Published in final edited form as:
Prog Neurobiol. 2013 January ; 100: 30–47. doi:10.1016/j.pneurobio.2012.09.003.

Emerging Roles of Nrf2 and Phase II Antioxidant Enzymes in Neuroprotection

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Abstract

Phase II metabolic enzymes are a battery of critical proteins that detoxify xenobiotics by increasing their hydrophilicity and enhancing their disposal. These enzymes have long been studied for their preventative and protective effects against mutagens and carcinogens and for their regulation via the Keap1 (Kelch-like ECH associated protein 1) / Nrf2 (Nuclear factor erythroid 2 related factor 2) / ARE (antioxidant response elements) pathway. Recently, a series of studies have reported the altered expression of phase II genes in postmortem tissue of patients with various neurological diseases. These observations hint at a role for phase II enzymes in the evolution of such conditions. Furthermore, promising findings reveal that overexpression of phase II genes, either by genetic or chemical approaches, confers neuroprotection *in vitro* and *in vivo*. Therefore, there is a need to summarize the current literature on phase II genes in the central nervous system (CNS). This should help guide future studies on phase II genes as therapeutic targets in neurological diseases. In this review, we first briefly introduce the concept of phase I, II and III enzymes, with a special focus on phase II enzymes. We then discuss their expression regulation, their inducers and executors. Following this background, we expand our discussion to the neuroprotective effects of phase II enzymes and the potential application of Nrf2 inducers to the treatment of neurological diseases.

Keywords

phase II genes; Keap1/Nrf2/ARE; inducers; effectors; acute neurological diseases; neurodegenerative diseases

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1 Introduction

Once they enter cells or tissues, drugs or xenobiotics induce a series of compensatory cellular reactions. The purpose of these endogenous reactions is to reduce the potential injury caused by these compounds via their metabolism and excretion. These procedures occur in a stepwise fashion and are mediated by a group of enzymes known as drug metabolizing enzymes (DMEs). Based on the sequential nature of catalysis, DEMs are categorized into three groups, the phase I, II, and III enzymes, respectively. Each group of the DMEs serves distinct roles. Briefly, phase I enzymes oxidize drugs or xenobiotics, while phase II enzymes conjugate products of phase I reactions. In contrast, phase III enzymes transport or extrude the final metabolites out of cells. The distribution and functions of phase I, II, and III enzymes are summarized in Table 1 (Nakata *et al.*, 2006; Xu *et al.*, 2005).

The expression of phase I genes is governed by a number of nuclear receptors, including aryl hydrocarbon receptors (AhR) and orphan nuclear receptors such as constitutive androstane receptors and pregnane X receptors. Each of these receptors binds a particular consensus sequence on its target genes (Xu *et al.*, 2005). For instance, AhR regulates its target genes mainly by interacting with the xenobiotic response element (XRE). The major regulator of phase II genes is nuclear factor erythroid 2 related factor 2 (Nrf2), which binds the antioxidant response elements (ARE) consensus (Itoh *et al.*, 1997). Transcriptional regulation of phase III genes is not fully understood. On the other hand, there is some overlap between the expression regulation of phase I, II, and III enzymes. For example, 3-methylcholanthrene, a carcinogen, induces not only phase I genes, but also phase II and phase III genes (Rushmore and Kong, 2002). One explanation is that the intermediate products of phase I enzymes serve as potential inducers of Nrf2 pathway/phase II enzymes. Another study suggests that DME genes share some common cis-acting elements or trans-acting factors. For instance, AhR can bind both XRE and ARE consensus sequences (Kohle and Bock, 2009). The regulation of phase I, II, and III genes is generally complicated and beyond the scope of this review. Herein, we will mainly focus on phase II enzymes and the Nrf2 pathway, as it is the main regulator of phase II genes (Jancova *et al.*, 2010).

2 Regulation of Nrf2/ARE pathway

The Nrf2/ARE pathway is a major determinant of phase II gene induction. The importance of Nrf2 is evident from reports showing that the levels of phase II genes, such as glutathione *s*-transferase (GST) and NAD(P)H: quinone oxidoreductase 1 (NQO1), are significantly reduced in Nrf2-deficient mice and that the induction of phase II genes is abolished by Nrf2 disruption (Ramos-Gomez *et al.*, 2001). It has long been known that Keap1 is a negative regulator of the Nrf2/ARE pathway by the formation of a Keap1/Nrf2 complex in the cytoplasm. This complex sequesters Nrf2 away from the nucleus and prevents its downstream effects on phase II genes. However, recent studies also implicate Keap1-independent means of regulating Nrf2/ARE. Therