*raf* | 8 | 3.6 | 0.25 | 3558 | 18 | 1.6 | 0.22 | 7427 |
| U31668 | E2F-5 transcription factor | 8 | 2.8 | 0.06 | 2242 | 18 | 1.7 | 0.16 | 933 |
| U04835 | cAMP-responsive element modulator | 8 | 2.0 | 0.20 | 2242 | 10 | 2.7 | 1.1 | 756 |
| | Antioxidant/reducing potential/metabolism | | | | | | | | |
| AI233261 | Glutamate-cysteine ligase, modifier subunit ($\gamma$GCS) | 8 | 13.0 | 0.13 | 945 | 18 | 5.1 | 0.21 | 208 |
| U73174 | Glutathione reductase 1 | 8 | 3.3 | 0.15 | 1667 | 14 | 2.0 | 0.32 | 1324 |
| U63923 | Thioredoxin reductase 1 | 8 | 2.6 | 0.16 | 10344 | 16 | 3.4 | 0.56 | 3426 |
| M26594 | Malic enzyme 1 | 8 | 8.3 | 0.23 | 1264 | 18 | 10.1 | 0.35 | 227 |
| AI169802 | Ferritin H subunit H | 7 | 1.8 | 0.23 | 50557 | 10 | 1.3 | 0.13 | 18452 |
| J02791 | Acyl-coenzyme A dehydrogenase, medium chain | 8 | 3.2 | 0.22 | 925 | 12 | 3.2 | 0.87 | 42 |
| M60322 | Aldose reductase 1 | 8 | 5.1 | 0.15 | 24812 | 18 | 2.4 | 0.18 | 9958 |
| AA799452 | Transaldolase | 8 | 3.6 | 0.25 | 21356 | 18 | 1.8 | 0.23 | 19236 |
| M96633 | Mitochondrial intermediate peptidase | 8 | 2.8 | 0.30 | 699 | 9 | 1.5 | 0.24 | 452 |
| AA945054 | Cytochrome b5 | 8 | 2.6 | 0.40 | 1902 | 14 | 1.6 | 0.27 | 2179 |
| S53527 | S-100 calcium binding protein $\beta$ | 8 | 78.7 | 1.15 | 1167 | 10 | 2.3 | 0.68 | 67 |
| U26714 | Solute carrier family 29 (iron-regulated transporter) | 6 | 21.3 | 0.37 | 402 | 18 | 5.9 | 0.27 | 102 |
| | Inflammation | | | | | | | | |
| AI176170 | FK506 binding protein | 6 | 1.5 | 0.20 | 19602 | 18 | 1.3 | 0.06 | 18354 |
| AA818025 | CD59 antigen | 8 | 25.9 | 0.04 | 1128 | 18 | 1.4 | 0.08 | 10966 |
| U03388 | Cyclooxygenase 1 | 8 | 3.9 | 0.23 | 2083 | 18 | 5.6 | 0.50 | 312 |
| | Housekeeping | | | | | | | | |
| V01217 | $\beta$ actin | | | | 186225 | | | | 164621 |
| X02231 | Glyceraldehyde-3-phosphate-dehydrogenase | | | | 405 | | | | 348 |
| U07181 | Lactate dehydrogenase B | | | | 13108 | | | | 11253 |

Genes with fold change>1.3 are listed. R, relative rank; CV, coefficient of variation; BS, basal signal intensity from GFP-infected cultures. Data are shown from n=2 glial-enriched cultures and n=3 mixed cultures.
(Shih, Johnson et. al. 2003)

## 1.4 Nrf2 Mediated Protection from Neuroinflammation

### 1.4.1 Nrf-2 Deficient Mice are more Susceptible to Oxidative and Electrophilic Stress

In initial experiments using Nrf2 deficient mice (Nrf2-KO), Chan and Kan and co-workers observed normal erythropoiesis and growth and development. However upon challenge, they observed increased susceptibility to butylated hydroxytoluene-induced lung and acetaminophen-induced liver injuries when compared to WT mice (Chan et al., 2001; Chan and Kan, 1999; Chan et al., 1996; Enomoto et al., 2001). Moreover, others showed the lack of induction of NQO1 and GSTs in liver from these mice (Itoh et al., 1997). Many other studies using Nrf2-KO mice, have demonstrated the significance of Nrf2 in prevention of cellular oxidative and electrophilic stress and prevention of diseases (Kensler et al., 2007). For instance, emphysema, acute lung injury (ALI), pleurisy, asthma, sepsis, uveitis, skin tumorigenesis and ultraviolet B irradiation were more severe, and in some cases had increased lethality, in the Nrf2-KO mice compared with WT. In many cases the Nrf2-KO mice had an enhanced inflammatory response as indicated by increased and/or persistent presence of neutrophils, macrophages or lymphocytes, perhaps exacerbating the already injured tissue (Braun et al., 2002; Itoh et al., 2004; Kong et al., 2011; Mochizuki et al., 2005; Nagai et al., 2009; Reddy et al., 2009a; Reddy et al., 2011; Reddy et al., 2009b; Schafer et al., 2010). Our laboratory has evaluated models of PD (MPTP, 6-hydroxydopamine), HD (malonate, 3-nitroproprionic acid), and epilepsy (kainic

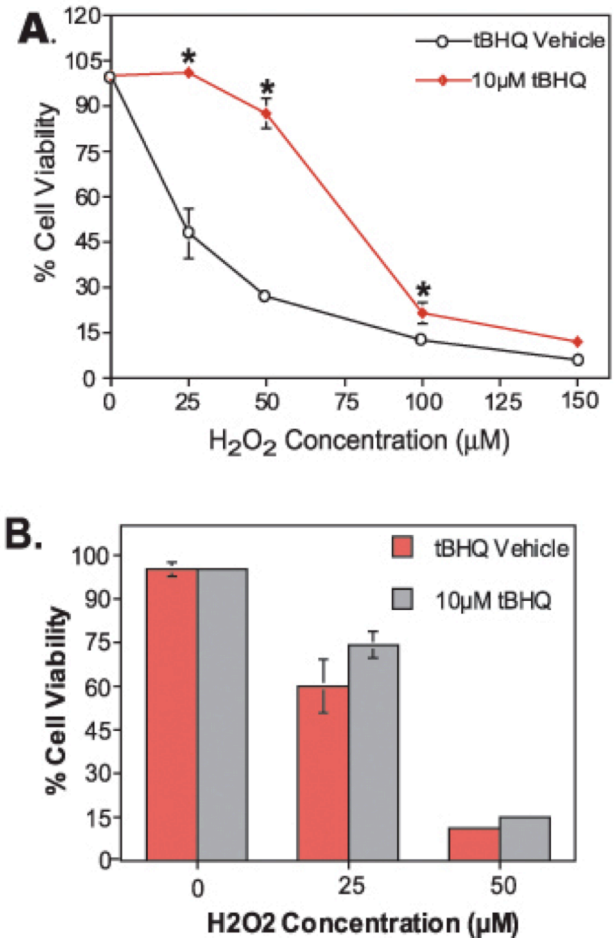
acid) in Nrf2-KO mice (Calkins et al., 2005a; Chen et al., 2009a; Jakel et al., 2007a; Kraft et al., 2004b; Kraft et al., 2006; Vargas et al., 2008b). In all cases, the Nrf2-KO mice had increase neurotoxicity compared to control mice. Similar to the above observation regarding the inflammatory response, there was also an enhanced/persistent increase in neuroinflammation.

### 1.4.2 Nrf2 Protection in Neurodegenerative Diseases

Activation of the Nrf2-ARE pathway in primary brain cell cultures has been shown to block neurotoxicity resulting from hydrogen peroxide, glutathione depletion, lipid peroxidation, intracellular calcium overload, excitotoxins, and disruption of the mitochondrial electron transport chain (Kraft et al., 2004b; Lee et al., 2003c; Shih et al., 2003b). Neuronal protection from hydrogen peroxide toxicity was Nrf2-dependent since tBHQ treated neuronal cultures from Nrf2-KO mice were susceptible to hydrogen peroxide toxicity (Fig. 1.4 A-B). In these models, Nrf2-ARE activation also leads to increased cellular energetics and redox potential, inhibitory neurotransmitter signaling, and metabolic processes (Kraft et al., 2004b; Lee et al., 2003c; Li and Jaiswal, 1992; Nguyen et al., 2004; Prestera et al., 1995; Shih et al., 2003b; Thimmulappa et al., 2002). It should be noted that Nrf2-driven genes are preferentially activated in astrocytes (Johnson et al., 2002a; Kraft et al., 2004a; Lee et al., 2003b) and that this Nrf2 activation in glial cells not only precludes oxidative damage in astrocytes but also confers protection to neighboring neurons (Calkins et al., 2005b; Jakel et al., 2007b; Kraft et al., 2004a; Shih et al., 2003a). Based on these

observations, transgenic mice engineered to overexpress Nrf2 in astrocytes were generated by placing Nrf2 under control of the astrocyte-selective GFAP (glial fibrillary acidic protein) promoter (Vargas et al., 2008a). The GFAP-Nrf2 mice have significantly increased resistance to acute *in vivo* models of PD (Chen et al., 2009b), HD (Calkins et al., 2010) and AD (Joshi et al., unpublished data). In addition, crossing the GFAP-Nrf2 mice with multiple mouse models of ALS, generated by overexpressing human mutant of superoxide dismutase 1 ($hSOD1^{G93A}$ and $hSOD1^{H46R/H48Q}$), resulted in a significant extension of lifespan in the ALS models (Vargas et al., 2008a). Finally, unpublished data from the laboratory crossing the GFAP-Nrf2 mice with the mutant alpha-synuclein (A53T) mouse, a genetic model of PD, resulted in a dramatic increase in the lifespan of these mice (45% increase in lifespan; Gan et al., unpublished data). These studies were focused on Nrf2 in the astrocyte. No studies have been done looking at the absence or activation of Nrf2 in microglia in *in vivo* inflammatory models.

**Figure 1.4. tBHQ-mediated attenuation of $H_2O_2$-induced cell death is Nrf2-dependent.** *(A)* Nrf2-WT or (*B)* Nrf2-KO primary cortical neurons (5 DIV) were pretreated with vehicle or 10 μM tBHQ for 48h. Cultures were then exposed to $H_2O_2$ for 24h and cell viability was assessed using the soluble MTS assay ($n$=4; mean SE; *$p$<0.05, compared with vehicle).




(Kraft et al., 2004b)

### 1.4.3 Nrf2 and Neuroinflammation

Microglia, the resident macrophages of the central nervous system (CNS), generate ROS such as $O_2^{\bullet -}$ and RNS via NADPH oxidase and nitric oxide synthase, respectively. ROS/RNS species are extremely injurious to cellular macromolecules such as protein, lipid organelles and DNA, and thus contribute to damage observed in cells and tissue (Block et al., 2007; Li et al., 2005). Microglia have also been localized to sites of injury and pathology in human postmortem tissue from PD, HD and ALS and multiple sclerosis (MS) lesions. Increased oxidized end products such as 8-hydroxy-2-deoxyguanosine, 4-hydroxy-2-nonenol, and protein carbonyls were also documented in these human tissues (Amor et al., 2010; Innamorato et al., 2009). This implies that activated microglia contribute to the pathogenesis of these chronic neurodegenerative diseases and potentially others, including ischemia and HIV-associated dementia. Whilst they are an important first line of defense against microorganism invasion and injury, continuous activation of microglia can have deleterious affects on other cells in the CNS. In fact, continuous activation generates intracellular ROS that act as second messengers self-perpetuating the pro-inflammatory signaling mechanisms in the microglia (Mander et al., 2006; Pawate et al., 2004; Qin et al., 2004). Without intervention, microglia activation can deplete intracellular antioxidants and the innate inflammatory response may continue (Aronis et al., 2005; Block et al., 2007). Thus activation of the Nrf2-ARE signaling pathway in microglia may be an important therapeutic target for protection in

neurodegenerative disorders.

The immunomodulatory potential of Nrf2 has been described in experiments using cultured human blood monocytes and neutrophils as well as in mouse peritoneal macrophages, mouse and rat microglia. Treatments with various Nrf2 activating compounds, including adenosine, tert-butyl hydroquinone (tBHQ), diethyl maleate, CDDO-Im and sulforaphane (SFN) and astrocyte-conditioned media all resulted in Nrf2-activation and attenuation of LPS-induced inflammation. Moreover, levels of ROS and cytokines measured after the LPS treatments were decreased in the Nrf2-activated cultures and as expected, when Nrf2 was disrupted, attenuated inflammation was not observed (Ishii et al., 2000; Lin et al., 2008; Thimmulappa et al., 2007). It is postulated that crosstalk occurs between Nrf2 and the pro-inflammatory transcription factor, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). Kong and co-workers observed attenuation of the NF-κB signaling pathway after treatments with Nrf2-activators, phenethyl isothiocynate, curcumin and SFN (Li et al., 2008). How Nrf2 activation inhibits NF-κB signaling is nebulous and remains to be determined. On the contrary, it has also been demonstrated that NF-κB can disrupt Nrf2 signaling by competing for the transcription co-factor cAMP response element-binding (CREB) protein (CBP) and by recruiting histone deacetylase 3 (HDAC3) resulting in local hypoacetylation and inhibition of Nrf2 transcriptional mechanisms (Liu et al., 2008).

Unfortunately, many of the microglia studies described above, used the murine immortalized microglia cell line BV-2. These cells were generated by infection with v-raf/v-myc carrying retrovirus and thus do not fully recapitulate a non-transformed primary microglia with regards to redox-cellular homeostasis and associated signaling mechanisms. For instance, when Rohl *et al.* (2010), treated primary astrocyte cultures with conditioned media from LPS treated primary microglia, they observed increased expression of anti-inflammatory and oxidative stress response genes in the astrocyte (Rohl et al., 2010). However, when these same investigators used the C6-astroglioma cell line with the same conditioned media, the anti-inflammatory and oxidative response genes did not increase. This suggests the importance of using primary derived cell cultures based on the differences in stress responses in primary cultures versus cells lines (Rohl et al., 2010; Rohl et al., 2008). Interestingly, the latter studies also suggest interaction between the microglia and astrocytes may attenuate oxidative stress in the astrocyte via the Nrf2-ARE signaling pathway.

Experiments by Cuadrados group have demonstrated that Nrf2 can regulate neuroinflammation in mice injected with LPS and MPTP (a mouse model of PD) (Innamorato et al., 2008; Rojo et al., 2010). In the LPS experiments, hippocampi from Nrf2-KO mice injected with LPS had greater microgliosis compared to WT hippocampi. In addition, when WT mice were injected with SFN and LPS, they observed a 2-3-fold induction of HO-1 and an attenuation of the microglia response

measured by presence and markers of inflammation. The latter experiment suggested that SFN increased Nrf2 activity and subsequently reduced neuroinflammation (Innamorato et al., 2008). A major issue with these studies was that they did not use the Nrf2-KO mice. It would be far more convincing if SFN and LPS was administered to Nrf2-KO mice and the reduced neuroinflammatory response was not observed.

In Nrf2-KO mice injected with MPTP, the striatum-substantia nigra axis exhibited more severe dopaminergic dysfunction and microglial activation than in the WT mice (Chen et al., 2009a; Rojo et al., 2010). Although, the extent of peripheral infiltration of macrophages was not different between these cohorts, it did appear that the resident microglia in the Nrf2-KO mice were more characteristic of a classical activated macrophage phenotype, while the WT mice that of an alternative activated macrophage (Rojo et al., 2010).

## 1.5 Inflammatory Mediated Oxidative Stress in Multiple Sclerosis

### 1.5.1 Multiple Sclerosis

*The Disease*

MS is a chronic autoimmune demyelinating disease of the CNS characterized by multifocal lesioning in the white matter. MS negatively affects many biological systems presenting with common symptoms that include but are not limited to monocular visual impairment with pain (optic neuritis), tingling, burning or

numbness of skin sensation (parethesias), weakness, impaired coordination and urinary bladder dysfunction (Calabresi, 2004). Initiation of this disease is thought to originate in the peripheral lymph system where auto-reactive CD4+ T helper 1 (Th1), Th2, Th17 and CD8+ cytotoxic T lymphocyte (CTL) cells are activated, adhere and migrate across the blood-brain barrier and attack the myelin sheathing wrapped around the neuronal axons (Brinkmann et al., 2010) (Nylander and Hafler, 2012). Exposure to myelin-like proteins may be the initiating inducers for the immune system attack against myelin. Although not precisely known, the auto antigen that binds to the T-cell receptor is now presumed to be more related in side-chain spatial structure than to specific amino acids sequences found in myelin-associated proteins including proteolipid protein (PLP), myelin oligodendrocyte glycoprotein (MOG) and/or myelin basic protein (MBP) (Dhib-Jalbut, 2007). Studies using protein databases identified seven viral peptides and one bacterial peptide that were capable of activating reactive T-cell clones. These peptides included sequences found in Epstein-Barr virus (EBV), *Herpes simplex* (HSV), adenovirus 12, human papilloma virus (HPV), influenza A and the bacterial peptide *Pseudomonas aeruginosa.* Early exposure to epitopes found in these pathogens similar to self-antigens (a phenomenon known as molecular mimicry) may then trigger the onset of MS (Dhib-Jalbut, 2007). Autoreactive cells must then be reactivated by resident microglia/macrophages in the CNS for the process of adaptive immunity to reinitiate. In addition to an adaptive immune response, massive micro- and

astrogliosis is prevalent in reactive MS lesions, as is loss of oligodendrocytes and neurons (Brinkmann et al., 2010; Dhib-Jalbut, 2007)

*Etiology*

It is estimated that more than 2.1 million persons worldwide have MS (NMSS, 2012) . Women have a greater risk factor for this disease, as the prevalence of MS in women to men is 2:1 (Calabresi, 2004) . Interestingly, individuals farthest from the equator appear to be at greater risk of developing MS suggesting an environmental component in its etiology. As mentioned previously, early infection with the viruses HSV, EBV, adenovirus 12, HPV and influenza A, may also be an environmental risk factor (Handel et al., 2010). There are classifications of MS with relapsing-remitting MS (RRMS) being the predominant. Eight-five percent of MS patients present with RRMS characterized by an initial episode of symptoms associated with demyelination followed by a recovery period. Heterogeneous relapses occur and in 42-57% of cases, there is incomplete recovery resulting in impairment and permanent disability. In some cases (3-40%), RRMS progresses to secondary progressive MS (SPMS) whereby patients steadily regress and succumb to the disease (Compston and Coles, 2002).

*Multiple Sclerosis Therapies*

Current therapies for MS are aimed at inhibiting or modulating the adaptive immune system (in addition to drugs involved in managing symptoms). These

include the beta interferons (IFNß), glatiramer acetate (GA), mitoxantrone and natalizumba (Graber et al., 2010). The precise mechanisms of IFNß and GA are unknown. However, INFß appears to inhibit Th1-cell activation and proliferation as well as strengthen the integrity of the BBB. GA is a compound similar in structure to amino acids found in myelin basic protein and skews the immune response to myelin antigen toward anti-inflammatory Th2 cell development. The broad-spectrum mitoxantrone is used sparingly as it is cardiotoxic, however this drug induces apoptosis in active cells of the immune system. The latest of these therapies is the monoclonal antibody, natalizumba. It is a specific antibody against very late antigen-4 (VLA-4) an adhesion protein found on cell membrane surfaces of activated T-cells. Natalizumba thus targets activated T-cells by inducing apoptosis and preventing these cells from migrating across the BBB (Graber et al., 2010).

*Oxidative Stress in Multiple Sclerosis*

Oxidative stress is also documented in the pathogenesis of MS. Here, activation of macrophages, microglia, and astrocytes produce ROS such as super oxide radicals and RNS, which are injurious to cells and also contribute to the cellular and tissue damage observed in the MS lesion (Liu et al., 2001; van Horssen et al., 2008; Zeis et al., 2008). For instance, increased iNOS, nitrotyrosine in protein and oxidative stress were found in MS lesions, as were elevated levels of anti-oxidant enzymes. Moreover and interestingly, in one study, a biopsy taken from white matter that was distant from an inflammatory lesion had elevated expression of nNOS and

nitrotyrosine that was associated with the toxicity to oligodendrocytes (Zeis et al., 2009). In addition, expression levels for HO-1 and other anti-oxidant genes were increased suggesting a more widespread pathological process.

Activation of the Nrf2-ARE signaling pathway in the CNS may thus be a novel therapeutic approach in modulating MS (Fig. 1.5). As described earlier, autoreactive T-cells are capable of activating the innate immune response in the CNS. ROS and RNS have deleterious affects on neurons and oligodendrocytes resulting in toxicity and cell death. The Nrf2-ARE driven gene expression may then increase cellular redox homeostasis and antioxidant capacity in the CNS and directly or indirectly protect neurons and oligodendrocytes from ROS or RNS toxicity. Likewise, preventing over activity of microglia by increasing intracellular antioxidants via Nrf2 activation may also be beneficial (Fig.1.6). The central hypothesis for this thesis is: The absence of Nrf2 exacerbates and the cell specific overexpression of Nrf2 in microglia attenuates experimental autoimmune encephalomyletis (EAE), a mouse model of MS. Consequently, the specific aims of this thesis are to:

**1) DETERMINE IF THE ABSENCE OF NRF2 EXACERBATES EXPERIMENTAL AUTOIMMUNE ENCEPHALOMYLELITIS (EAE) A MOUSE MODEL FOR MS**

What role (if any) does Nrf2 have in modulating inflammation associated with multiple sclerosis? WT and Nrf2-KO mice will be used in these studies.

**2) EVALUATE IF OVEREXPRESSION OF NRF2 IN MICROGLIA PROTECTS AGAINST NEUROTOXICITY AND EAE?**

CD11b-cre (selective cre expression in microglia/macrophages) and floxed Nrf2 transgenic mice with selective overexpression and deletion of Nrf2 in microglia/macrophages, will be generated for these experiments.

**Figure 1.5 Activation of the Nrf2-ARE Signaling Pathway in the CNS and Protection from Oxidative Stress in MS.** In MS, autoreactive T-cells such as CD4+ Th1, CD8+ CTLs and B-cells are activated in the peripheral lymph system, migrate to and infiltrate the BBB. Microglia/macrophages of the innate immune response are subsequently activated and the ROS and RNS generated are cytotoxic to neurons and oligodendrocytes resulting in tissue damage. Cellular debris from damaged tissue can activate microglia resulting in a feed forward loop of reactive microgliosis. Increased expression of antioxidants and antioxidant enzymes via activation of the Nrf2-ARE signaling pathway can restore cellular redox homeostasis and detoxify ROS and RNS thus protecting cells in the CNS in an autocrine or paracrine manner.

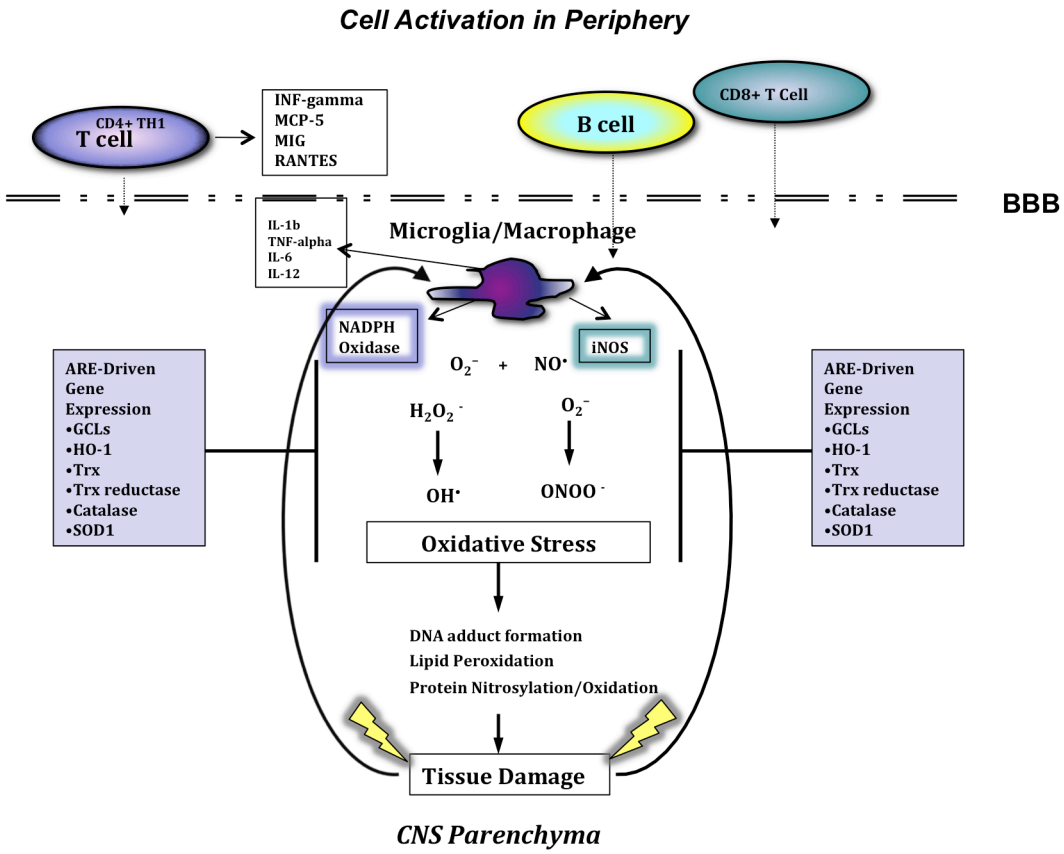
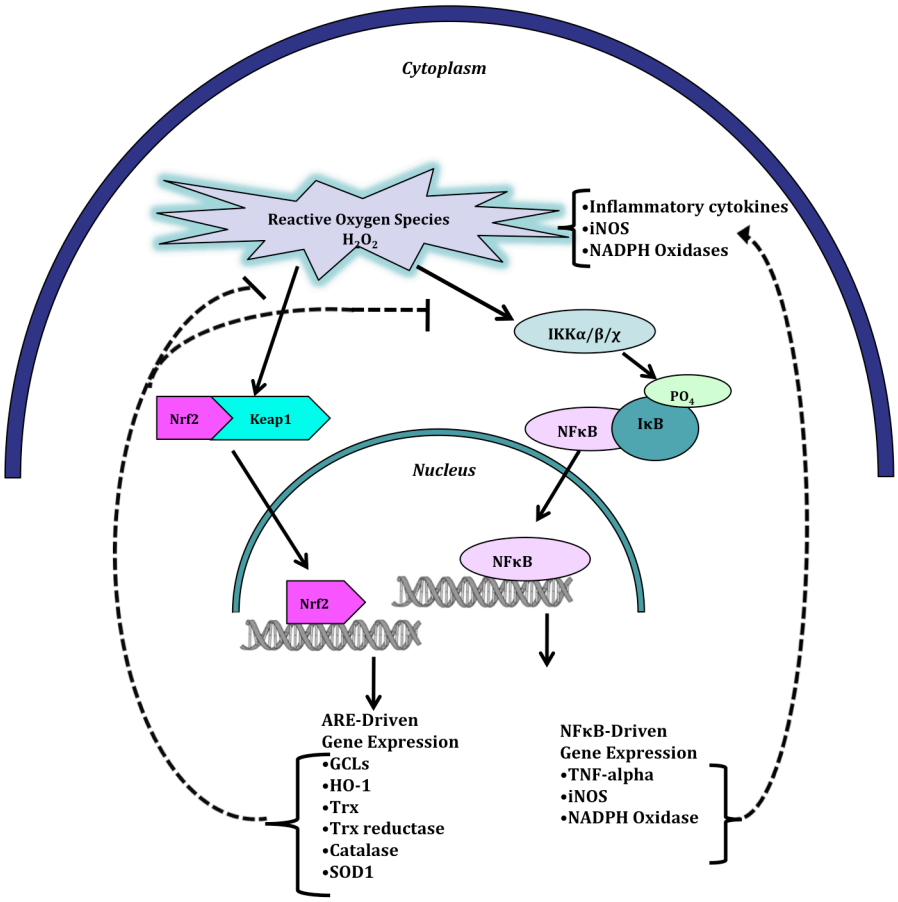


**Figure 1.6 Activation of Nrf2 Restores Cellular Redox Homeostasis and Reverts Reactive Microglia/Macrophage Back to Resting Stages.** Intracellular ROS and RNS act as second messengers and can activate kinase cascades and transcription factors such as NFκB that drive inflammatory gene expression. However, persistent stress can deplete the cell's antioxidant defense system and amplification of the inflammatory response can have deleterious effects on neurons and oligodendrocytes. Activation of the Nrf2-ARE pathway amplifies antioxidants and antioxidant enzymes restoring cellular redox homeostasis and reverting the microglia/macrophage back to resting and basal functioning stages. Note-NFκB is sequestered in the cytoplasm by an inhibitor protein, IκB. Upon phosphorylation by IκB kinases (Iκκα/β/χ), IκB releases NFκB through proteolytic mechanisms. NFκB then translocates to the nucleus driving the expression of inflammatory genes.



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# CHAPTER 2

## The Absence of the Pro-Antioxidant Transcription Factor Nrf2 Exacerbates Experimental Autoimmune Encephalomyelitis[1]

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[1] Johnson DA, Amirahmadi S, Ward C, Fabry Z, Johnson JA (2010). The Absence of the Pro-Antioxidant Transcription Factor Nrf2 Exacerbates Experimental Autoimmune Encephalomyelitis. *Toxicological Sciences*, 114 (2), 237-46.

## 2.1 Abstract

Multiple sclerosis (MS) is an autoimmune disease characterized by peripheral activation of CD4+ T cells that migrate into the central nervous system (CNS) and mount an autoimmune neuroinflammatory attack on myelin and oligodendrocytes. Secondary to these events, however equally destructive, is the generation of inflammatory-mediated reactive oxygen and nitrogen species generated by persistently activated microglia and astrocytes. Nuclear factor-erythroid 2–related factor 2 (Nrf2) is a basic leucine zipper transcription factor that regulates genetic expression of many protective antioxidant and detoxication enzymes. Here we describe the Nrf2 modulation of innate and adaptive immune responses in an acute autoimmune model of MS, experimental autoimmune encephalomyelitis (EAE). Wild-type (WT) mice and Nrf2 knockout mice were immunized with myelin oligodendrocyte glycoprotein (MOG 35-55) and monitored daily for clinical scores of disease. Disruption of Nrf2 resulted in a more severe clinical course, a more rapid onset, and a greater percentage of mice with the disease. Furthermore, increased immune cell infiltration and glial cell activation in spine was observed. In conjunction, we observed increased inflammatory enzyme (iNOS, phox-47, gp91-phox, and phox-67), cytokine (IFN- gamma, IL1-b, TNF-alpha, and IL-12), and chemokine (BLC and MIG) gene expression levels in the Nrf2-deficient mice compared to the WT mice, supporting the notion that Nrf2 can modulate an autoimmune neuroinflammatory response. Our results show that the absence of Nrf2 exacerbates the development of EAE and thus suggests that

activation of Nrf2 may attenuate pathogenesis of autoimmune diseases such as MS, as well as other neurodegenerative diseases that present with neuroinflammation.

## 2.2 Introduction

The National Multiple Sclerosis Society estimates that over 2 million persons worldwide are affected with the autoimmune demyelinating disease known as multiple sclerosis (MS). MS is regarded as a heterogenic disease that presents with variable symptoms and severity ranging from loss of vision to debilitating ataxia (Noseworthy et al., 2000). In most cases, MS is considered to be a chronic inflammatory disease of the central nervous system (CNS) with intervals of remission followed by relapse. However, acute progressive cases are documented. Onset of this disease initiates outside of the CNS through activation of CD4+ T cells by myelin-like antigenic peptides. These cells then migrate across the blood brain barrier (BBB) initiating focal inflammation. In addition, activation of infiltrating macrophages, CD8+ T cells, B cells, as well as resident microglia and astrocytes have all been implicated in the pathology of MS. Sclerotic lesions associated with this disease result from an attack on the myelin sheathing surrounding the neuronal axons and on the oligodendrocytes, resulting in axonal retraction and subsequent astrogliosis. Current therapies for this disease primarily focus on prevention of the penetration of immune cells across the BBB (Dhib-Jalbut, 2007; Noseworthy et al., 2000). However, oxidative stress is also documented in the pathogenesis of MS. Here, activation of macrophages, microglia, and astrocytes produce reactive oxygen

species (ROS) such as super oxide radicals and reactive nitrogen species (RNS), which are injurious to cells and also contribute to the cellular and tissue damage observed in the MS lesion (Liu et al., 2001; van Horssen et al., 2008; Zeis et al., 2008).

Many studies clearly demonstrate that the pro-antioxidant transcription factor, nuclear factor-erythroid 2–related factor 2 (Nrf2), promotes cell survival or tumor prevention via disruption of the Keap1-Nrf2 cytosolic complex, an event mediated by electrophilic or free radical molecules (Chan et al., 2001; Durchdewald et al., 2007; Kang et al., 2004; Lee et al., 2005; Li et al., 2005; Liu et al., 2008). The actin-bound Keap1 sequesters Nrf2 in the cytosol and serves as an E3 ligase, shuttling the continuously ubiquinated Nrf2 to the proteasome for degradation (Zhang and Hannink, 2003). The turnover of Nrf2 is thus rapid, and its short half-life in cell lines and macrophages is estimated to be less than 20 min. Hence, basal levels of Nrf2-driven genes can be quite low. Upon cytosolic activation of Nrf2, the transcription factor releases from Keap1, translocates to the cell nucleus, and binds to the antioxidant response element (ARE; Itoh et al., 2003, 1997). The ARE is a cis-acting DNA-responsive element located in the promoter region of a battery of genes whose functions are to promote cell survival. Clusters transcribed include genes that (1) metabolize and conjugate xenobiotics or electrophiles, such as the phase II enzymes NAD(P)H-quinone oxidoreductase (NQO1), glutathione S-transferases (GSTs), and UDP-glycosyltransferase 1A6 (UGT), and (2) boost a cells antioxidant potential that include enzymes and proteins, such as glutamate-cysteine ligase (glutathione [GSH]

production), heme oxygenase-1, thioredoxin reductase-1 (TXNRD1), thioredoxin, and ferritin (Kraft et al., 2004; Lee et al., 2003; Li et al., 2002; Shih et al., 2003). Nrf2 is thus considered an important mediator of cellular oxidative stress particularly in diseases that present with neuroinflammation such as Parkinson's disease, Alzheimer's disease, and MS.

Modulation of the innate immune response by Nrf2 has been observed in mice with lung inflammation or acute lung injury (ALI). Previous studies showed that if lungs of mice were instilled with LPS or carrageenan, there was an increased presence and persistence of inflammatory cells in Nrf2-KO mice when compared to WT controls. This corresponded to increased activation of nuclear factor KB in the Nrf2-KO mice. (Itoh et al., 2004; Thimmulappa et al., 2006a). Expression of genes associated with inflammation, such as tumor necrosis factor-alpha (TNF-alpha), IL-1beta, and IL-6, were also dramatically increased. Sepsis-induced mortality in these same mice was greater than in a wild-type (WT) cohort. This response correlated to the lack of Nrf2, since pretreatment with an activator of Nrf2, the triterpenoid analogue CDDO-imidazolide (CDDO-Im), failed to attenuate the innate inflammatory response and mortality in the Nrf2-knockout (KO) mice. However, administration of a cellular antioxidant, N-acetyl-cysteine, was able to partially protect (Thimmulappa et al., 2006b, 2007).

Previous studies in our laboratory have demonstrated the protective effects of Nrf2

in mouse cortical cultures treated with H2O2 as well as in mouse models of neurodegeneration, including Parkinson's disease, Huntington's disease, and amyotrophic lateral sclerosis. Moreover, an increased occurrence of activated microglia and astrocytes was evident in the brains or spines from Nrf2-KO mice with these diseases compared with WT controls (Calkins et al., 2005; Chen et al., 2009; Jakel et al., 2007; Kraft et al., 2004, 2006; Vargas et al., 2008). These observations further underline the significance of Nrf2 in the modulation of neuroinflammation as well as the protection from oxidative stress.

The present study attempts to understand the role of Nrf2 in an autoimmune inflammatory model of MS. Nrf2-KO and WT mice were immunized with MOG 35-55 to induce experimental autoimmune encephalomyelitis (EAE). Clinical scores of disease and disease onset were monitored, and cellular and molecular mechanisms of the disease in Nrf2-KO and WT mice were analyzed.

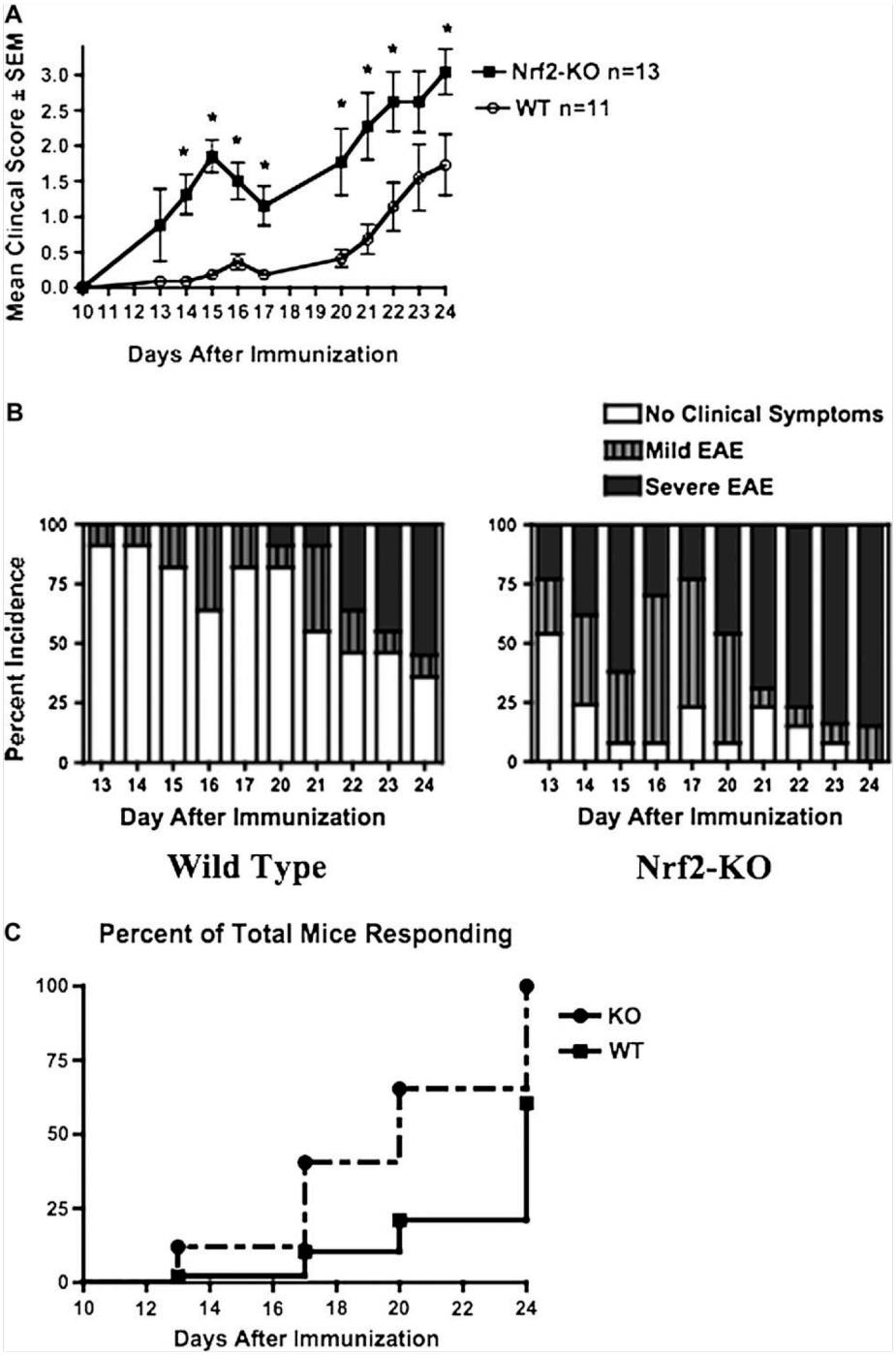
## 2.3 Results

### 2.3.1 Nrf2-KO mice with EAE have higher clinical scores of disease and incidence

To understand the significance of Nrf2 in modulating inflammation and demyelination, Nrf2-KO and WT mice were immunized with MOG 35-55 emulsified in CFA or CFA alone. Clinical scores of the disease were assessed starting at day 13 after the initial immunizations, and the onset and severity of EAE were monitored.

Mice lacking Nrf2 showed a more severe course of disease than the WT mice (Fig. 2.1A). When mice were categorized as having a mild (clinical score 0 and < 2) or severe (clinical score > 2) course of disease, the percentage of Nrf2-KO mouse had greater incidence in both mild and severe EAE at all time points after disease onset (Fig. 2.1B). Further, the Nrf2-KO mice had an earlier onset of EAE and had a greater percentage of the mice responding to the immunization of MOG 35-55 (100% of Nrf2-KO mice responding vs. 64% of WT) (Fig. 2.1C). It is noted that male and female mice were randomly and evenly distributed among the experimental treatment groups, and no significant gender differences in clinical scores, percent incidence, or severity were observed.

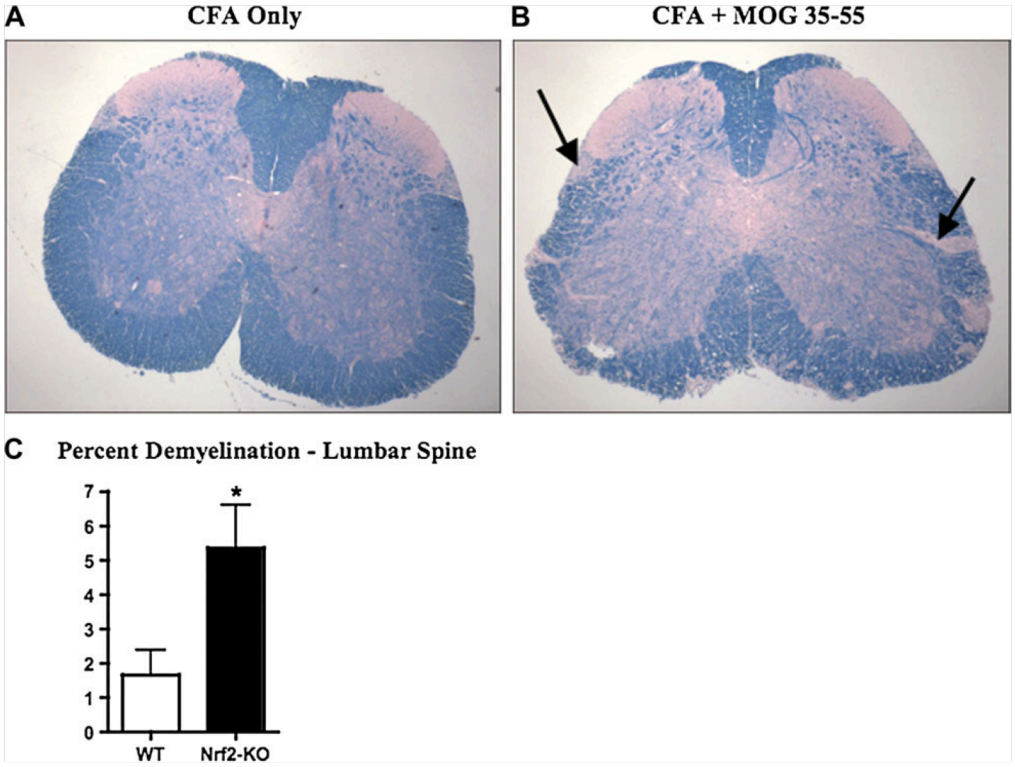
**Figure 2.1 Mice lacking the transcription factor Nrf2 show a more severe response to EAE.**
(A) Clinical scores of disease as described in the Materials and Methods were monitored daily after injection of 100 μg of MOG-35-55 emulsified in CFA. (B) Percent incidence of the severity of disease was documented as follows—0, no clinical score; 0.5–1.5, mild EAE; and 2.0 or above, severe EAE. (C) Onset of EAE was tracked, and the percent of total numbers of mice responding to immunization with MOG 35-55 is represented for WT and Nrf2-KO mice. Onset curves are significantly different, $p < 0.003$ (v2 1/4 8.853). Data in (A) are presented as mean ± SEM. *Significantly different than WT control($p<0.05$).



### 2.3.2 Histopathological Analysis of Demyelination and Cell Infiltration in Nrf-KO and WT mice with EAE

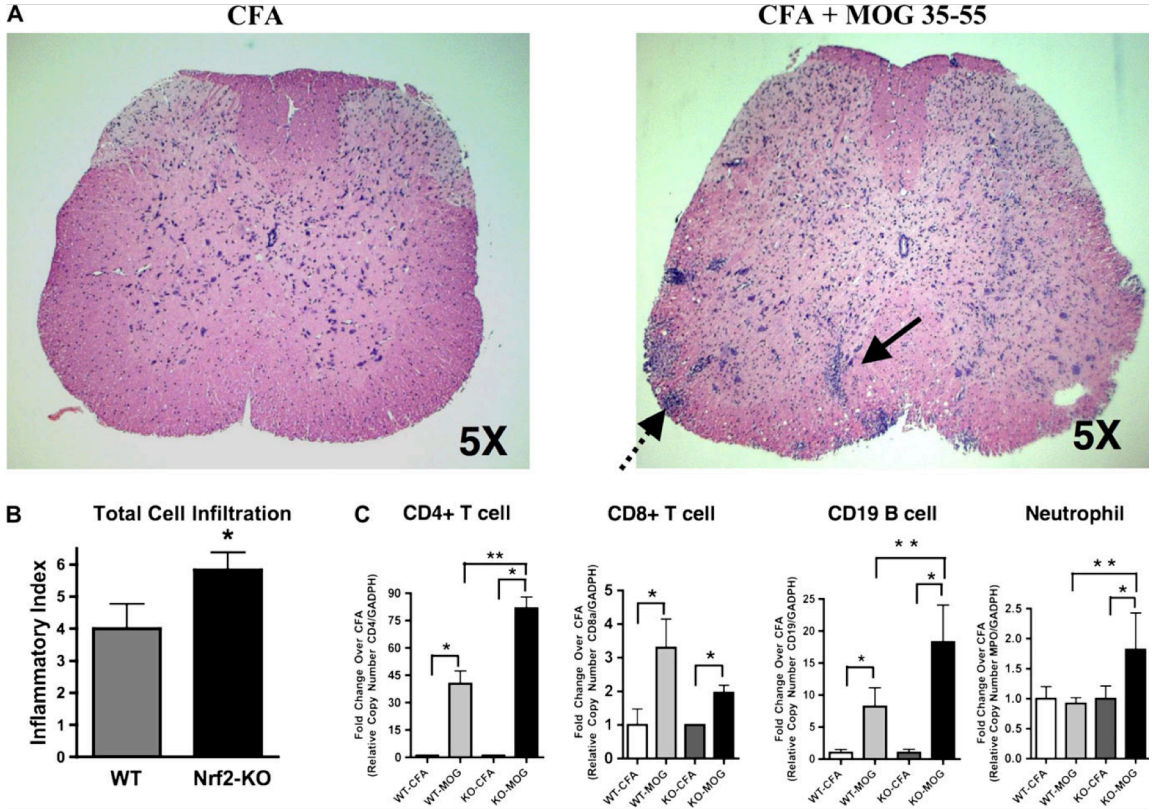
A hallmark of tissue damage in EAE is loss of myelin in white matter regions of spinal cord. Lumbar spine regions from day 14 after MOG 35-55 immunizations were assessed for demyelination by staining with luxol fast blue (LFB). Lumbar spinal cord sections from both Nrf2-KO and WT mice with EAE showed loss of LFB stain in multifocal areas of the lumbar spine. Demyelination, shown by the loss of LFB stain in multifocal areas of the lumbar spine, was then measured and analyzed using ImageJ (Fig. 2.2A and 2.2B). Here, Nrf2-KO mice showed a 5.4% loss of myelin, which was significantly greater than the 1.7% loss seen in WT mice (Fig. 2.2B). Quantitative measurement of gene expression levels for MOG was determined by qPCR from whole spine total RNA. Although not significant, a trend showing a greater reduction in MOG expression in Nrf2-KO (35%) compared with WT mice ( 26%) versus with their respective CFA-treated control mice, was observed (data not shown).

**Figure 2.2 Nrf2-KO mice have greater demyelination than WT mice with EAE.** (A and B) LFB staining of representative paraffin-embedded sections from lumbar spine of nondiseased (left panel) and diseased (MOG-induced EAE, right panel) mice. Bolded black arrows point to areas exemplifying demyelination. (C) Quantification of demyelination of sections described in (A and B) using ImageJ processing software and represented as mean ± SEM (WT, n = 4; Nrf2-KO, n = 5). *Significantly different than WT control ($p<0.05$).



Based on the previous data showing a greater amount of demyelination in the Nrf2-KO mouse with EAE, we wanted to determine the cellular composition of infiltrating immune cells and to what extent these cells were involved in the pathological process. At day 14–16 after immunization, a time point corresponding to the early stages of disease onset, Nrf2-KO and WT mice from all experimental groups were sacrificed for histopathological and molecular examination. Representative sections of lumbar spinal cord stained with H&E from mice with and without EAE (MOG 35-55 treated) are shown (Fig. 2.3A). Cell infiltration was observed in all mice with EAE as measured by the number of perivascular cuffings and the amount of subpial infiltration; however, there was a significant increase in the inflammatory index of Nrf2-KO compared with WT mice (Fig. 2.3B). Total RNA from whole spinal cord was also harvested and assessed for what cell types and to what extent the cells had breached and infiltrated the BBB. By measuring gene expression using qPCR, we were able to identify and quantify CD4+ T cell, CD8+ T cell, and CD19+ B cell lymphocytes. All mice with EAE disease had significantly augmented expression levels of these genes compared with the nondiseased CFA-immunized mice (Fig. 2.3C). Further, gene expression levels for CD4+ T cell, CD19, a cellular marker for B cells, as well as myeloperoxidase, a neutrophil cell marker, were significantly greater in the Nrf2-KO mice compared with WT, both with EAE (Fig. 2.3C).

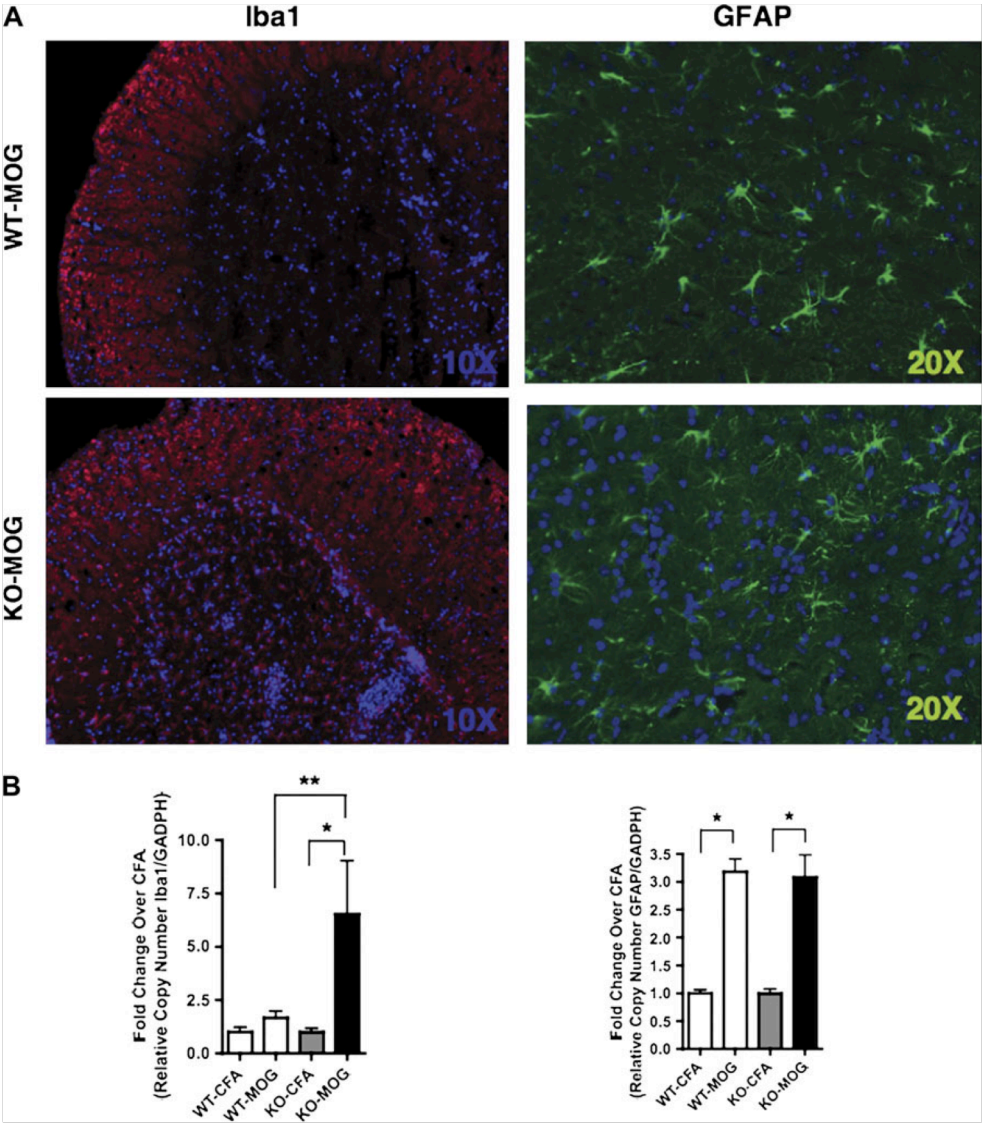
**Figure 2.3 Nrf2-KO mice with EAE show a greater total cell infiltration.**
(A) Representative paraffin-embedded spinal cord lumbar sections (20 lm) stained with H&E from CFA and MOG 35-55–immunized mice (x50). The solid and stippled black arrows display the perivascular and subpial cell infiltration, respectively. (B) Inflammatory index reflecting quantification of total cell infiltration in lumbar spinal regions from Nrf2-KO (n = 6) and WT (n = 6). *Significantly different from corresponding WT mice ($p < 0.05$). (C) Quantitative PCR analysis of whole spine extracts for mRNA levels of CD4, CD8, CD19, and myeloperoxidase (MPO). All data were standardized to GAPDH mRNA and presented as the mean fold change ± SEM when compared with the CFA-immunized mice (WT-CFA, n=5; WT-MOG, n=6; KO-CFA, n=5; KO-MOG, n=5). GraphPad Prism 4 (GraphPad Software) statistical software was used to analyze and plot two-way ANOVA followed by Bonferroni post hoc tests. *Significantly different than CFA-immunized mice within respective genotype cohort ($p < 0.05$). **Significantly different than MOG 35-55–immunized WT mice ($p<0.05$).



### 2.3.3 Evidence of Astrogliosis in Lumbar Spines of Nrf2-KO and WT Mice with EAE

Activation of astrocytes and microglia is associated with degeneration of neurons and oligodendrocytes, directly or indirectly or both. Lumbar spinal cord from WT and Nrf2-KO mice with EAE were stained for the microglial cell marker Iba1 and the astrocytic cell marker GFAP. Compared with their nondiseased (CFA alone) littermate controls, both WT and Nrf2-KO mice showed increased activation of both cell types (Fig. 2.4A; data not shown for CFA alone). Staining for Iba1, however, indicated more robust microglial activation in the Nrf2-KO mice than WT (Fig. 2.4A). In fact, gene expression levels for Iba1 were significantly higher in the diseased Nrf2-KO mice when compared with both nondiseased Nrf2-KO and diseased WT mice (Fig. 2.4B). Staining and gene expression levels for GFAP showed increased and significant activation in both Nrf2-KO and WT mice (Fig. 2.4A and 2.4B).

**Figure 2.4 Evidence of astrogliosis and microgliosis in mice with EAE.** (A and B) Representative immunofluorescent staining of lumbar spine for the cellular markers GFAP (astrocytes) and Iba1 (microglia) from mice immunized with CFA only or CFA and MOG 35-55. (B) Quantitative PCR analysis of whole spine extracts for mRNA levels of GFAP and Iba1. All data were standardized to GAPDH mRNA and presented as the mean fold change ± SEM when compared with the CFA-immunized mice (WT-CFA, n=4-5; WT-MOG, n=6; KO-CFA, n=5; KO-MOG, n=5). GraphPad Prism 4 (GraphPad Software) statistical software was used to analyze and plot two-way ANOVA followed by Bonferroni post hoc tests. *Significantly different than CFA-immunized mice within respective genotype cohort ($p < 0.05$). **Significantly different than MOG 35-55– immunized WT mice ($p < 0.05$).



### 2.3.4 Altered Expression Levels of Proinflammatory, Cytokine and Chemokine Genes in Nrf2-KO and WT mice with EAE

To better understand the biological and molecular mechanisms responsible for the differences in the clinical scores as well as the more robust presence of microglia in Nrf2-KO mice with EAE, the genetic profiles of proinflammatory enzymes and canonical cytokines and chemokines were assessed. Whole spine extracts of RNA from diseased and nondiseased mice in both Nrf2-KO and WT were analyzed by qPCR. Results showed that the proinflammatory enzyme, inducible nitric oxide synthase (iNOS), was significantly increased 23- and 96-fold in WT and Nrf2-KO mice with EAE, respectively, compared with the littermate controls (CFA only) (Fig. 2.5A). The 96-fold induction of iNOS in the Nrf2-KO mice was also significantly greater than in WT mice. Subunits of the multimeric enzyme, NADPH oxidase, a significant contributor of ROS ($O^{\cdot}$), were also analyzed. Gene expression levels for the subunits, phox-47, phox-67, and gp91-phox, were all increased in the diseased mice; gp91-phox showed the greatest induction in Nrf2-KO mice with EAE at 156-fold. Furthermore, all subunits were also significantly increased in Nrf2-KO versus WT mice with EAE (Fig. 2.5A). Gene expression levels for all cytokines, chemokines, and proinflammatory enzymes were similar and not significantly different between CFA- treated WT and Nrf2-KO (data not shown).

Levels for cytokines and chemokines were also monitored in these same spine extracts. The canonical cytokines, INF-gamma, IL1-b, TNF-alpha, and IL-12, were all significantly increased in both Nrf2-KO and WT-diseased mice. All but TNF-alpha were significant in the Nrf2-KO compared with WT mice with EAE (Fig. 2.5B). Two chemokines, monokine induced by interferon- gamma (MIG) and B cell attractant chemokine (BLC), which attract monocytes/macrophages and lymphocytes to sites of injury, respectively, were also analyzed. Similar to the cytokines analyzed, gene expression levels for MIP1-gamma and BLC with EAE were significantly increased in the Nrf2-KO mice when compared with WT (Fig. 2.5B). In particular, BLC and MIG were nearly an order of magnitude greater in the Nrf2-KO mice with EAE.

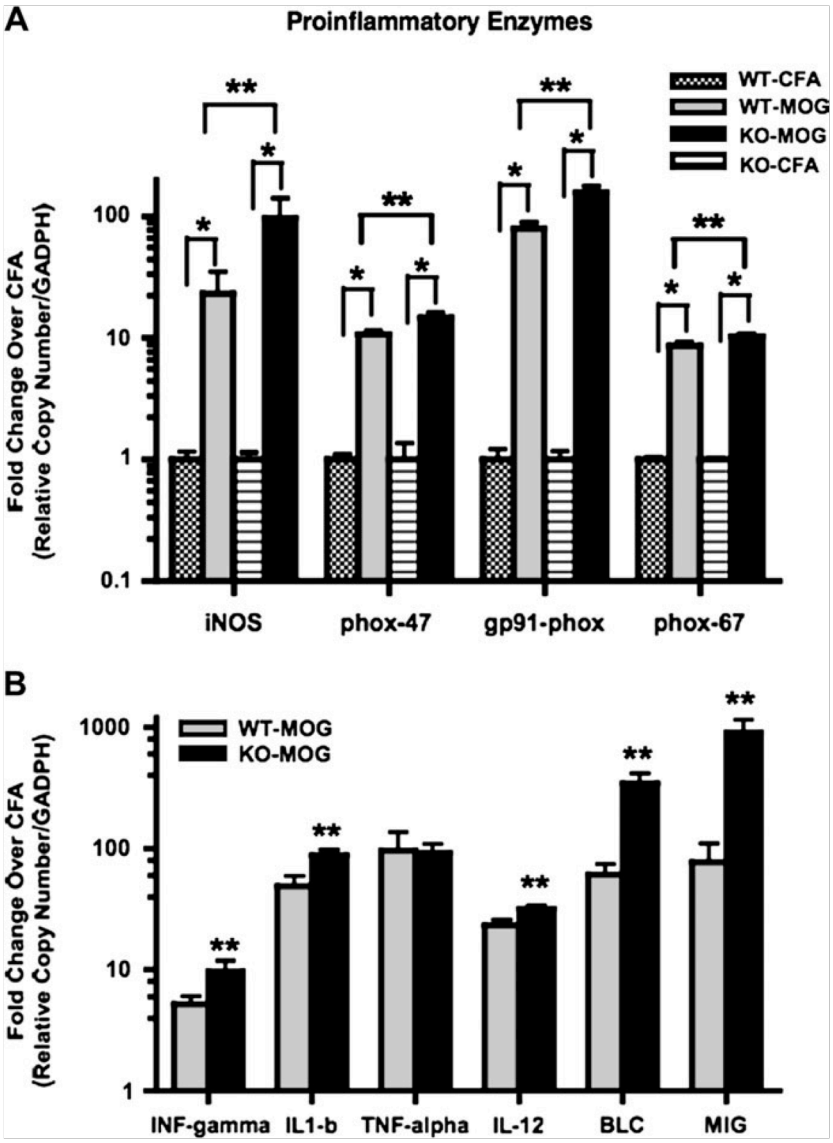



**Figure 2.5 Gene expression levels for proinflammatory enzymes, cytokines, and chemokines are greater in Nrf2-KO than WT with EAE.**

(A and B) At days 14–16, total RNA from whole spine was extracted from mice immunized with CFA or CFA and MOG 35-55. RNA was reverse transcribed and the cDNA product was subject to quantitative PCR analysis for mRNA expression levels of the following gene clusters—proinflammatory enzymes: iNOS, phox-47, gp91-phox, and phox-67; cytokines: IFN-gamma, IL1-b, TNF-alpha, and IL-12; and chemokines: BLC and MIG. All data were standardized to GAPDH mRNA and presented as the mean fold change ± SEM when compared with the CFA- immunized mice (WT-CFA, n=5; WT-MOG, n=6; KO-CFA, n=5; KO- MOG, n=5). GraphPad Prism 4 (GraphPad Software) statistical software was used to analyze and plot two-way ANOVA followed by Bonferroni post hoc tests. *Significantly different than CFA-immunized mice within respective genotype cohort ($p < 0.05$). **Significantly different than MOG 35-55– immunized WT mice ($p < 0.05$).

## 2.4 Discussion

Inflammation is an essential defense mechanism against a wide selection of pathogens as well as tumor formation. However, when the inflammatory process fails to subside and becomes dysregulated, such as in sepsis or during neurodegeneration, or if immune cells begin to attack self, then destructive and irreversible tissue damage can occur (Innamorato et al., 2008, 2009; Johnson et al., 2008). The data presented herein demonstrate that the transcription factor Nrf2 can modulate innate and adaptive immune responses in an acute autoimmune model of MS, EAE. Disruption of Nrf2 resulted in a more severe clinical course, a more rapid onset, and a greater percentage of mice with the disease. Furthermore, increased immune cell infiltration and glial cell activation in spine was observed, indicative of a greater pathological response in the Nrf2-KO mice with EAE. In conjunction, we observed increased inflammatory, cytokine, and chemokine gene expression levels in the Nrf2-deficient mice compared with the WT mice, supporting the notion that Nrf2 can modulate an inflammatory response.

Our data are consistent with previous studies researching the role of Nrf2 in modulation of inflammation in models outside the CNS. When LPS was injected ip or after cecal ligation/ puncture, a more dramatic rate of mortality in Nrf2-KO versus WT mice was observed. Septic Nrf2-KO mice had serum TNF- alpha levels that were significantly greater than in WT, suggesting that mice lacking Nrf2 had a more severe innate immune response to the bacteria or endotoxin (Thimmulappa et al.,

2006a). In nonlethal inflammatory studies, lungs from Nrf2-KO or WT mice were instilled with LPS or carrageenan, or mice had ip injections of LPS or TNF-alpha. In all cases, Nrf2-KO mice had a greater influx of inflammatory cells in bronchoaleveolor lavage fluids (Itoh et al., 2004; Thimmulappa et al., 2006a). Carrageenan, a potent activator of COX-2, also showed increased ALI in Nrf2-KO mice. The latter data are interesting since resolution of inflammation is also thought to occur through the COX-2 generation of cyclopentanone 15d-PGJ2, an endogenous electrophile and activator of Nrf2. Previous reports had shown that 15d-PGJ2 increased expression of Nrf2-driven genes in peritoneal macrophages. In fact, 15d-PGJ2 has been shown to covalently bind to Keap1, whereby Nrf2 is then activated and expression of ARE-driven genes again is increased. Enzymatic inhibition of 15d-PGJ2 production using NS-398, a selective COX-2 inhibitor, exacerbated ALI in WT mice to the severity observed in Nrf2-KO. Moreover, exogenous administration of 15d-PGJ2 reversed ALI in WT but not in Nrf2-KO mice, supporting the notion that Nrf2 does indeed modulate inflammatory response (Itoh et al., 2004).

Microarray analysis of lung tissue from LPS-injected Nrf2- KO mice showed greater gene expression levels of several cytokines, chemokines, and adhesion molecules, at 30 min and 1 h. We also observed increased gene expression levels for cytokines and chemokines of which IL-12, BLC, and MIG were significantly increased in Nrf2-KO mice and were common to the LPS study described above (Thimmulappa et al., 2006a). In contrast, our gene expression studies were performed on whole spine

samples taken at 14–16 days after initial injection of MOG 35-55. We chose this time point because onset of EAE begins at or near day 14 and inflammatory responses are continuously occurring. Perhaps, analysis of cytokine and chemokine profiles at earlier time points is warranted, as it appears from the LPS studies that gene expression levels are dramatically changed within minutes after injection. Interestingly, TNF-alpha levels, although significantly increased in both WT and Nrf2-KO with EAE, were consistently increased in both cohorts. Studies done in mice lacking TNF-alpha showed delays in remyelination. It was suggested that TNF-alpha may actually play role in the regeneration and repair of oligodendrocytes (Arnett et al., 2001). Although we would expect significant increases in the Nrf2-KO mice with EAE, perhaps the TNF-alpha levels, in this respect, are actually protective.

The immunomodulatory potential of Nrf2 was demonstrated in cultured human blood monocytes and neutrophils as well as mouse peritoneal macrophages treated with various Nrf2- activating compounds, including tert-butyl hydroquinone, diethyl maleate, CDDO-Im, and sulforaphane (Ishii et al., 2000; Liu et al., 2008; Thimmulappa et al., 2007). In some of these studies, activation of Nrf2 attenuated inflammation induced by subsequent treatments with LPS. ROS measured after the LPS treatments, was decreased in Nrf2-activated macrophages and, as expected, when Nrf2 was disrupted in these cultures, loss of the protection against inflammation was observed (Thimmulappa et al., 2007). Further reinforcing evidence that the transcription factor Nrf2 can prevent oxidative stress in

inflammation was demonstrated in a study using peritoneal macrophage cultures from mice lacking glutathione peroxidase-1 (GPX) (Fu et al., 1999). GPX is a selenium-dependent enzyme that catalyzes the reduction of hydrogen peroxides to water and lipid hydroperoxide radicals to alcohols using GSH for donation of electrons in these processes. Nrf2 regulates gene expression levels of GPX. In this study, GPX-KO and WT macrophages were treated with LPS, interferon-gamma (IFN-gamma), and diquat, a super oxide anion-generating molecule. Peritoneal macrophages from GPX-KO mice showed significantly enhanced levels of oxidative stress as detected by levels of NO and protein carbonyls (Fu et al., 2001). Our studies did indeed see increased gene expression levels of enzymes involved in the generation of ROS and RNS in mice with EAE supporting these concepts. More specifically, iNOS and the multimeric subunits of NADPH oxidase were all significantly increased in Nrf2-KO when compared with WT mice. Our future studies will include direct measurement of oxidative stress in a cell- specific manner.

Implications that Nrf2 modulates autoimmune diseases are found in studies investigating aged Nrf2-KO mice. Systemic lupus erythematous and autoimmune nephritis diseases have been documented in female Nrf2-KO mice that were over 12 months of age (Li et al., 2004; Yoh et al., 2001). Auto-antibodies against double-stranded DNA and Smith antigen were detected in the serums from these mice. In addition, deposition of IgG and IgM was noted in kidney, liver, heart, and brain. Since Nrf2-deficient mice have a decreased capacity to compensate for conditions of

oxidative stress, acute and chronic cell damage can occur. Increased damage resulting from lipid peroxidation, as well as modifications of DNA and proteins, can trigger immunologic reactions leading to dysregulated autoimmune responses (Li et al., 2004). Of note, the studies described in this manuscript were performed using Nrf2-KO mice that were 2–5 months of age, a period of time that is well before the onset of endogenous autoimmune disease.

Further evidence of increased neuroinflammation in Nrf2-KO mice was demonstrated by heightened reactive microgliosis in spines from Nrf2-KO mice with EAE. Iba1-positive microglia were clearly visible in immunostained sections of spine, as was increased gene expression levels for Iba1. However, GFAP staining for reactive astrocytes was increased similarly in spines from WT and Nrf2-KO with EAE. Interestingly, many of the GFAP-positive cells from Nrf2-KO appeared damaged as if sick or unhealthy. We speculated that these Nrf2-deficient astrocytes, unable to mount an adequate response to the increased oxidative stress generated by reactive microglia, had become diseased themselves. In fact, in active and chronic active MS lesions, NQO1, an Nrf2-driven antioxidant enzyme, was significantly upregulated in hypertrophic astrocytes and in myelin-laden macrophages, underlining the importance of Nrf2 activation as an endogenous response to oxidative stress (van Horssen et al., 2006, 2008). Previous studies from our laboratory have described similar neuroinflammatory responses. Kainate-induced loss of neurons in the hippocampus of Nrf2-KO mice had enhanced microgliosis. It

was postulated that this heightened microglia response exacerbated the neuronal damage that was observed (Kraft et al., 2006). Similar to that observed in our EAE studies, the processes of reactive astrocytes from Nrf2-KO mice appeared broken and damaged. In another study, astrocyte-selective activation of Nrf2 was able to confer protection to motor neurons in a mouse model of amyotrophic lateral sclerosis and was followed by a delayed astrocyte and microglia activation (Vargas et al., 2008). A more recent study by Innamorato et al. (2008) found that Nrf2-KO mice injected with LPS were hypersensitive and experienced a greater influx or activation of macrophage/microglia in the hippocampus, which was not prevented by pretreatments with an Nrf2-activating compound, sulforaphane (Innamorato et al., 2008). Enhanced adaptive immune responses were also observed in spines from Nrf2-KO mice with EAE. Gene expression levels for CD4, CD8, and CD19 (B cell)-positive lymphocytes were increased in WT and Nrf2-KO mice with EAE. CD4 and CD19 expression levels were greater in the Nrf2-KO and were significantly different. With respect to generation of oxidative stress, these lymphocytic cells contribute by chemotactically attracting microglia/macrophages to sites of disease or damaged tissue. Reactive microgliosis drives progressive neurotoxicity via secretion of reactive oxygen and nitrogen species such as super oxide, $O_2^{\bullet -}$, and nitric oxide, NO. Robust changes seen by measuring expression levels of specific genes associated with inflammatory cells allow for a quantitative measure of cell infiltration observed in the spines from mice with EAE that cannot be achieved simply by immunostaining. In all cases discussed, Nrf2 was implicated in the mitigation of

neuroinflammation as well as neuroprotection.

In conclusion, we postulate that the absence of Nrf2-mediated protective mechanisms against oxidative stress exacerbates EAE-induced neuroinflammation due to ongoing cellular and tissue damage by the inflammatory-mediated generation of ROS and RNS. Damaged tissue in turn reactivates resident microglia and astrocytes, and continuous activation of an adaptive immune response can also result. Alleviation of neuroinflammatory- induced oxidative stress via activation of Nrf2 may thus prove to be an important adjuvant therapy in the treatment of MS as well as other neurodegenerative diseases.

## 2.5 Experimental Procedures

### 2.5.1 Animals

All experiments were performed using Biozzi ABH mice back- crossed onto Nrf2-KO mice for a minimum of seven generations producing congenic strains of mice containing over 99% of the recipient genome. The Biozzi ABH mice were graciously provided by Dr Ian Duncan, School of Veterinary Medicine, University of Wisconsin, Madison (Note: these mice were originated by Dr David Baker, Institute of Neurology, University College London). ABH mice are widely used in studying diseases of autoimmunity. These mice have a unique major histocompatibility complex haplotype designated H-2dq1, yielding them highly susceptible to antigenic

agents (Amor et al., 2005). Nrf2-KO mice were generated by displacement of the basic leucine zipper/DNA-binding domain of Nrf2 with a B-galactosidase cassette (Chan et al., 1996) and were kindly provided by Dr Yuet Wai Kan, Howard Hughes Medical Institute, University of California, San Francisco. For sake of simplicity, the ABH 3 Nrf2-KO mice are hereafter referred to as Nrf2-KO.

### 2.5.2 Induction of EAE and Clinical Scoring

EAE was induced in 8- to 16-week-old male and female Nrf2-KO mice and WT littermate controls by sc injecting 100 μg of MOG 35-55 peptide (AnaSpec, Inc., San Jose, CA) emulsified in complete Freund's adjuvant (CFA) including Mycobacterium tuberculosis (SIGMA, St Louis, MO) into the scapular region. This was concomitant with ip injections of 200 ng of pertussis toxin (SIGMA) on days 0 and 2. EAE was monitored daily for clinical scores of disease until completion of the experiment using the following scoring system—1, complete tail atony; 2, hind limb weakness; 3, hind limb paralysis; 4, complete hind limb paralysis and front limb weakness; and 5, moribund. If mice with EAE were deemed to be within two scorings, a 0.5 value was added to the lower of the clinical score. Total body weights were also monitored daily. It is noted that all animals were treated and cared for in accordance with and approval of the University of Wisconsin's Institutional Animal Care and Use Committee.

### 2.5.3 LFB Staining and Measurement of Demyelination

At 14–16 days after EAE induction, mice were transcardially perfused with 0.1M PBS. Spinal cords were removed from the spinal column by liquid expulsion, fixed in 10% formalin, and embedded in paraffin. Spinal cords were cut, and middle lumbar and lower sacro-lumbar regions were included on each slide. Sections (10 μm) were cut and stained with Luxol Fast Blue (LFB) to detect myelin. Four intact cross-sections from each mouse (four to five mice per group) were photographed on an Olympus BX40 microscope (Olympus America Inc., Melville, NY) using a CMOS Pro 1000 series digital camera (Sound Vision Inc., Wayland, MA). Adobe Photoshop images were imported into the ImageJ image processing and analysis program (http://rsb.info.nih.gov/ij/index.html). Normally myelinated areas and areas of demyelination in the dorsal region were measured on each section. Areas from each animal were totaled, and the percent demyelination was calculated. GraphPad Prism 4 (GraphPad Software) statistical software was used to plot data and to calculate unpaired t test– generated p-values for comparison of demyelination between groups of mice ($p < 0.05$ was considered significant).

### 2.5.4 Cell Infiltration

Cell infiltration. Paraffin sections of spine from the above experiment were also stained with hematoxylin and eosin (H&E) for analysis of total cell infiltration in perivascular and subpial regions. High-resolution images of sections were captured

at 3200X magnification using the Mosaic Scanning System (Zeiss Microscopy, Germany). Paneled sections were then stitched, and the total cell infiltration was blindly assessed and scored by adding the total number of areas of perivascular cuffing to the arbitrary scoring system for subpial cell infiltration that consisted of the following: 0, no infiltration; 1, minor infiltration; 2, numerous infiltrating cells; 3, most of the pia involved; and 4, all of the pia involved. Statistical analysis was performed as described above.

### 2.5.5 Immunohistochemistry

Paraffin sections of spine from the above experiment were cut into 10-lm sections, rehydrated, and immunohistochemically stained using rabbit anti-ionized calcium-binding adaptor molecule 1 (Iba1) antibody (1:250; Wako Pure Chemical Industries, Osaka, Japan) or rabbit anti-Glial fibrillary acidic protein (GFAP) antibody (1:2000; Dako). Antigen retrieval was performed prior to staining for MOG by boiling sections for 20 min in 0.01 M sodium citrate, pH 6.0. All sections were then blocked with buffer containing 0.4% Triton X-100, 10% goat serum or horse serum, and 0.5% bovine serum albumin in PBS. Sections were incubated with primary antibodies diluted in blocking solution overnight at 4°C. Secondary staining of sections was performed using the appropriate antibodies conjugated to AlexafluorÒ 488 and 647 (Molecular Probes, Inc., Eugene, OR) that were diluted in blocking solution (1:250) and incubated for 1 h at room temperature. Hoescht 33258 (5 lg/ml, SIGMA) was added to the secondary antibody solutions to stain cell nuclei.

Sections were washed with PBS and mounted with Fluoro-Gel (EMS). Control staining was performed using appropriate IgG antibodies in place of the primary antibody. Sections were visualized using a Zeiss epifluorescent microscope (Carl Zeiss, Germany) and photographic images of immunofluorescence captured using AxioVision 4 software (Carl Zeiss).

### 2.5.6 qPCR

In some experiments, total RNA was extracted from whole spinal cords at day 14–16 after induction of EAE. Spines were expelled from spinal column and stored in RNA-later™ (Ambion, Austin, TX) for at least 24 h at 4°C until further processing. Spinal cords were then homogenized in Trizol reagent (Invitrogen, Inc.), and total RNA was extracted following the manufacturer's instructions. Integrity and concentrations of RNA were determined using the RNA 6000 Nano chip and analyzed by the Agilent 2100 Bioanalyzer (Agilent Technologies, Foster City, CA). Only samples that carried an RNA Integrity Number of 8.0 or greater were used for further analysis. RNA (1 lg) was reverse transcribed using an Oligo-dT 15 primer in accordance with the Reverse Transcription System (Promega Corporation, Madison, WI). PCR amplification and quantification (qPCR) of resulting cDNA was performed in real time using the LightCycler® 480 System (Roche Applied Science), using Cycler480 SYBR Green I Master (Roche Applied Science), and following manufacturer's instructions. All PCR product quantification was subject to relative standard curves that yielded amplification efficiencies greater than 1.79 and less than 2.19 and with

error less than 0.2. Primers used for the qPCR analysis are listed in the Supplementary table 1.

## 2.6 Supplementary Data

### Supplemental Table 2.6.1

| Gene of Interest | Forward Primer (5'-3') | Reverse Primer (5'-3') |
|---|---|---|
| **Cellular Markers** | | |
| *GFAP* | CGA GTC CCT AGA GCG GCA AAT G | CGG ATC TGG AGG TTG GAG AAA GTC |
| *Iba1* | GGA TTT GCA GGG AGG AAA AG | TGG GAT CAT CGA GGA ATT G |
| *CD4* | GGG CTG TGG CAG TGT CTA CT | GCC AGG AAC ACT GTC TGG TT |
| *CD8* | TCC TGG GAT CAC CAG CAT GCT TTA | TTT GAC AGT CAG CGT CTT CCT CCA |
| *CD19* | AGT GAT GCA AAT ATG TCC AGC CGC | AGA GGC AGA CAG TGT TTC CCA GTT |
| *MPO* | GGA AGG AGA CCT AGA GGT TGG | TAG CAC AGG AAG GCC AAT G |
| **Inflammatory Enzymes** | | |
| *iNOS* | CAG GAG GAG AGA GAT CCG ATT TA | GCA TTA GCA TGG AAG CAA AGA |
| *gp91-$^{phox}$* | CAG GAG TTC CAA GAT GCC TG | GAT TGG CCT GAG ATT CAT CC |
| *phox-47* | GGA CAC CTT CAT TCG CCA TA | CTG CCA CTT AAC CAG GAA CAT |
| *Phox-67* | CAG CCA GCT TCG GAA CAT G | GAC AGT ACC AGG ATT ACA TC |
| **Cytokines** | | |
| *INF-gamma* | AGC GGC TGA CTG AAC TCA GAT TGT | ACT GCT TTC TTT CAG GGA CAG CCT |
| *IL1-b* | TGG AGA GTG TGG ATC CCA AGC AAT | TGT CCT GAC CAC TGT TGT TTC CCA |
| *TNF-alpha* | CTG AGG TCA ATC TGC CCA AGT AC | CTT CAC AGA GCA ATG ACT CCA AAG |
| *IL-12* | ATC GTT TTG CTG GTG TCT CC | GGA GTC CAG TCC ACC TCT ACA |
| **Chemokines** | | |
| *BLC* | TGA GGC TCA GCA CAG CAA | TGA GGC TCA GCA CAG CAA |
| *MIG* | CTT TTC CTT TTG GGC ATC AT | GCA TCG TGC ATT CCT TAT CA |

## 2.7 Acknowledgements

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# CHAPTER 3

## Microglial Specific Overexpression of Nrf2 Protects Against Neuronal Toxicity and Experimental Autoimmune Encephalomyelitis

## 3.1 Abstract

Microglia are the resident macrophages in the central nervous system. They are important mediators in protection against microbial infection by releasing toxic substances and subsequently phagocytize and clear pathogens and damage tissue from pathogen-induced cytotoxic injury. Conversely, microglia protect neurons by secretion of trophic factors necessary for neuronal survival. However overactive microglia also release a plethora of neurotoxic factors that include IL-1ß, TNF-α, NO, $NOO^-$ and $O_2^{\bullet -}$, perpetuating a toxic cycle of injury to the neurons. Reactive microgliosis is implicated in the pathology and progression of many neurodegenerative diseases (PD, AD, ALS and HD) and injuries to the CNS (spinal cord injury, cerebral artery occlusion and MS). Nrf2, a major transcription factor involved in the maintenance of cellular homeostasis, prevention of oxidative stress and cell survival, has been shown to be protective in mouse models of many of these neurodegenerative diseases. These studies, however, have focused primarily on Nrf2 activation in astrocytes as necessary for protection of neurons. The studies presented here explore the Nrf2-ARE signaling pathway in preventing reactive microgliosis in primary cortical microglia cultures, organotypic spinal cord slices, and in EAE, a mouse model of MS. The data demonstrate that cell specific overexpression of Nrf2 or treatment with tBHQ, an Nrf2 activator, attenuated LPS-mediated microglial activation in both primary cortical microglial cultures and spinal cord slices. In addition, neurotoxicity was reduced in primary cortical

neurons co-cultured with Nrf2-activated microglia. Mice overexpressing Nrf2 in microglia/macrophages had significantly reduced EAE clinical scores when compared to WT mice. Contrary to these findings, there were no differences in EAE clinical scores between mice deficient in Nrf2 in microglia/macrophages and WT mice. These findings indicate that overexpression of Nrf2 in microglia/macrophages results in attenuation of reactive microgliosis. Nrf2 activation in microglia may thus be a viable therapeutic target to alleviate the damaging effects of inflammation in diseases of the brain and spinal cord.

## 3.2 Introduction

Microglia are the resident macrophages of the central nervous system (CNS). Originally defined by del Rio-Hortega in 1932, microglia comprise up to 12% of cells in the brain with regional heterogeneity and varying population densities (Kettenmann et al., 2011). In the healthy CNS environment, microglia are deemed quiescent having ramified cellular appearances with small soma that project branched and arborized processes. These "resting" cells however, delicately survey their surroundings for the presence of exogenous or endogenous danger signals. The daily housekeeping activities of the resting microglia remain largely unknown (Kettenmann et al., 2011). However, it is speculated they amend small undetectable neuronal insults by supplying trophic support and protection, and contribute to neuronal plasticity by the phenomenon known as synaptic stripping (Kettenmann et al., 2011; Paolicelli et al., 2011).

Dangerous toxic or foreign substances, including environmental chemicals, viruses, bacteria, or cellular components from brain injury or degenerating neurons, can activate pattern recognition receptors (PRRs) on microglia. This transforms microglia morphology to amoeboid-like structures. At this stage the microglia are termed "activated" and through cell signaling mechanisms secrete inflammatory cytokines (e.g. TNF-alpha, IL6, PGE2, IL1beta), chemokines (e.g. RANTES, MIP1, IL-8) as well as generate reactive oxygen (ROS) and/or nitrogen (RNS) species (e.g. superoxide, peroxynitrite and nitric oxide) by activation of NADPH oxidase and

iNOS (inducible nitric oxide synthase) respectively. These activated microglia protect neurons by maintaining the cellular environment through mitigation of the innate immune response, clearance of toxic cellular debris and secretion of trophic factors necessary for neuronal survival (Block et al., 2007; Kettenmann et al., 2011).

Upon removal or disruption of the danger signal the microglia "turn off" and return to their resting stage; albeit these "experienced" cells may acquire a more rapid and pronounced response if re-exposed to the same triggering substance. In addition, several factors such as IL-10, TGF-beta and endogenous cannabinoids exert anti-inflammatory signals that regulate and prevent microglial over-activation (Kettenmann et al., 2011).

Continuous microglial activation, however, can result in toxicity and disastrous effects on neurons and oligodendrocytes. Environmental toxins such as LPS, paraquat, rotenone and diesel exhaust particles (DEP), as well as cellular components released from dying neurons (e.g. laminin, MMP3, $\alpha$-synuclein and neuromelanin) can activate microglia. Microglia release of toxic factors such as superoxide, NO (nitric oxide) and TNF-alpha can have deleterious effects on neurons. These factors, in addition to direct neurotoxic insults or cell death by neurological diseases, can perpetuate a cycle of microglial activation and subsequent release of additional neurotoxic factors progressing the already dying population of neurons. Indeed, the presence of over-activated microglia, or reactive microgliosis, in

neurodegenerative disease pathologies is evident. Diseases such as multiple sclerosis (MS), Alzheimer's Disease (AD), Parkinson's Disease (PD), amyotrophic lateral sclerosis (ALS) and Huntington's Disease (HD), all present pathologically with reactive microgliosis (Amor et al., 2010; Kettenmann et al., 2011; Scott et al., 2003).

Intracellular ROS and RNS in microglia act as second messengers that activate kinase cascades and transcription factors such as NFκB that drive protective inflammatory gene expression. Persistent oxidative stress however can deplete the cellular antioxidant defense systems resulting in persistent activation of inflammation and subsequent toxicity to neurons and oligodendrocytes (Block et al., 2007). Thus microglial-activation of the Nrf2-ARE signaling pathway can increase levels of antioxidants and antioxidant enzymes restoring cellular redox homeostasis, reverting the microglia/macrophage back to resting and basal functioning stages. Increased cellular antioxidant capacity in the microglia may thus be an important therapeutic approach to quench and reverse reactive microgliosis or maintain these cells in their basal resting stage.

Nuclear factor erythroid-derived related factor 2 (Nrf2) is one of the major transcription factors involved in the maintenance of cellular homeostasis, prevention of oxidative stress and cell survival (Kensler et al., 2007). It is a member of the cap-n-collar (CNC) protein family that comprises a basic leucine zipper DNA

binding domain. Nrf2 is tightly controlled in the cytoplasm tethered to the cytoskeleton via a high-affinity interaction with Kelch-like ECH-associated protein, Keap1. Keap1, an E3-ligase, targets Nrf2 for proteasomal degradation. However, electrophiles, oxidative stress, or other cellular stressors and inducers can reactively modify cysteine residues on Keap1, mediating a conformational change and subsequent release of Nrf2. Nrf2 possesses nuclear localization signals and thus translocates to the nucleus. Together with small Maf proteins, Nrf2 binds to the anti-oxidant response element (ARE) located in the promoter regions of a battery of genes with various target gene functions that promote cell survival (Kensler et al., 2007; Sykiotis and Bohmann, 2010). These include, but are not limited to, glutathione homeostasis (e.g. GCLM, glutamate cysteine ligase modifier subunit; GCLC, glutamate cysteine ligase catalytic subunit; GR, glutathione reductase), metabolic detoxification (e.g. NQO1, NADPH-quinone oxidoreductase 1; GSTs, glutathione transferases), and direct antioxidants (e.g. Trx, thioredoxin; TR, thioredoxin reductase; Prxs, peroxiredoxins) (Lee et al., 2003; Li et al., 2002; Shih et al., 2003).

Previous studies in our laboratory have demonstrated the importance of Nrf2 in preventing or attenuating diseases in mouse models of ALS, PD, HD and epilepsy (Calkins et al., 2005; Chen et al., 2009; Kraft et al., 2006; Vargas et al., 2008). Many of these studies have focused on the importance of Nrf2 activation in the astrocyte as being neuroprotective. In addition, our lab has shown that lack of Nrf2

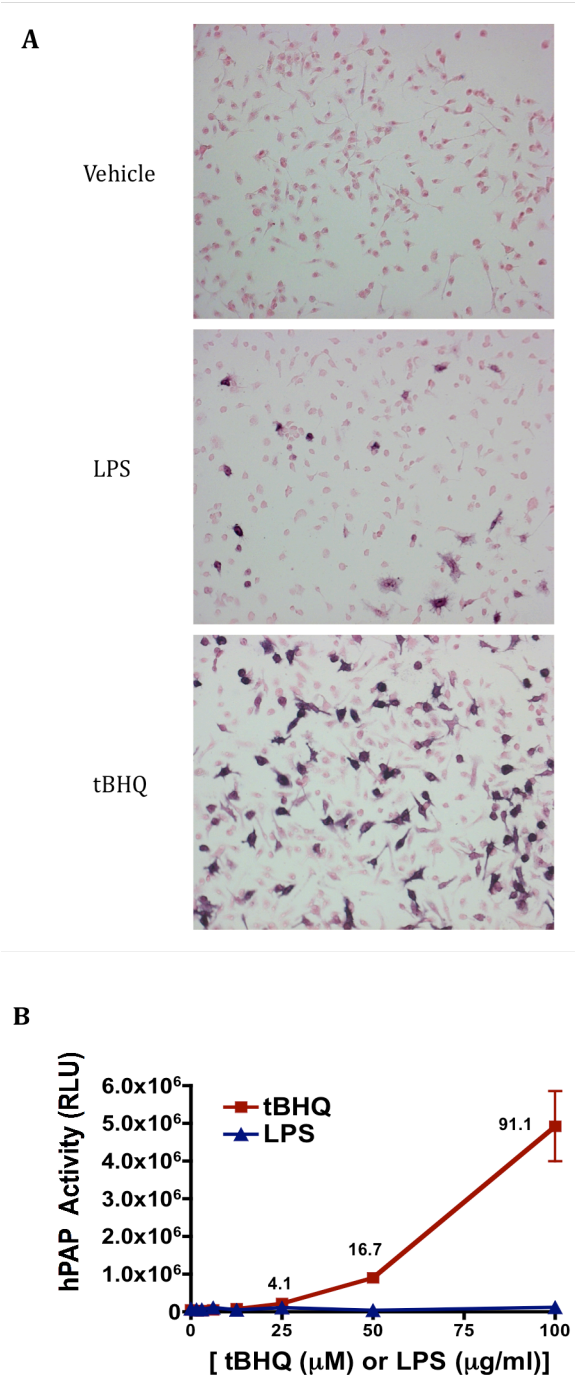
exacerbated experimental autoimmune encephalomyelitis (EAE), a mouse model of MS, and augmented inflammatory responses in the CNS (Johnson et al., 2010). Furthermore, other laboratories have implicated a role for Nrf2-mediated suppression of the inflammatory response in PD models (Rojo et al., 2010), sepsis (Kong et al., 2011; Thimmulappa et al., 2006), and acute lung injury (Cho et al., 2002; Mochizuki et al., 2005; Reddy et al., 2009a; Reddy et al., 2011; Reddy et al., 2009b) using the Nrf2 knockout mice. Activation of the Nrf2-ARE signaling pathway in microglia cell lines and primary cultures of microglia has been demonstrated (Min et al., 2008; Ni et al., 2010; Rohl et al., 2008; Rojo et al., 2010). What remains to be determined, however, is if and how activation of Nrf2 in microglia alleviates microglia overactivation and prevents deleterious effects on neurons. The studies presented herein attempt to address these questions. We present data suggesting that indeed overexpression of Nrf2 attenuates microglia activation in models of inflammation *in vitro* and lessens the severity of disease in the EAE mouse model of MS.

## 3.3 Results

### 3.3.1 Activation of the Anti-oxidant Response Element in Primary Cortical Microglia Cultures

To examine whether microglia activate the ARE, microglia from the cortices of ARE-hPAP transgenic reporter mice were cultured. The ARE-hPAP mice carry 51 base pairs of the promoter region of the NQO1 gene which includes an ARE and which is coupled to the human placental alkaline phosphatase (hPAP) reporter gene (Johnson et al., 2002). Cultures treated with 40μM tert-butylhydroquinone (tBHQ), a well known inducer of the Nrf2-ARE pathway, for 24 hours and showed significant activation as seen by increased histochemical staining for hPAP activity (Fig. 3.1A). In contrast, LPS treated cultures were treated (10 μg/ml) and showed few cells staining positive. This may be attributed to the high dose of LPS used and borderline toxicity associated with oxidative stress. Vehicle treated cultures, had little or no hPAP activity (Fig. 3.1A). Cell lysates from these same cultures were treated with increasing doses of tBHQ (0-100 μM) or LPS (0-100 μg/ml) for 24 hours and analyzed using a chemiluminescent substrate for hPAP activity. In tBHQ treated microglia cultures, a dose response of hPAP activity was observed with fold activations as high as 91-fold. No activation was seen in LPS treated cultures (Fig. 3.1B).

**Figure 3.1 Activation of ARE-hPAP in Primary Microglia Cultures Derived from Mouse Cortices** (*A*) Primary cortical microglia were treated with either vehicle, 40 µM tBHQ or 10 µg/ml LPS. After 24 hours, cells were stained with NBT/BCIP for the visualization of alkaline phosphatase activity. (*B*) Cell lysates from primary microglia cultures treated with tBHQ or LPS as indicated and assayed for hPAP activity using CSPD substrate and Emerald enhancer. hPAP activity is expressed as mean relative luminescent units (RLU) ± SD (n=5).



### 3.3.2 Expression Levels of Genes Associated with Inflammation and Anti-Oxidation in Primary Cortical Microglia Cultures Treated with tBHQ or LPS

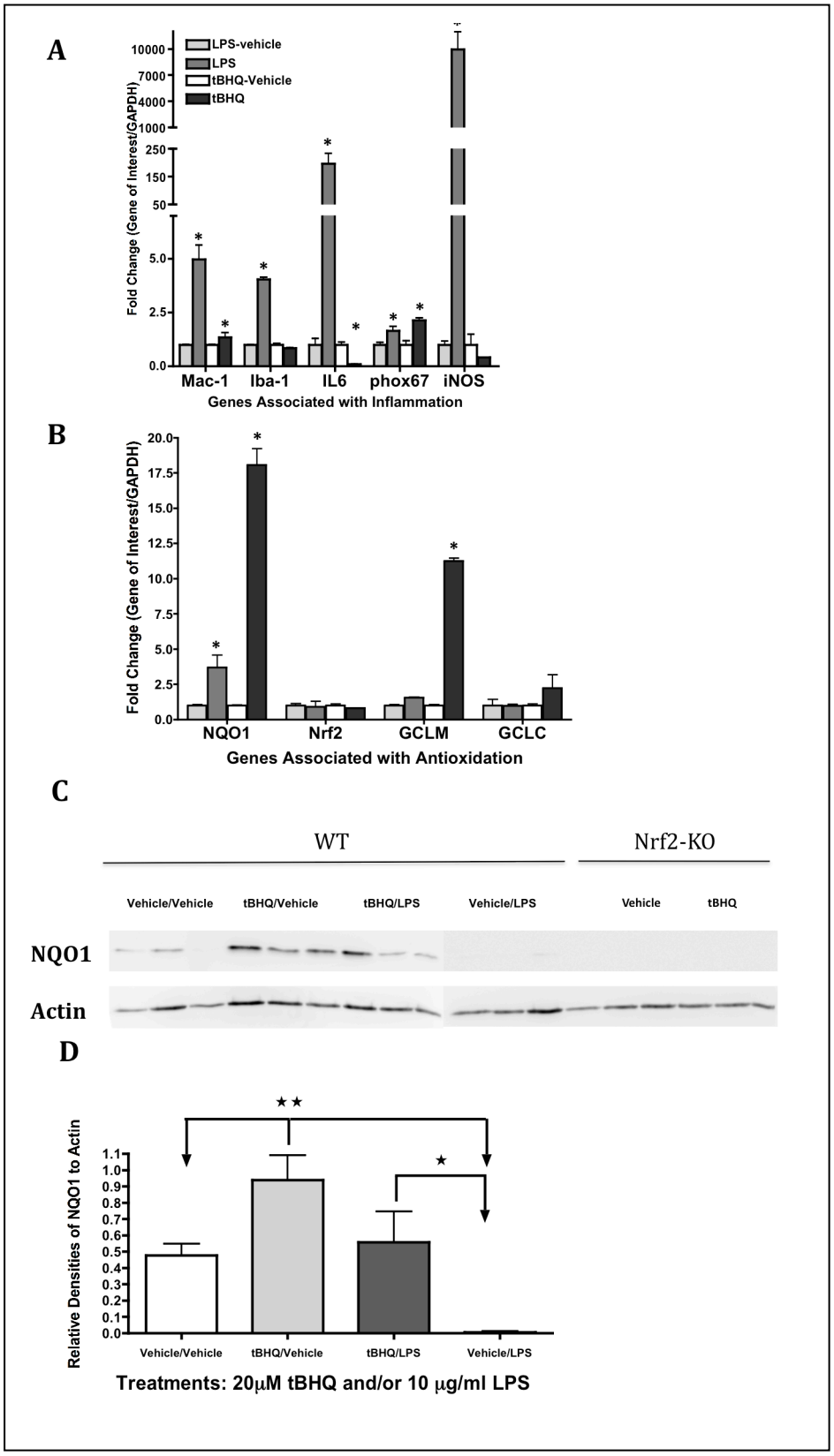
We examined expression profiles for genes associated with inflammation and oxidative stress in primary cortical microglia cultures treated with either 40 μM tBHQ or 10 μg/ml LPS. RNA was reversed transcribed and subject to qPCR analysis. Results for LPS treated cultures showed significant changes in mRNA for the microglial markers *Mac-1* and *Iba1*, a prototypic cytokine *IL-6*, and *Phox67*, a subunit for the multimeric enzyme NADPH Oxidase, when compared to vehicle treated cultures (Fig. 3.2A). When microglia were treated with tBHQ, gene expression levels for both *Mac-1* and *Iba1* had modest fold inductions that were significant when compared to the respective vehicle. Of note, LPS treatments increased *IL-6* levels by 195-fold and *iNOS* by 10,000-fold (Fig. 3.2A). In contrast, tBHQ treatment had no effect on *iNOS* and significantly reduced *IL-6*. With regards to genes associated with antioxidation, tBHQ significantly increased *NQO1*, *GCLM* and *GCLC* expression levels (Fig. 3.2B). *NQO1* showed a small but significant increase in LPS-treated cultures. Nrf2 gene expression did not change with either LPS or tBHQ treatment (Fig. 3.2A-B).

Expression levels for NQO1 protein were evaluated in the primary microglia cultures. The promoter region of the NQO1 gene includes an ARE and thus increased protein levels for this gene would suggest activation of Nrf2 in the microglia cultures. Primary cortical microglia cultures were treated with vehicle,

vehicle and 40 μM tBHQ, 40 μM tBHQ and 10 μg/ml LPS, or vehicle with 10 μg/ml LPS, all for 24 hours. Protein levels for NQO1 were significantly increased in tBHQ treated cultures in the absence or presence of LPS when compared to vehicle/vehicle or vehicle/LPS treatments as determined by measurement of NQO1 band intensities relative to actin (Fig. 3.2C-D). Interestingly, the level of NQO1 protein was dramatically reduced by LPS treatment. This could contribute to the increase in NQO1 mRNA following LPS (Fig. 3.2B) as the cell attempts to compensate for the loss of NQO1 protein. In addition, primary microglia from Nrf2-KO mice were cultured at this same time and treated with vehicle or 40 μM tBHQ. As expected, basal levels of NQO1 protein were reduced compared to WT cultures (the bands were barely visible on the western blot) and no obvious increase was observed following tBHQ treatment (Fig. 3.2C).

**Figure 3.2 Expression Profiles for Inflammatory and Anti-oxidant genes and NQO1 Protein Levels in Primary Microglia Treated with tBHQ and/or LPS**

Primary microglia cultures from mouse cortices were treated with 40 μM tBHQ or 10 μg/ml LPS for 24 hours. Total RNA was isolated and reverse transcribed and the resultant cDNA subject to real time quantitative PCR for measurement of mRNA expression levels for genes associated with inflammation: (*A*) *Mac1, Iba1, IL6, phox 67* and *iNOS*; (*B*)or associated with anti-oxidation: *NQO1, Nrf2, GCLM, GCLC*. All data were standardized to GAPDH mRNA and presented as mean fold change ± SD (n=3). GraphPad Prism 4 (GraphPad Software) statistical software was used to analyze and plot one-way ANOVA followed by Bonferroni post-tests. * Significantly different than respective vehicle treated cultures ($P<0.05$). (*C*) Immunoblot images of NQO1 protein from WT or Nrf2-KO primary cortical microglia and treated with Vehicle, 40 μM tBHQ and/or 10μg/ml LPS for 24 hours. (*D*) Densitometric analysis of NQO1 protein bands from the immunoblot images decribed above. Densities were analyzed for statistical differences using unpaired Student t-tests and plotted by GraphPad Prism 4 (GraphPad Software) statistical software. ★Significantly different than Vehicle-LPS treated cultures. ($P<0.05$). ★★Significantly different than Vehicle/Vehicle and Vehicle/LPS.treated cultures ($P<0.05$).


A
LPS-vehicle
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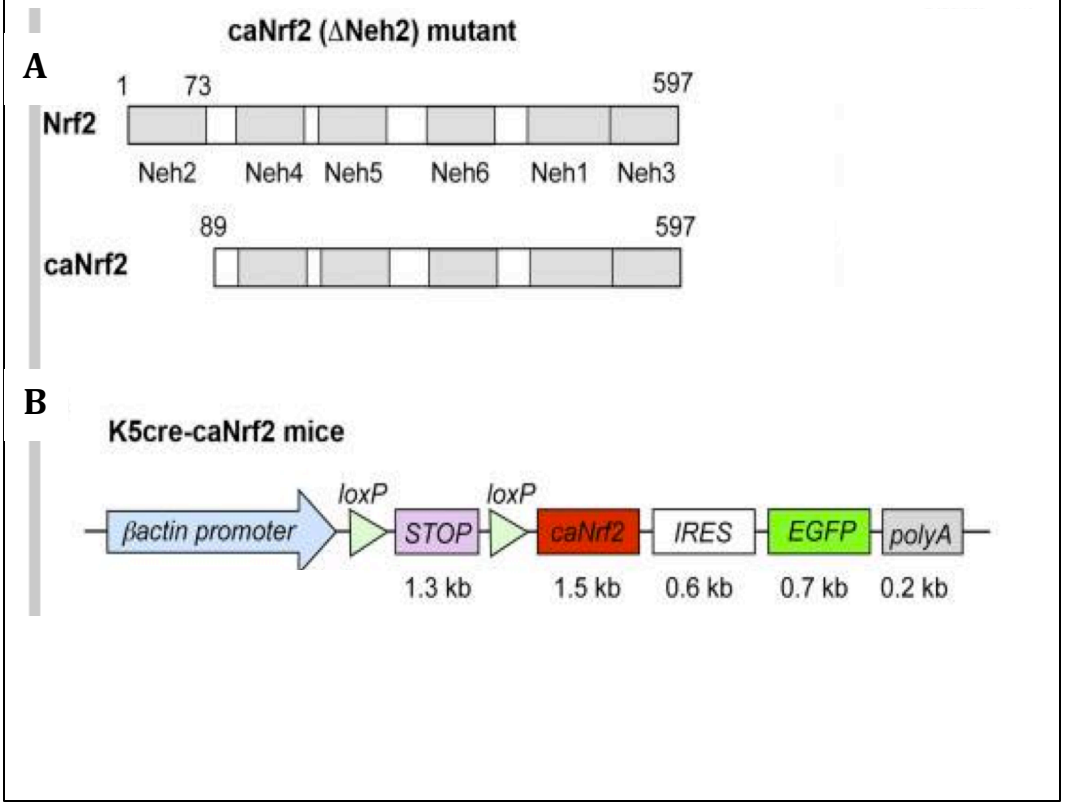
Fold Change (Gene of Interest/GAPDH)
Mac-1
Iba-1
IL6
phox67
iNOS
Genes Associated with Inflammation
B
Fold Change (Gene of Interest/GAPDH)
NQO1
Nrf2
GCLM
GCLC
Genes Associated with Antioxidation
C
WT
Nrf2-KO
Vehicle/Vehicle
tBHQ/Vehicle
tBHQ/LPS
Vehicle/LPS
Vehicle
tBHQ
NQO1
Actin
D
Relative Densities of NQO1 to Actin
Vehicle/Vehicle
tBHQ/Vehicle
tBHQ/LPS
Vehicle/LPS
Treatments: 20μM tBHQ and/or 10 μg/ml LPS

### 3.3.3 Genetic Manipulation of Nrf2 Affects Expression Levels of Glutathione Synthesizing Genes and Glutathione Levels in Primary Mouse Cortical Microglia

Glutathione (GSH) is a major cellular antioxidant important for cellular redox-homeostasis. GSH detoxifies destructive ROS and RNS generated by activated microglia. GSH depletion can result in toxicity and cell death. As described earlier, tBHQ treatment of primary microglia cultures with tBHQ activated the ARE and induced Nrf2-dependent genes (*GCLC* and *GCLM*) involved in GSH synthesis. In these next experiments, we investigated the affects of genetic manipulation of Nrf2 on the expression levels of *GCLC* and *GCLM* and intracellular levels of glutathione. Primary cortical microglia were cultured from WT and Nrf2-KO mice as well as transgenic mice selectively over-expressing Nrf2 in microglia. These latter mice were generated using a cre-lox system (Fig. 3.3A-B). Briefly, mutated Nrf2 (caNrf2) was generated by deletion of the Neh2 site, the Nrf2-Keap1 binding domain, of the Nrf2 gene. Without Neh2, Nrf2 continuously translocates to the nucleus and constitutively activates ARE-driven genes. Inserting the caNrf2 gene downstream from a floxed transcription/translation stop cassette subsequently generated the transgene used to generate the transgenic mice. In addition, a CMV enhancer as well as the ßactin promoter was used to drive transcription of the caNrf2 gene (Fig. 3.3B). The CMV-caNrf2 mice were bred with mice carrying the cre recombinase gene driven by a microglia specific promoter from the *CD11b* gene. Thus, double transgenic mice (CD11bCre x CMV-caNrf2) should cell specifically over-express Nrf2

in microglia.

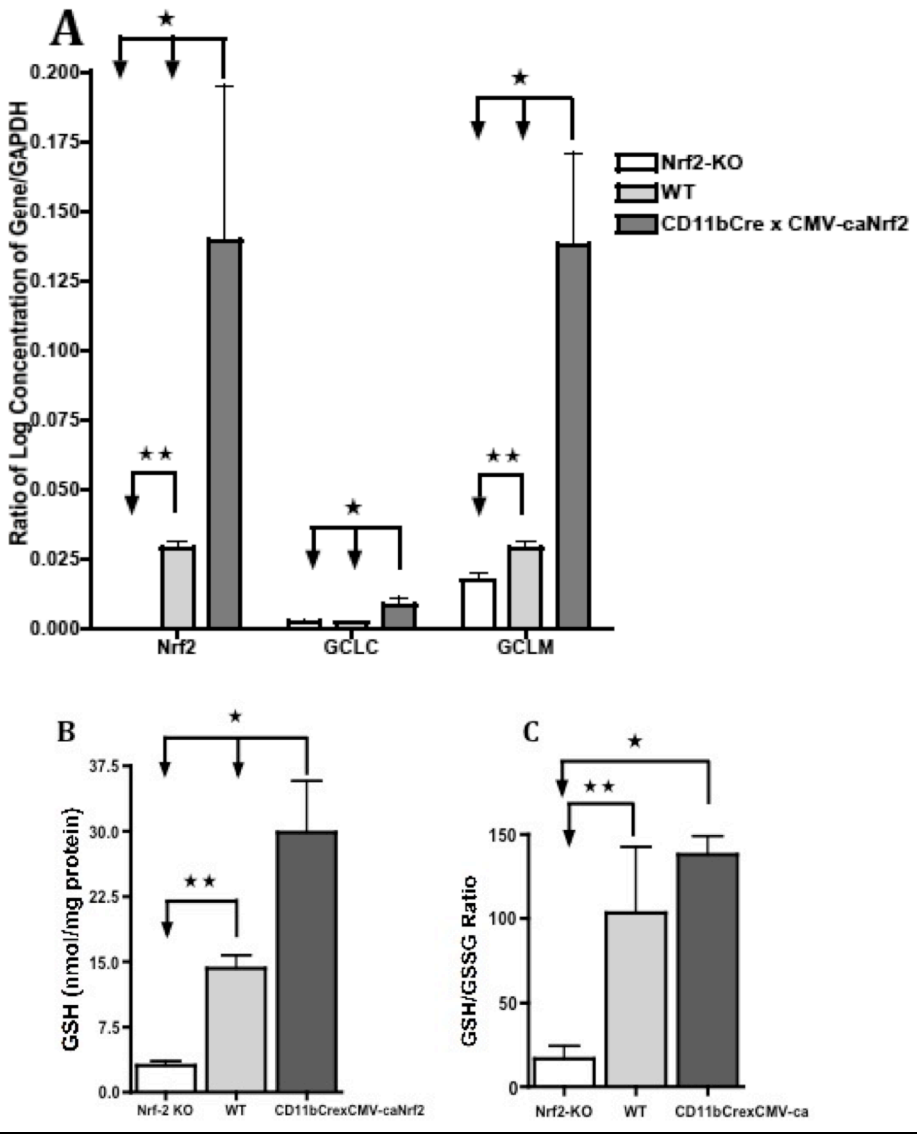
**Figure 3.3 Generation of CMV-β-actin-caNrf2 transgenic mice (CMV-caNrf2)** (*A*) Functional domains of Nrf2 and caNrf2 (deltaNeh2 mutant). (*B*) Scheme of the transgene used for generation of caNrf2 mice. Note: a second mouse line was generated containing a CMV enhancer sequence plus the chicken βactin promoter.




Quantitative PCR results demonstrated significantly increased expression levels in microglia isolated from the CD11b-Cre x CMV-caNrf2 mice for *GCLC, GCLM* and *Nrf2* when compared to WT and Nrf2-KO primary microglia (Fig. 3.4A). *Nrf2* and *GCLM* expression levels in the WT microglia were also significantly higher than in the Nrf2-KO microglia. As expected, expression levels for *Nrf2* correlated with *Nrf2*-gene dosage, as did expression levels for *GCLM* (Fig. 3.4A).

**Figure 3.3 Nrf2 Gene Dose-dependent Expression of GSH Synthesizing Genes and GSH Levels in Primary Cortical Microglia**

(*A*) Primary cortical microglia were cultured from Nrf2-KO, WT and cell specific Nrf2 over-expressing mice. Total RNA was isolated and reverse transcribed and the resultant cDNA subject to real time quantitative PCR for measurement of mRNA expression levels for *Nrf2, GCLC* and *GCLM*. Data are expressed as mean ± SD (n=4-6). (*B-C*) Reduced (GSH) and oxidized (GSSG) glutathione from microglia cell lysates were measured by normal phase (ion exchange) HPLC. Data are expressed as mean ± SEM (Nrf2-KO n=9; WT n=10; CD11bCre x CMV-caNrf2 n=4). ⋆p< 0.05 compared with Nrf2-KO and WT microglia cultures. ⋆⋆p< 0.05 compared with Nrf2-KO microglia cultures. All data were subject to one-way ANOVA followed by Newman-Keuls Multiple Comparison Test and plotted using GraphPad Prism software.



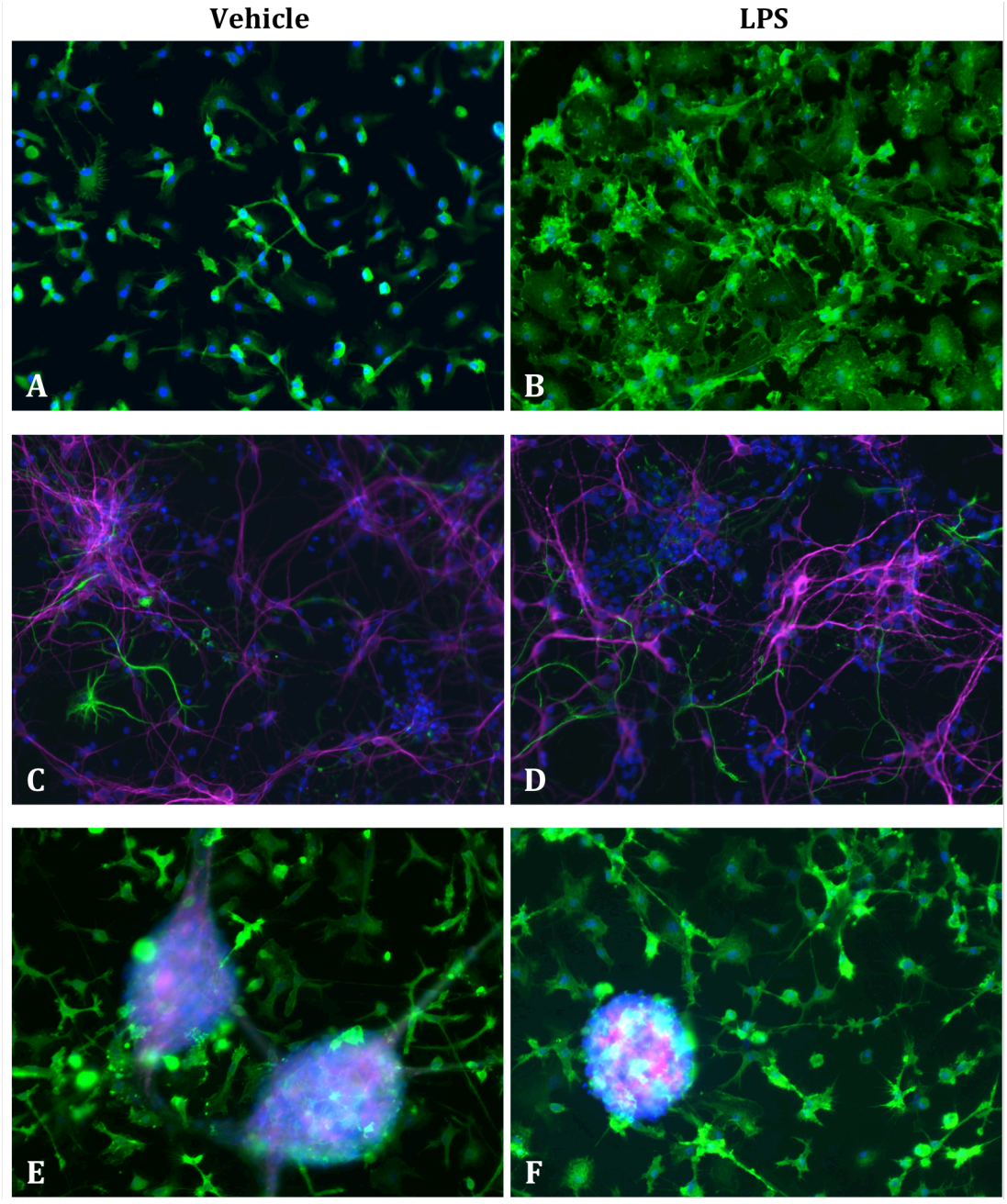
GSH (reduced) and GSSG (oxidized GSH) levels in primary microglia cultures from CD11b-cre x CMV-caNrf2 mice were determined by HPLC. There was a significant GSH levels (29.9 ng/mg protein) were significantly higher in the double transgenic microglia cultures compared with WT (14.22 ng/mg protein) and Nrf2-KO (3.1 ng/mg protein) (Fig. 3.4B). Moreover, ratios of oxidized to reduced GSH (GSH/GSSG), an indicator of levels of stress or antioxidant capacity of cells, were significantly greater in both WT and CD11b-Cre/CMV-caNrf2 microglia cultures when compared to Nrf2-KO (Fig. 3.4C). Thus indicating a higher anti-oxidant capacity in the WT and double transgenic microglia cultures. Interestingly, the CD11b-Cre xCMV-Nrf2 microglia had a slightly higher GSH/GSSG ratio than the WT cultures.

### 3.3.4 Direct Co-cultures of Neurons With Mouse Primary Microglia

The experiments described above confirmed that: 1) the Nrf2-ARE pathway is capable of being activated in primary microglia cultures; 2) primary microglia cultures were capable of expressing inflammatory-mediated and anti-oxidant genes; and 3) GSH levels in primary microglia are regulated by Nrf2-dependent gene expression of GCLC and GCLM in a gene dose-dependent manner. We next wanted to examine whether activating Nrf2 in microglia would attenuate microglial activation and associated neurotoxicity. First, however, it was important to assess the necessary conditions for co-culturing microglia with neurons, as microglia are

notorious for spontaneous activation dependent on the culture conditions and matrices on which they are grown (Kettenmann et al., 2011). In the first attempt at co-culturing, 100,000 or 200,000 primary cortical neurons were seeded atop a confluent layer of primary cortical microglia cells in either 6-well plates or chambered microscope slides. Primary microglia and neurons alone were also plated. The following day, all cultures were treated with either vehicle or 10 μg/ml LPS for 24 hours. Immunohistochemical staining was used to identify microglia (Iba1), neurons (MAP-2) and astrocytes (GFAP) to determine the population of contaminating astrocytes. Primary microglia alone showed a robust activation by LPS as exhibited by highly ramified cell morphologies and increased Iba1-reactive staining compared to the vehicle treated control cultures (Fig. 3.5A-B). Neuron-enriched primary cortical cultures contained mostly neurons with some GFAP-positive cells. LPS treatments in these cultures, was not toxic nor did it activate the astrocytes that were present (Fig. 3.5C-D). Interestingly, neurons that were plated directly atop the confluent primary microglia aggregated into spherical clusters regardless of the numbers of cells seeded or treatment group (Fig. 3.5E-F). Because of the failure of the neurons to disperse across the cultured microglia matrices, we did not further pursue this mode of co-culture.

**Figure 3.5 LPS-treated Primary Microglia Cells Show Robust Activation and is Not Toxic to Neuronally-enriched Primary Cortical Cultures.**
(*A-B*) Primary microglia cultures from mouse cortices were treated with vehicle or 10 μg/ml LPS for 24 hours. Cultures were stained for IBa1 (microglia in green) and Hoescht 33258 (cell nuclei in blue). *(C-D)* Primary neuron-enriched cultures from mouse cortices with either vehicle or 10 μg/ml LPS for 24 hours. Cultures were stained with MAP-2 (neurons in pink), GFAP (astrocytes in green), Hoescht 33258 (cell nuclei in blue). (*E-F*) Representative image of 200,000 neuronally-enriched cells seeded atop confluent primary microglia and treated with either vehicle or LPS for 24 hours. Cultures were stained with MAP-2 (neurons in pink), GFAP (astrocytes in green), Hoescht 33258 (cell nuclei in blue). Note images of 100,000 neuronally-enriched cells appeared as (*E-F*), data not shown.

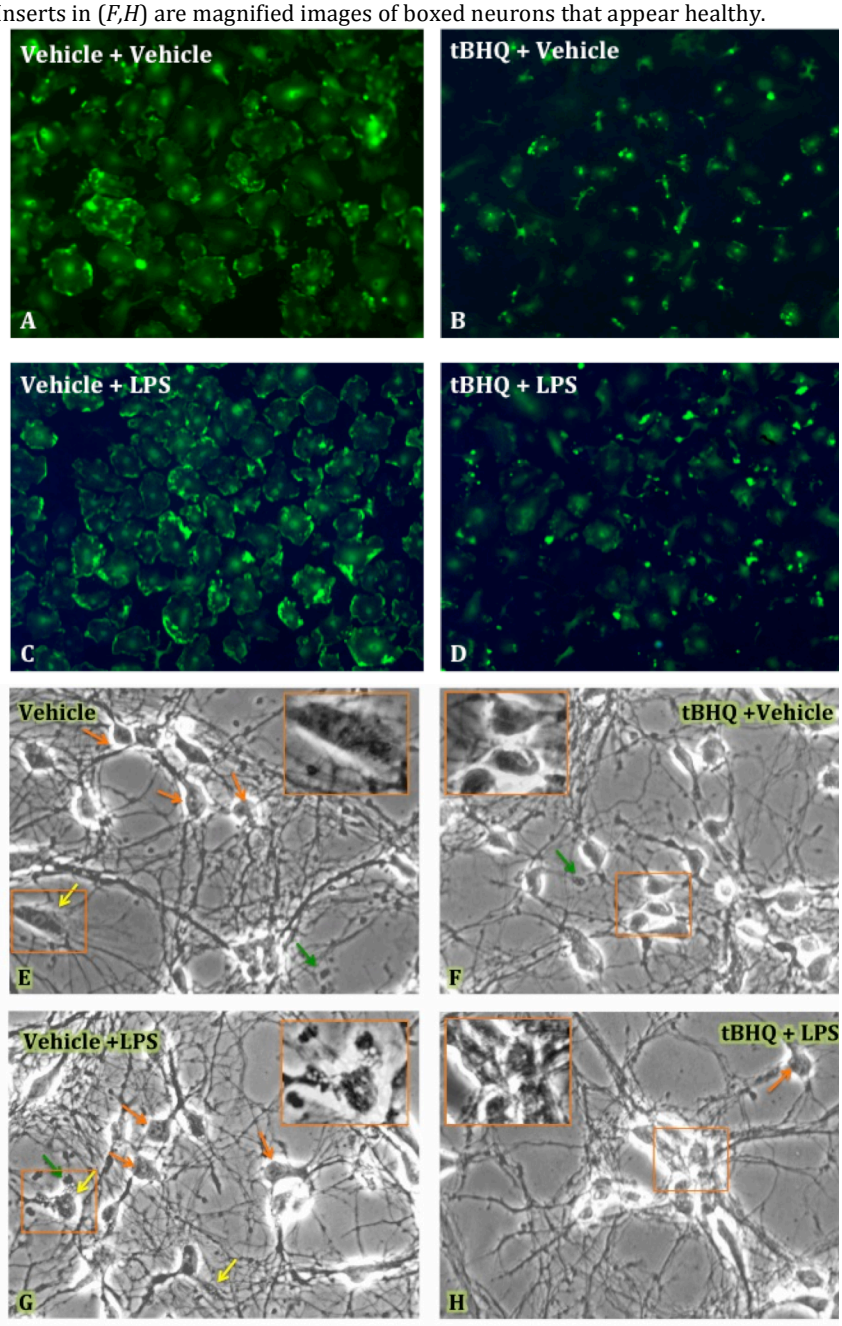


### 3.3.5 Pretreatment with tBHQ Moderately Attenuates LPS-Mediated Activation of Primary Microglia Cultures

Based on the preceding co-culture experiments proved futile, we investigated a transwell co-culturing system. Primary microglia were grown on porous membranes (the top compartment) of a transwell insert and placed in a culture dish that had a bottom layer of neuronal-enriched primary cortical cultures. The microglia cultures were pretreated with either vehicle or 20 μM tBHQ for 24 hours, followed by vehicle or 500 ng/ml LPS for another 24 hours. As discussed earlier, microglia can be easily activated by the culturing conditions alone. In fact, amoeboid shaped microglia, indicative of activation, were observed in vehicle treated cultures alone, similar to that of the LPS treated cultures (Fig. 3.6A and C). Interestingly, the primary microglia that were pretreated with tBHQ had attenuated activation in both vehicle and LPS treated cultures, although some activation was observed (Fig. 3.6B and D). Additionally, neuronal apoptosis was observed by phase-contrast imaging of the bottom layer of neuronal-enriched cultures, which corresponded to the degree of microglia activation in the respective transwells. Neurons that were co-cultured with microglia treated with Vehicle or LPS, presented with cellular membrane blebbing, pyknotic nuclei and apoptotic bodies, which were attenuated in the presence of tBHQ (Fig. 3.6E-H).

**Figure 3.6 Activation of Mouse Primary Cortical Microglia Induces Neuronal Apoptosis that is Moderately Attenuated by Pretreatments with tBHQ**

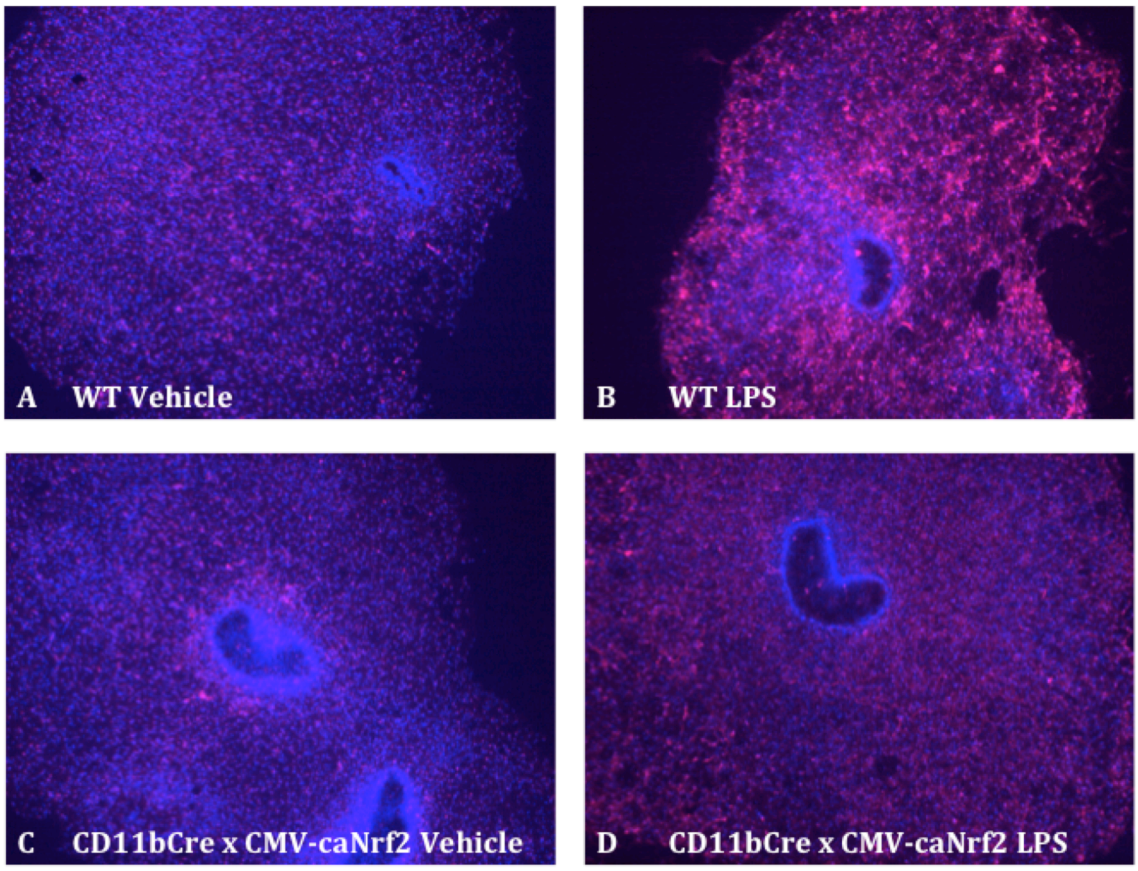
Primary mouse microglia were co-cultured with neurons using a transwell system. Microglia were grown in a transwell insert that was placed on top of primary cortical neurons. (*A,C*) Microglia were pretreated with vehicle for 24 hours followed by vehicle or 500ng/ml LPS for 24 hours; or (*B,D*) with 20 μM tBHQ for 24 hours followed by vehicle or LPS and subsequently IHC-stained for Iba-1 (green). (*E,G*) Phase-contrast images of primary cortical neurons that were co-cultured with microglia transwell inserts corresponding to (*A,C*). (*F,H*) Phase-contrast images of primary cortical neurons that were co-cultured with microglia transwell inserts corresponding to (*B,D*). Orange arrows indicate pyknotic nuclei. Yellow arrows indicate membrane blebbing. Green arrows indicate apoptotic bodies. Inserts in (*E,G*) are magnified images of boxed neurons that appear apoptotic. Inserts in (*F,H*) are magnified images of boxed neurons that appear healthy.



### 3.3.6 Organotypic Spinal Cord Slice Cultures from Microglia Over-expressing Nrf2 and Treated with LPS Have Markedly Decreased Activation

Based on the transwell co-culture experiments described above, it is apparent co-culturing microglia can be difficult due to their sensitivity and reactivity based on the growth matrices alone (vehicle treated co-cultures showed activated microglia in Fig. 3.6A). We investigated a strategy to avoid this phenomenon by using organotypic spinal cord slice cultures (slices of primary tissue grown in culture) from WT and CD11bCre xCMV-caNrtf2 mice. Organotypic cultures require less cellular manipulation and the cytoarchitecture and cellular components normally present in an *in vivo* environment are preserved. Perhaps, these conditions could provide a stable environment for the microglia and less basal activation. Spinal cord slices were chosen versus brain slices for experiments because the spinal cord is most affected in experimental autoimmune encephalomyelitis (EAE), a mouse model of MS, and this model presents with massive microgliosis. Organtotypic spinal cord slices were isolated and cultured on porous membranes. After two weeks in culture, tissues were treated with either vehicle or 30 μg/ml LPS for 72 hours. Immunohistochemical staining for Iba1 showed intense staining in the WT LPS treated slices. Whereas in the CD11b-Cre/CMV-caNrf2 LPS treated slices, there was a remarkable decrease in staining (Fig. 3.7A-D). This observation was noted in separate spinal cord cultures from three CD11b-Cre x CMV-caNrf2 mouse pups.

**Figure 3.7 Overexpression of Nrf2 Attenuates LPS-mediated Activation of Microglia in Organotypic Spinal Cord Slices.** Primary spinal cord slices from WT/WT or CD11b-Cre x CMVcaNrf2 mice were organotypically cultures on pourous membranes. *(A,C)* After 14 days, cultures were treated with vehicle; or *(B,D)* 30 µg/ml LPS for 72 hours. Cultures were stained with Iba1 (microglia in pink) or Hoescht 33258 (cell nuclei in blue).

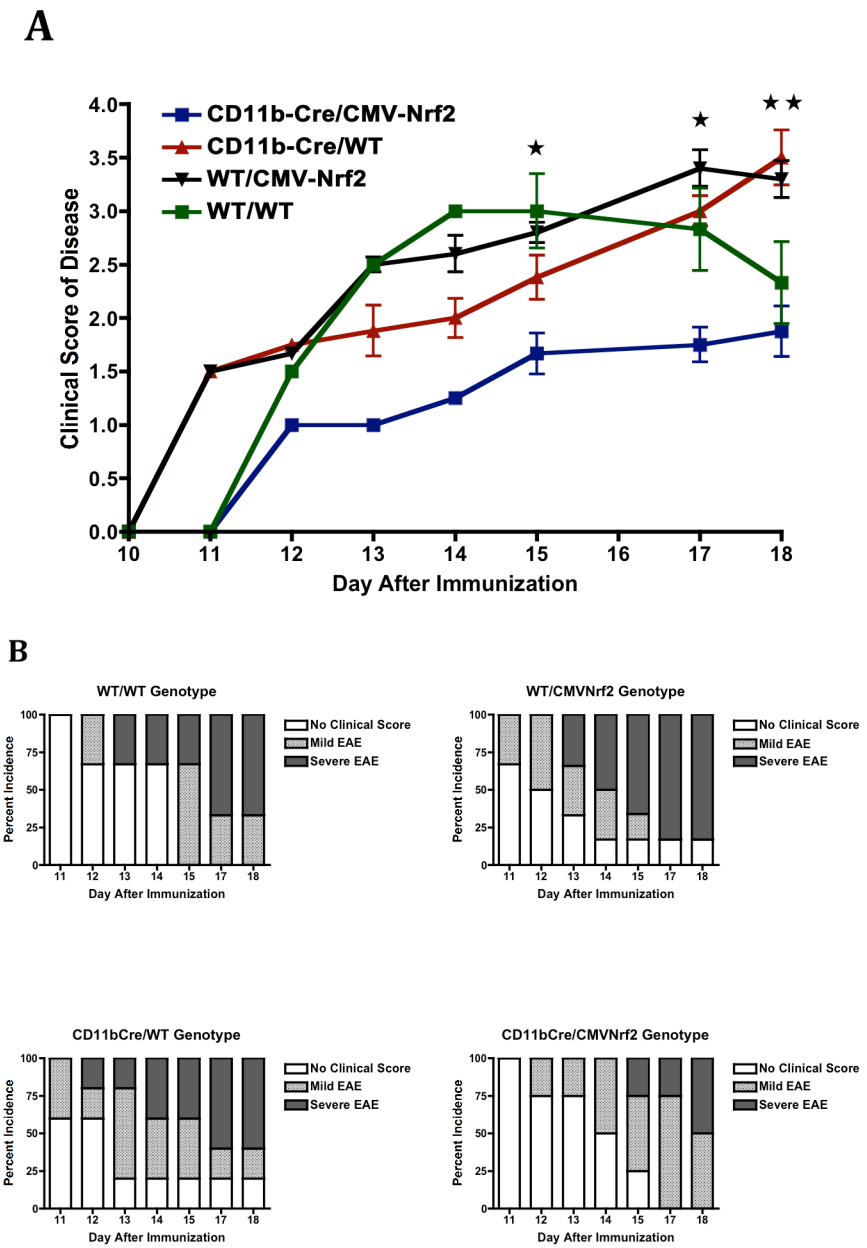


### 3.3.7 Mice with Experimental Autoimmune Encephalomyelitis Show Decreased Clinical Scores of Disease When Overexpressing Nrf2 in Microglia/Macrophages

Data from the organotypic spinal cord cultures described above were encouraging and lead us to investigate the impact of overexpression of Nrf2 in microglia on EAE. CD11b-Cre/CMV-Nrf2 and littermate control mice, WT/WT, CD11b-Cre/WT, WT/CMV-Nrf2, were immunized with MOG 35-55 to induce EAE, and clinical scores of disease were monitored. Overexpression of Nrf2 specifically in microglia/macrophages had a positive affect on clinical scores of disease. CD11b-Cre/CMV-Nrf2 mice displayed significantly lower disease scores 15, 17 and 18 days after immunization when compared with CD11b-Cre/WT and WT/CMV-caNrf2 littermate controls (Fig. 3.8A). In addition, CD11b-Cre/CMV-caNrf2 mice had significantly lower disease scores 15 and 17 days after immunization when compared to WT/WT mice (Fig. 3.8A). Of note, the WT/WT cohort had only three mice and this small number may not be statistically powerful enough to account for biological variation in these or any type of animal experiments. When analyzing percent incidence of mice with no clinical score, mild EAE (>0, ≤2.0) and severe EAE (>2.0), the percent incidence of severe EAE in the CD11b-Cre/ CMV-Nrf2 was less at all time points compared with all other mouse cohorts (Fig. 3.8B).

**Figure 3.8 Mice with Experimental Autoimmune Encephalomyelitis and Overexpressing Nrf2 Specifically in Microglia/Macrophages have Attenuated Clinical Scores of Disease**

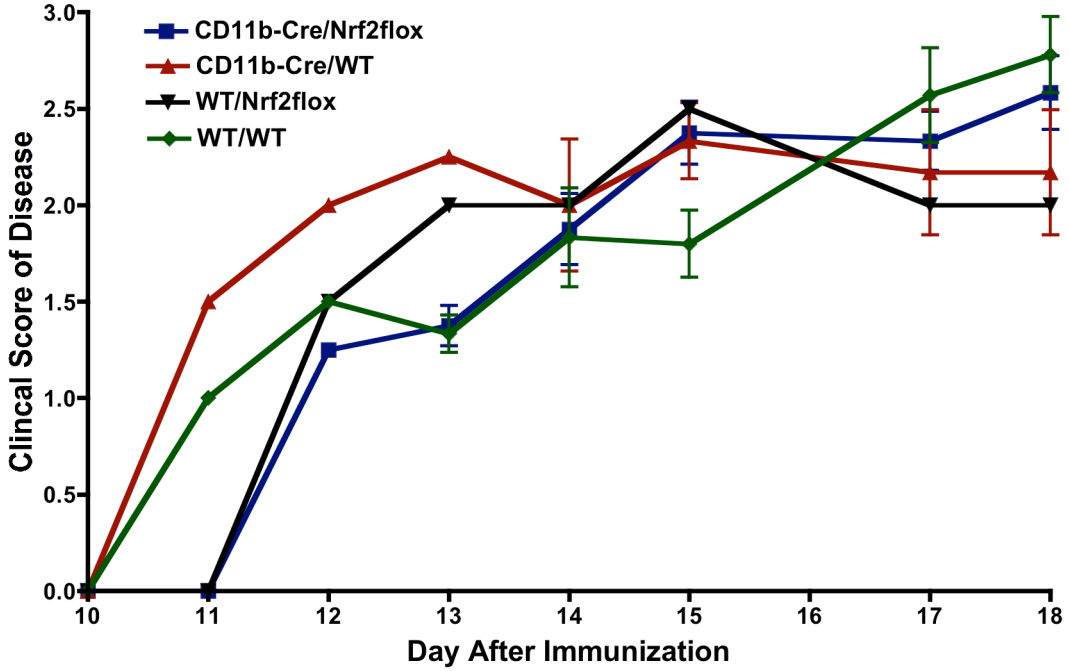
(*A*) WT/WT, CD11b-Cre/WT, WT/CMV-caNrf2 and CD11b-Cre/CMV-caNrf2 mice were immunized with 150 µg MOG 35-55 emulsified in Fruend's complete adjuvant (CFA). Clinical scores of disease were monitored as described in materials and methods. (*B*) Percent incidences corresponding to the severity of the disease were calculated as follows: 0 - No Clinical Score; 0.5 to 2.0 - Mild EAE; 2.5 or above- Severe EAE. ). Data in (*A*) are presented as mean ± SEM. ⋆ Significantly different than WT/WT (n=3), CD11b-CRE/WT (n=4), WT/CMV-caNrf2 control (n=5), ($P < 0.05$). ⋆⋆ Significantly different than CD11b-CRE/WT (n=4) and WT/CMV-caNrf2 control (n=5), ($P < 0.05$).



### 3.3.8 Nrf2-deficient Microglia in Mice with EAE Does Not Affect Clinical Scores of Disease

Since overexpression of Nrf2 in microglia appears to have a protective effect on mice with EAE, we wanted to investigate EAE in mice specifically lacking Nrf2 only microglia/macrophages. CD11b-Cre mice were crossed with Nrf2-flox knockout mice (as described in materials and methods). The double transgenic mouse, CD11b-Cre/Nrf2-flox$^{f+/f+}$, should specifically knock down Nrf2 in microglia/macrophages. These mice and their littermate controls, WT/WT, CD11b-Cre/WT, WT/Nrf2-flox $^{f+/f+}$, were immunized with 100 μg of MOG 35-55 emulsified in CFA and clinical scores were monitored. Interestingly, there were no differences in clinical scores amongst the mouse groups (Fig. 3.9).

**Figure 3.9 Clinical Scores of Disease are Similar in Mice with EAE and Deficient in Nrf2 in Microglia/Macrophages**
WT/WT, CD11b-Cre/WT, WT/Nrf2-flox and CD11b-Cre/Nrf2-flox mice were immunized with 100 µg MOG 35-55 emulsified in Fruend's complete adjuvant (CFA). Clinical scores of disease were monitored as described in materials and methods.




## 3.4 Discussion

Both exogenous and endogenous factors can trigger inflammatory responses in the CNS as well as cellular debris from neurotoxic insults. Activated microglia release a plethora of neurotoxic factors that include IL-1ß, TNF-α, NO, $NOO^-$ and $O_2^{\bullet -}$. Together these perpetuate a toxic cycle of injury to neurons. These studies demonstrated how cell-specific overexpression of Nrf2 in microglia attenuated

microglial activation by LPS and prevented neurotoxcity. Furthermore, transgenic mice overexpressing Nrf2 in microglia/macrophages were more resistant to inflammatory mediated EAE.

Many experiments studying activation of the Nrf2-ARE signaling pathway in microglia have used the immortalized cell line BV2 (Innamorato et al., 2008; Konwinski et al., 2004). Although these cells may have similar characteristics to microglia, they may not fully recapitulate microglia *de nova*. Furthermore, experiments that have used primary cortical microglia to study the activation of Nrf2, have cultured them in incubating conditions that consist of 5% $CO_2$ in air. Unfortunately, these experiments were performed using hyperoxic conditions as air consists of 21% $O_2$. Since these primary microglia may be already oxidatively stressed, it may be difficult to interpret the existing data (Min et al., 2008; Min et al., 2006). Our primary cortical microglia were cultured in 5% $CO_2$ and 5% $O_2$, conditions that better mimic *in vivo* gas levels. Similar to the aforementioned studies, we also observed Nrf2 activation in our primary microglia cultures treated with tBHQ as indicated by robust hPAP reporter gene activation. LPS-treated cultures however did not have increased hPAP activity and only weak change in cytochemical staining. The latter observations may be explained by the high concentration of LPS (10 μg/ml) used to treat the cultures resulting in borderline toxicity. In our past experience, primary neuronal or astrocytic cultures treated with experimental compounds that did not activate Nrf2 at low concentrations will

modestly do so at near toxic concentrations (data not shown). Interestingly, tBHQ treated primary microglia cultures appeared to consist of two populations of microglia; some cells stained positive for hPAP activity, while others did not suggesting differential activation of Nrf2 in these cell types. It is possible that pro- and anti-inflammatory microglial phenotypes may exist, similar to the M1 or classical, and M2 or alternative phenotypes described for subsets of macrophages (Kofler and Wiley, 2011; Murray and Wynn, 2011). In fact, Rojo *et. al.*, describe that in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) neurotoxin induced model of PD, Nrf2-KO mice had greater microgliosis with increased inflammatory markers for the classical pathway (M1) when compared to the WT. Conversely, the anti-inflammatory markers for the alterative pathway (M2) were decreased (Rojo et al., 2010). These observations warrant further investigation.

Likewise, we wanted to verify inflammatory gene expression in our mouse primary microglia cultures. Indeed our LPS treated cultures responded with robust gene expression for genes associated with inflammation. Treatments of microglia with tBHQ resulted in a slight but significant increase in both *Mac1* and *Phox67,* and a marked and significant decrease in *IL-6* gene expression. With regards to IL-6, a recent study by Hoetzennecker *et. al.* (2012) suggests that under oxidative conditions, Nrf2 strongly induces the activating transcription factor 3 (ATF3), which subsequently binds to the IL-6 promoter and suppresses its transcription. If this is

correct, we should see higher basal levels of IL-6 expression in our future experiments in the Nrf2-KO microglia.

Previous studies have indicated that treatments of microglia cultures with sulforaphane (SFN), dimethylfumarate (DMF), adenosine or methyl mercury can activate the Nrf2-ARE signaling pathway and/or upregulate anti-oxidant gene expression levels for genes such as *NQO1, HO-1, GCLM* and *GCLC* (Innamorato et al., 2008; Konwinski et al., 2004; Min et al., 2008; Ni et al., 2010; Wierinckx et al., 2005). In some experiments, pretreatment with SFN or DMF attenuated the LPS-mediated production and release of TNFα, IL-1ß, IL-6 and NO (Wierinckx et al., 2005), or inhibited oxidative stress as measured by the fluorescent substrate 2',7'dichlordihydrofluorescein diacetate ($H_2$DCFDA) (Innamorato et al., 2008; Min et al., 2008). We verified gene expression for *NQO1, GCLM* and *GCLC* using mouse primary cortical microglial cultures. Treatments of our microglia cultures with tBHQ significantly increased gene expression levels associated with prevention of oxidative stress, with the exception of *Nrf2*. LPS treated microglia cultures only increased gene expression for *NQO1.* The mechanism(s) for this reason remain to be determined. Since LPS activates NFκB, one could hypothesize that the NQO1 promoter contains an NFκB response element leading to its expression. Further studies using primary cortical microglia cultures also need to be performed to analyze for markers of activation and assess the Nrf2-mediated attenuation of microglia using WT and Nrf2-KO cultures.

Increased NQO1 protein levels in tBHQ treated microglia cultures corresponded to *NQO1* gene expression levels. Lack of NQO1 protein in tBHQ treated Nrf2-KO microglia cultures verified Nrf2-dependent activation of this gene and protein expression. Interestingly, LPS treatments did not increase protein expression of NQO1 as would be expected as indicated by increased *NQO1* gene expression. In fact, it suppressed NQO1-expression by tBHQ. As discussed earlier, this observation may be explained by the presence or polarization of the microglia to a classical (M1) phenotype, thereby shutting down the Nrf2-ARE signaling pathway. Alternatively, this may contribute to the increase in NQO1 mRNA as the cell attempts to compensate for the loss of NQO1 protein by increasing gene expression. Future experiments with CD11b-Cre/CMV-Nrf2, WT and Nrf2-KO primary microglia cultures treated with LPS will aid in understanding this phenomenon.

Based on the glutathione data as well as increased gene expression levels of anti-oxidant genes from tBHQ treated primary microglia cultures, we evaluated microglial activation of Nrf2 for prevention of neurotoxicity. The first attempts at co-culturing neurons atop microglia proved futile as described earlier (Fig. 3.4). Since growing microglia on transwell membranes lead to their activation without treatment, this technique was also not suitable for subsequent experiments. These data do, however, suggest that Nrf2 activation in activated microglia returns them to a partial resting phenotype. Previous studies have focused on pretreatment of

cellswith Nrf2 activators to prevent microglial activation. This inadvertent and novel observation implies that Nrf2 blocks both microglial activation and also reverses microglia activation. More detailed experiments will evaluate this observation in the future.

Organotypic spinal cord slices are an ideal *in vitro* system for recapitulating *in vivo* cell types and cellular architecture, and require less manipulation that may disrupt and activate microglia. LPS treated WT microglia spinal cord slices showed robust activation as demonstrated by Iba1 staining. LPS treated spinal cord slices from CD11b-Cre/CMV-Nrf2 mice, however, did not stain to the same extent, supporting the previous observation that activation or overexpression of Nrf2 can attenuate the activation of microglia.

Mouse models of PD, epilepsy, and MS exhibited enhanced microgliosis in Nrf2-KO mice than in WT (Chen et al., 2009; Johnson et al., 2010; Kraft et al., 2006; Rojo et al., 2010). In addition, Innamorato *et. al.* demonstrated that LPS-mediated neuroinflammation was more severe in the hippocampus of the Nrf2-KO mice, as indicated by increased F4/80 (microglial marker), iNOS, IL-6 and TNF-$\alpha$ protein levels, when compared to WT littermate controls. They later treated WT mice with SFN, an activator of the Nrf2-ARE pathway, and observed increased heme oxygenase-1, a reduced presence of microglia in the hippocampus and attenuation of iNOS, IL-6 and TNF-$\alpha$. Whilst these observations appear to be mediated by the

activation of Nrf2, additional experiments using Nrf2-KO mice under these same conditions should be performed. In addition, it should be noted that in other studies, rats fed SFN had increased activation of Nrf2 in the endothelial cells and tight junction proteins, both of which contribute to the blood brain barrier (BBB) (Noyan-Ashraf et al., 2005; Zhao et al., 2007). It is possible then, that the attenuated inflammation observed after LPS treatments in SFN treated mice, may be a result of fewer macrophages infiltrating the BBB. Lastly, it would also be important to measure the amount of neuronal loss in these mice to determine if the attenuated inflammation also prevented neurotoxicity.

We previously demonstrated that the global knockout of Nrf2 exacerbated experimental autoimmune encephalomyelitis resulting in more severe microgliosis (Johnson et al., 2010). We speculate this results from increased oxidative stress perpetuating inflammation and subsequent neurotoxicity. In this study we induced EAE in CD11b-Cre/CMV-Nrf2 mice and their single transgenic and WT littermate controls, to see if overexpression of Nrf2 in microglia/macrophages could impact the severity of the disease. Indeed, cell specific overexpression of Nrf2 in microglia/macrophages significantly decreased the clinical scores compared to littermate controls. Future experiments will determine the pathological, cellular and molecular events that are involved in protection from EAE by overexpression of Nrf2 in microglia. Conversely, no differences were observed between the clinical scores in WT mice and mice with Nrf2-deficient microglia/macrophages with EAE.

These findings, together with previous studies demonstrating that global KO of Nrf2 (all cell types lack Nrf2) exacerbates this disease, suggests that the presence of Nrf2 in other cell types must also be important. One caveat to these experiments is that the cre recombination of the floxed Nrf2 gene may not be of efficiency. Thus cellular levels of Nrf2 may not be reduced enough in the microglia/macrophages to modify the inflammatory response in EAE compared to WT mice. We have designed a PCR technique to determine the level of floxed alleles relative to non-floxed alleles. However, this cannot be done on tissue since all the other cells would have the non-floxed allele. Thus, the extent of floxing will be determined in primary microglial cultures.

Neuroinflammation is present in many neurodegenerative diseases resulting in enhanced neurotoxicity. Endogenous or exogenous factors may compound and contribute to the self-perpetuating cycle of inflammation and subsequent neuronal toxicity. One therapeutic intervention would be to activate the Nrf2-ARE signaling pathway in microglia boosting the anti-oxidant capacity of these cells and maintain microglia in a resting but basally functional state. The *in vitro* studies presented here support the hypothesis that activation or overexpression of Nrf2 in microglia can attenuate microglia activation and prevent neurotoxicity. In addition, the overexpression of Nrf2 in microglia/macrophages attenuated EAE, albeit Nrf2-defiency in microglia had no effect. This therapeutic approach may thus have future

potential in alleviating damaging from inflammation in diseases such as PD, ALS, MS and epilepsy, and thus contribute to neuronal protection.

## 3.5 Experimental Procedures

### 3.5.1 Animals

All animals were treated and cared for in accordance with and approval of the University of Wisconsin's Institutional Animal Care and Use Committee (IACUC). In addition, all efforts were afforded to assure minimal numbers of mice used in each experiment and to minimize or prevent any discomfort the mice may have experienced.

*ARE-hPAP*

Are-hPAP transgenic reporter mice were generated by genomic random insertion of 51 base pairs of the rat NQO1 promoter region coupled to the human placental alkaline phosphatase reporter gene ((Johnson et al., 2002).

*CD11b-Cre*

CD11b-Cre transgenic mice were purchased from the European Mouse Mutant Archive. Briefly, these mice were generated by Boillee S *e.t al. al*. (Boillee et al., 2006) by cloning a 1.7 kb DNA fragment from the human CD11b-Cre promoter with 1.1 kb of the Cre-recombinase gene with a nuclear localization site and a trailing poly-

adenylation signal from the human growth hormone gene. The gene fragment was linearized and microinjection into fertilized F1:CBAxC57BL/6 eggs.

*Nrf2-flox*

Dr. Shyam Biswal, Johns Hopkins University, Department of Environmental Health Science kindly provided the Nrf2-flox mice. Nrf2-flox mice were generated by homologous recombination of the Nrf2 DNA binding domain (Exon 5) with a DNA fragment containing *loxP*-sites flanking Exon 5 (Reddy et al., 2011). Heterozygous mice (f/w) were crossed to generate mice homozygous for the Nrf2-flox gene (f/f), which we refer to as Nrf2-flox mice.

*CMV-caNrf2*

Dr. Sabine Werner, Department of Biology, Institute of Cell Biology, ETH Zurich generously provided the CMV-caNrf2 mice. Mice were generated by Schäfer *et. al*. (Schafer et al., 2010), inserting a transgene containing a mutated Nrf2 Neh2 domain preventing Nrf2 from binding to Keap1 (caNrf2). Upstream (5') to the caNrf2 cDNA are 50 base pairs of a CMV enhancer sequence, the *B-actin* promoter and a STOP cassette that is flanked by *loxP* sites in sequential order. In addition, downstream (3') to the caNrf2 lies an internal ribosomal entry site (IRES) and the eGFP cDNA gene. When the STOP cassette is "floxed out" by the enzyme Cre-recombinase, the caNrf2 gene is constitutively active (Fig. .

### 3.5.2 Primary Cell Cultures

*Primary Microglia Cultures*

Primary microglia cultures were obtained from postnatal day one (PND1) mice following a high-yield isolation protocol described by Saura *et. al.,* with slight modifications (Saura et al., 2003). Cerebral cortices were stripped of meninges, minced in DMEM:F12 (cellgro® by Mediatech, Inc.) containing 10% heat-inactivated FBS (Atlanta Biologicals, Inc.) and Pen (100 IU/mL)/ Strep (100 µg/mL) which we refer to as CDMEM:F12. Minced tissue was pelleted by centrifugation (500 g for 5'). The tissue pellet was resuspended in 10 ml of 0.25% trypsin containing 1mM EDTA in HBSS (Gibco®, Life Technologies) and incubated for 10 minutes at 37 °C. Tissue was again centrifuged and rinsed with CDMEM:F12. The repelleted tissue was resuspended in 4ml of CDMEM:F12, gently triterated to a single cell suspension and filtered through a 50 µm sterile cell strainer, and rinsed with an additional 4 ml of CDMEM:F12. Dependent on experimental design, cells were plated at 62,500 cells/$cm^2$ in CDMEM:F12 on 96-well or 6-well tissue cultures dishes, 0.02 µm Nunc Anapore membrane inserts, or 8-well Permanox™ Lab-Tek™ Chamber Slides for at least 15 days (at least 3-days after cells reach confluency) and incubated in a tri-gas humidified incubator set at 37 °C with 10% $O_2$ and 5% $CO_2$ in air. Full media changes occurred every 4-5 days. After 15 days, media was removed and saved, and cells were washed with DMEM:F12 (to eliminate serum) and incubated with 0.25% trypsin (in 1mM EDTA): DMEM:F12 at a ratio of 1:3 at 37 °C until the upper layer of cells detached (typically 25-60 minutes). The remaining attached cells consisted of

microglia and were cultured with half of the saved media from above and half fresh CDMEM:F12. Half media changes were conducted every 2-3 days. If the density of the microglia was deemed low, 200 ng/ml M-CSF was added to media for continued proliferation of the microglia cells. After robust microglia growth, a full media change was conducted (to remove M-CSF). Microglia were then rested for 2-3 days before using for experiments.

*Primary Cortical Cultures*

Primary cortical cultures were isolated as previously described (Johnson et al., 2002; Kraft et al., 2004). Briefly, cortical tissue was isolated from E15-16 mouse embryos and meninges removed. Tissue was minced into 1-2 $mm^2$ pieces in ice-cold HBSS, pelleted, and resuspended in 0.05% trypsin followed by incubation in a 37 °C shaking water bath. Tissue was triterated in complete EMEM, pelleted, and resuspended in Neuralbasal media supplemented with B27 (NBM-B27), and seeded at a density of 270,000 cells/$cm^2$ on poly-d-lysine coated 6-well plates or alternatively 100,000 and 200,000 cells plated directly on top of confluent microglia grown in 6-well dishes.

*Coculture Experiments*

Primary cortical microglia and neuronally-enriched cultures were grown as described above. In addition, neurons were seeded at 100,000 and 200,000 cells onto confluent microglia cultures grown on 8-well Permanox™ Lab-Tek™ Chamber

Slides. After 48 hours, cultures were treated with vehicle ($ddH_2O$) or 10 μg/ml LPS (Sigma-Aldrich, L2262, *Salmonella enterica* serotype typhimurium) and the following day fixed with 4% paraformaldehyde (PFA) in PBS for 20 minutes. Slides were immunohistochemically stained as described below. For transwell insert coculture experiments, primary cortical microglia were cultured on 0.02 μm Nunc Anapore membrane inserts until near confluency. After which, transwells were transferred to confluent neuronally-enriched in 6-well plates. Fresh NBM-B27 was added to microglial and neuronally-enriched cultures. Microglia were pretreated with vehicle (0.05% ethanol in $ddH_2O$) or 20 μM tBHQ for 24-hours. Media was changed to NBM-B27 without antioxidants and microglia treated subsequently treated with vehicle ($ddH_2O$) or 500 ng/ml LPS. Phase contrast images were obtained of the neuronal cultures and the transwell inserts were fixed in 4% PFA for 20 minutes. The transwell inserts were immmuno-histochemically stained as described below.

*Organotypic Spinal Cord Cultures*

Organotypic spinal cored cultures were prepared as described by Corse and Rothstein (Corse and Rothstein, 1995) with slight modifications. Spinal cords were carefully dissected from WT/WT, CD11b-Cre/WT, WT/Nrf2-flox and CD11b-Cre/Nrf2-flox individual pups PND1 and placed in Gey's balanced salt solution (GBSS) supplemented with 6.4 mg/ml glucose. Spinal cords were transversely cut into 350 μm slices using a McIlwain tissue chopper and returned to the GBSS with

glucose. Slices were then transferred to Millicell culture inserts (Millipore, PICM 03050) with 0.4 μm membrane pore size and bathed with culture media on the basolateral side of the membrane. The culture media consisted of 50% DMEM with glucose and glutamine (cellgro® by Mediatech, Inc., 10-012-CV), 25% HBSS, 25% heat inactivated horse serum (Atlanta Biologicals, Inc.) and Pen (100 IU/mL)/ Strep (100 μg/mL). Cultures were allowed a 14-day recovery period prior to experimentation. On day 14, cultures were treated with vehicle (dd$H_2O$) or 30 μg/ml LPS for 72 hours. This dosing scheme was based on previous experiments by Li *et. al.* (Li et al., 2008). After 72-hours, spinal cord slices were fixed with 4% PFA in PBS for 1.5 hours and immunohistochemically stained as described below.

### 3.5.3 hPAP Assay and Cytochemistry

Whole-cell extracts were prepared by lysing cells in 96-well plates. hPAP levels were quantified by measuring alkaline phosphatase activity. Briefly, cells were lysed in a Tris-5 mM magnesium saline solution containing 1% (wt/vol) CHAPS (TMNC buffer, Sigma-Aldrich). Extracts were incubated at 65°C for 30 min to heat inactivate endogenous alkaline phosphatase activity, then incubated at room temperature for 10 minutes with CSPD® chemiluminescent substrate for alkaline phosphatase and Emerald-II™ enhancer (Applied Biosystems). The resulting luminescent signal represents relative hPAP activity. To visualize hPAP activity, fixed cells (4% (wt/vol) paraformaldehyde for 20 min) were incubated in a 50 mM Tris and 5 mM magnesium saline solution (TMN), pH 10, at 65°C for 15 min. to heat

inactivate any endogenous alkaline phosphates activity. Cells were then stained for hPAP by replacing the TMN with a staining solution containing 1 mg/mL of NBT (nitro-blue tetrazolium chloride) and 1 mg/mL BCIP (5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt) (Pierce-Thermo Fisher Scientific) and incubated at 37°C for 30–45 min.

### 3.5.4 qPCR

Primary mouse microglia cultures were lysed in Trizol reagent (Invitrogen, Inc.) and total RNA extracted following the manufacturer's instructions. Integrity and concentrations of RNA were determined using the RNA 6000 Nano chip and analyzed by an Agilent 2100 Bioanalyzer (Agilent Technologies, Foster City, CA). Only samples that carried an RNA Integrity Number (RIN) of 8.0 or greater were used for further analysis. RNA (1 ug) was reverse-transcribed (RT) using an Oligo-dT 15 primer in accordance with the Reverse Transcription System (Promega Corporation, Madison, WI). PCR-amplification and quantification (qPCR) of resulting cDNA was performed in real time using the LightCycler® 480 System (Roche Applied Science) with Cycler 480 SYBR Green I Master Mix (Roche Applied Science) following manufacturer's instructions. All PCR product quantification was subject to relative standard curves that yielded amplification efficiencies greater than 1.79 and less than 2.19, and with error less than 0.2. Primers used for the qPCR analysis are listed in the following table.

| Gene of Interest | Forward Primer (5'-3') | Reverse Primer (5'-3') |
|---|---|---|
| **Cellular Markers** | | |
| *Iba1* | GGA TTT GCA GGG AGG AAA AG | TGG GAT CAT CGA GGA ATT G |
| *Mac-1* | | |
| **Inflammatory Enzymes** | | |
| *iNOS* | CAG GAG GAG AGA GAT CCG ATT TA | GCA TTA GCA TGG AAG CAA AGA |
| *Phox-67* | CAG CCA GCT TCG GAA CAT G | GAC AGT ACC AGG ATT ACA TC |
| **Cytokines** | | |
| *IL-6* | GCT ACC AAA CTG GAT ATA ATC AGG A | CCA GGT AGC TAT GGT ACT CCA GAA |
| **Anti-Oxidants** | | |
| *Nrf2* | TTC TTT CAG CAG CAT CCT CTC CAC | ACA GCC TTC AAT AGT CCC GTC GAG |
| *GCLC* | ACA TCT ACC ACG CAG TCA AGG ACC | CTC AAG AAC ATC GCC TCC ATT CAG |
| *GCLM* | GCC ACC AGA TTT GAC TGC CTT TG | TGC TCT TCA CGA TGA CCG AGT ACC |
| *NQO1* | GCG AGA AGA GCC CTG ATT GTA CTG | TCT CAA ACC AGC CTT TCA GAA TGG |

### 3.5.5 Western Blot Analysis

Protein samples were resolved on 12% sodium dodecyl sulfate–polyacrylamide gel and transferred to Hybond-P membrane (Amersham, Pittsburgh, PA). Membranes were blocked for 1h in Tris-buffered saline, 0.1% Tween-20 and 5% non-fat dry milk, followed by a 5 hour incubation with primary antibody (rabbit polyclonal anti-NQO1, abcam®, ab34173) diluted in the same buffer. After washing with 0.1% Tween in Tris-buffered saline, the membrane was incubated with peroxidase-conjugated secondary antibody (Amersham) for 1h, and then washed and developed using the ECL chemiluminescent detection system (Pierce). Densitometric analyses were performed using Photoshop and normalized against the signal obtained by reprobing the membranes with anti-actin (Sigma-Aldrich).

### 3.5.6 GSH/GSSG Measurements by HPLC

Reduced (GSH) and oxidized (GSSG) glutathione was measured by normal-phase (ion exchange) HPLC (Fariss and Reed, 1987; Vargas et al., 2011). Briefly, mouse primary microglial cultures were lysed in 3% perchloric acid (PCA) followed by centrifugation at 10,000 g for 10 minutes at 4 °C. Supernatant was removed and stored at -80 °C for future HPLC analysis. In addition, pellets were saved for determination of protein concentration. Supernatants were alkylated with iodoacetic acid derivatized with 1-flouro-2,4, dinitrobenzene (DNB). The S-carboxymethyl-N-dinitrophenyl-derivitized samples were then loaded onto a 3-aminopropyl-Spherisorb column (Waters Corporation, Milford, MA) and separated by a sodium acetate/methanol gradient using a Shimadzu Prominence HPLC system. Resolved GSH- and GSSG-analyte was detected at 365 nm using a Shimadzu Prominence SPD-20A UV/Vis detector. GSH and GSSG concentrations in the samples were determined by reference GSH and GSSG standard curves included in each run and subsequently normalized to respective tissue protein concentrations.

### 3.5.7 Immunocytochemistry/Immunohistochemistry

All primary cellular and organotypic cultures were blocked with buffer containing 0.4 % Triton X-100, 10% goat serum or horse serum, and 0.5% bovine serum albumin in PBS for one hour. Cultures were incubated with primary antibodies diluted in blocking solution overnight at 4°C. Primary antibodies consisted of Iba-1 at a 1:500 dilution (Wako Pure Chemical Industries, LTD.) and MAP-2 at a 1:500

dilution (Chemicon-Millipore™). Secondary staining of sections was performed using the appropriate antibodies conjugated to Alexafluor® 488 and 647 (Molecular Probes, Inc. Eugene, OR) that were diluted in blocking solution (1:250) and incubated for 1 h at room temperature. Hoescht 33258 (5 μg/ml, SIGMA, St. Louis, MO) was added to the secondary antibody solutions to stain cell nuclei. Cultures were washed with PBS and mounted with Fluoro-Gel (EMS). Control staining was performed using appropriate IgG antibodies in place of the primary antibody. Sections were visualized using a Zeiss epifluorescent microscope (Carl Zeiss, Germany) and photographic images of immunofluorescence captured using AxioVision 4 software (Carl Zeiss, Germany).

### 3.5.8 Induction of EAE and Clinical Scoring

Experimental autoimmune encephalomyelitis (EAE) was induced in eight- to sixteen-week old male and female respective transgenic mice and WT littermate controls by subcutaneously injecting 100 or 150 μg of MOG 35-55 peptide emulsified in complete Freund's adjuvant including *Mycobacterium tuberculosis* (Hooke Kit™ EK-0112, $MOG_{35-55}$/CFA Emulsion PTX 2.5X, Hooke Laboratories, Inc.) into the scapular region. This was concomitant with intraperitoneal (i.p.) injections of 2.5X formulation of pertussis toxin on days 0 and 2. Experimental control mice received the CFA only and pertussis toxin (Hooke Control Kit™ CK-0112, Hooke Laboratories, Inc.). EAE was monitored daily for clinical scores of disease until completion of experiment using the following scoring system: 1-complete tail atony;

2-hindlimb weakness; 3- hindlimb paralysis; 4-complete hind limb paralysis and front limb weakness and 5- moribund. If mice with EAE were deemed to be within two scorings, a 0.5 value was added to the lower of the clinical score. It is noted that all animals were treated and cared for in accordance with and approval of the University of Wisconsin's Institutional Animal Care and Use Committee (IACUC).

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# CHAPTER 4

## Conclusions and Future Directions

## 4.1 Conclusions and Closing Remarks

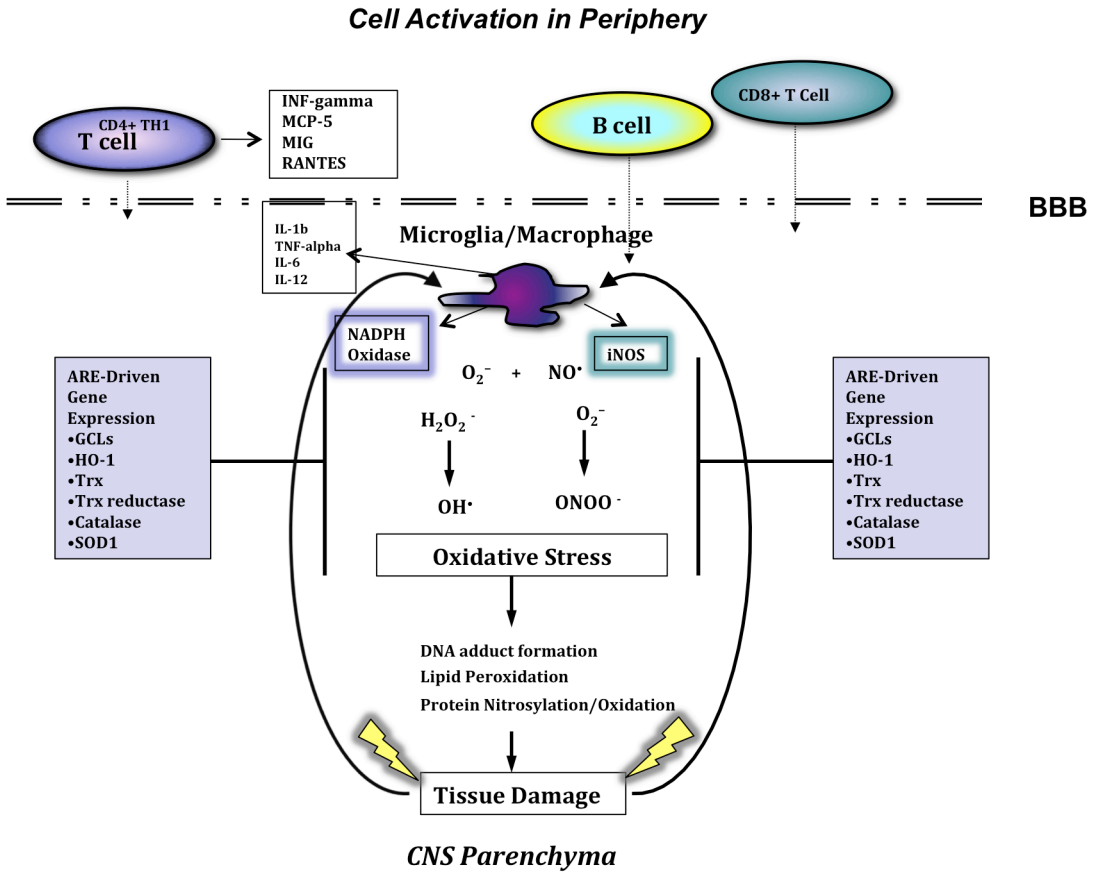
Based on the studies presented here, it is clear that the transcription factor Nrf2 is important in the modulation of inflammation and subsequent demyelination in a mouse model of the autoimmune disease MS. In addition, specific overexpression of Nrf2 in microglia attenuated microglia activation and associated neurotoxicity. Indeed, Nrf2 activation in microglia may thus be a viable therapeutic target in the alleviation of the damaging effects of inflammation in diseases such as PD, ALS, MS and epilepsy.

*Chapter 2*

In the EAE studies described in Chapter 2, we witnessed an exacerbation of disease in mice deficient in Nrf2, based on clinical scores, pathology and increased inflammatory markers and astro- and microgliosis (refer to Fig. 2.1A-C). Our data are consistent with previous studies researching the role of Nrf2 in modulation of inflammation in models outside the CNS. When LPS was injected i.p. or after cecal ligation/ puncture, a more dramatic rate of mortality in Nrf2-KO versus WT mice was observed. Septic Nrf2-KO mice had serum TNF-alpha levels that were significantly greater than WT mice, suggesting that mice lacking Nrf2 had a more severe innate immune response to the bacteria or endotoxin (Thimmulappa et al., 2006). In nonlethal inflammatory studies, lungs from Nrf2-KO or WT mice were instilled with LPS or carrageenan, or mice had i.p. injections of LPS or TNF-alpha. In all cases, Nrf2-KO mice had a greater influx of inflammatory cells from

bronchoaleveolor lavage fluids (Itoh et al., 2004; Thimmulappa et al., 2007). Based on these data, it is clear that Nrf2 modulates and inflammatory response. However, questions still remain as to what cell or cell types are important for the protection. Because EAE studies were performed in mice that had global knock out of Nrf2, it is possible that cells from the peripheral immune system, as well cells in the CNS, may all be affected by the absence of Nrf2. For instance, peripheral T cells and B cells may be more reactive and/or have greater clonal expansion capacity due to increased cell damage in the CNS. Neuronal cells and oligodendrocytes in the CNS may be more vulnerable to ROS and RNS since their antioxidant capacity is reduced. Boosting the overall anti-oxidant capacity in the CNS may certainly have protective effects on all cell types as well as quench the destructive toxins released from reactive microglia (Fig. 4.1).

**Figure 4.1 Activation of the Nrf2-ARE Signaling Pathway in the CNS and Protection from Oxidative Stress in MS.** In MS, autoreactive T-cells such as CD4+ Th1, CD8+ CTLs and B-cells are activated in the peripheral lymph system, migrate to and infiltrate the BBB. Microglia/macrophages of the innate immune response are subsequently activated and the ROS and RNS generated are cytotoxic to neurons and oligodendrocytes resulting in tissue damage. Cellular debris from damaged tissue can activate microglia resulting in a perpetual cycle of reactive microgliosis. Increased expression of antioxidants and antioxidant enzymes via activation of the Nrf2-ARE signaling pathway can restore cellular redox homeostasis and detoxify ROS and RNS thus protecting cells in the CNS in an autocrine or paracrine manner.



Based on the greater inflammatory response in Nrf2-KO mice with EAE, we hypothesize that lack of Nrf2 in the microglia/macrophage may be a predominant factor in modulating inflammation in this disease model (refer to figure Fig. 2.4A-B).

*Chapter 3*

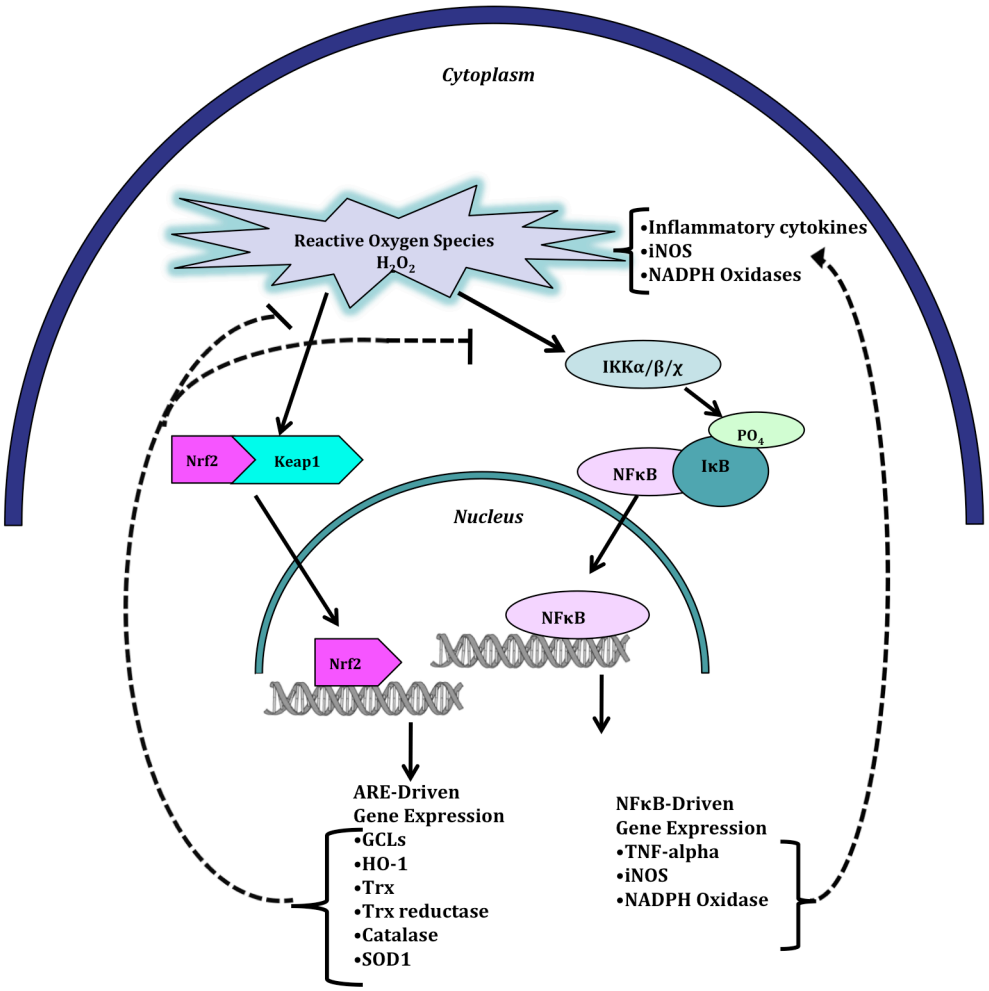
To better understand the role of Nrf2 in neuroinflammation these studies explored the Nrf2-ARE signaling pathway. Experiments were performed using primary cortical microglia cultures, organotypic spinal cord slices, and EAE. Cell specific overexpression of Nrf2 or treatment with tBHQ, an Nrf2 activator, attenuated LPS-mediated microglial activation in both primary cortical microglial cultures and spinal cord slices (Refer to Fig. 3.4A-D).

In addition, neurotoxicity was reduced in primary cortical neurons co-cultured with Nrf2-activated microglia. EAE induced in mice overexpressing Nrf2 in microglia/macrophages had significantly reduced clinical scores when compared to WT mice. Contrary to these findings, there were no differences in clinical scores between in mice specifically deficient in Nrf2 in microglia/macrophages and WT mice with EAE. Clearly overexpression of Nrf2 in microglia/macrophages results in attenuation of reactive microgliosis.

Based on this data presented in Chapter 3, we theorize the activation of Nrf2 can prevent overactivation of microglia, restore cellular redox homeostasis and

potentially revert the cells to resting and basal functioning stages (Fig. 4.2). Nrf2 activation can drive ARE-driven genes increasing intracellular anti-oxidant capacity in the microglia subsequently blocking the NFκB signaling pathway shutting down inflammatory gene expression and the perpetual cycle of inflammation. We were quite excited about the unexpected observation that the tBHQ treated microglia either attenuated activation or reverted the reactive microglia back to resting stages (recall that the of microglia plated in transwells became activated themselves).

**Figure 4.2 Activation of Nrf2 Restores Cellular Redox Homeostasis and Reverts Reactive Microglia/Macrophage Back to Resting Stages.** Intracellular ROS and RNS act as second messengers and can activate kinase cascades and transcription factors such as NFκB that drive inflammatory gene expression. However, persistent stress can deplete the cell's antioxidant defense system and amplification of the inflammatory response can have deleterious effects on neurons and oligodendrocytes. Activation of the Nrf2-ARE pathway amplifies antioxidants and antioxidant enzymes restoring cellular redox homeostasis and reverting the microglia/macrophage back to resting and basal functioning stages. Note-NFκB is sequestered in the cytoplasm by an inhibitor protein, IκB. Upon phosphorylation by IκB kinases (Iκκα/β/χ), IκB releases NFκB through proteolytic mechanisms. NFκB then translocates to the nucleus driving the expression of inflammatory genes.



## 4.2 Future Directions

### 4.2.1 Assessment of Oxidative Damage, Pathology and Inflammatory Markers in Transgenic Mice with EAE

To fully understand the role of overexpression of Nrf2 in microglia and attenuation of the EAE in the CD11b-Cre/CMV-Nrf2 mice, further experiments will be conducted. These will include:

1) Assessment of biological and molecular markers of oxidative/nitrosative stress in spinal tissue and slices. These markers will include immunohistochemical and western blot analysis for nitrotyrosines and 4-hydroxynonenol.
2) Spinal cord slices will be stained with luxol fast blue (LFB) for the assessment of demyelination. In addition, slices will be stained with H&E for analysis of peripheral cell infiltration. Silver staining will also be conducted to assess loss of neuronal axons. Motor neurons will counted to assess neurotoxicity
3) Markers of inflammation will be measured by qPCR as described in Chapter 2.
4) Inflammatory cytokines will be measured in serum and tissue by using western blot analysis and Elisa.

### 4.2.2 Cell Specific Pattern of Expression of ARE Activity *in vivo*

CD11b-Cre/CMV-Nrf2 mice will be crossed onto the ARE-hPAP reporter mice to generate triple transgenic mice. This will allow us to determine the cell specific patterns of expression of Nrf2 both basally and in mice with EAE.

### 4.2.3 Cell Flow Analysis of Peripheral Immune Cells in CD11b-Cre/CMV-Nrf2 and WT Mice with EAE.

CD11b is expressed in both microglia and macrophages (Kettenmann et al., 2011). Hence peripheral macrophages in CD11b-Cre/CMV-Nrf2 mice will also have increased expression of Nrf2 and reduced reactivity. Because of this, it is also possible that T cells and B cells in the peripheral system may be less reactive. Cell flow analysis will determine the population densities and reactive cell types between the double transgenic and WT mice with EAE.

### 4.2.4 Microglia cultures of CD11b-Cre/Nrf2-flox$^{f+/f+}$ to evaluate recombination efficiency

Based on the lack of differences in response to EAE between the CD11b-Cre/Nrf2-flox$^{f+/f+}$, transgenic littermate controls and WT mice, microglia will be cultured from these mice to determine the extent and efficiency of recombination of the Nrf2-floxed allele. Although the data would suggest that Nrf2 in other cell types must also be important, another caveat could be the efficiency/extent of "floxing out" Nrf2. The level of Nrf2 may not be reduced enough in the microglia/macrophages to

modify EAE. We have designed a PCR technique to determine the level of floxed alleles relative to non-floxed alleles. However, this cannot be done on tissue since all the other cells would have the non-floxed allele. Thus, the extent of floxing will be determined in primary microglial cultures. In addition, the loss of Nrf2 would attenuate tBHQ-mediated induction of Nrf2-driven genes. Thus, the lack of or reduced induction of NQO1, GCLM and GCLC will be evaluated.

### 4.2.5 Role of IL-6 in microglia-and microglia phenotypes

Treatments of microglia with tBHQ showed a marked and significant decrease in *IL-6* gene expression. With regards to IL-6, a recent study by Hoetzennecker *et. al.* (2012*)*, suggests that under oxidative conditions, Nrf2 strongly induces the activating transcription factor 3 (ATF3), which subsequently binds to the IL-6 promoter suppressing its transcription (Hoetzenecker et al., 2012). If this is accurate, we should see higher basal levels of IL-6 expression in our future experiments in the Nrf2-KO microglia. Microglia will be cultured from Nrf2-KO, WT and CD11b-Cre/CMV-Nrf2 mice to confirm these data and determine if lack of Nrf2 activated *IL-6* gene expression.

### 4.2.6 Nrf2 Overexpression in Microglia and Other Models of Neurodegenerative Disease

Our laboratory has evaluated the role of Nrf2 in astrocytes and how these astrocytes affect models of neurodegenerative disease (PD, AD, ALS, HD). However reactive microgliosis is present and implicated in the pathology and progression of many neurodegenerative diseases as well as in injuries to the CNS (spinal cord injury, cerebral artery occlusion and MS). Based on the data generated in this thesis, future experiments using transgenic mice overexpressing Nrf2 in microglia/macrophages will be used to evaluate if Nrf2 can prevent or attenuate reactive microgliosis and subsequent neurodegeneration in mouse models of PD, AD, and ALS.

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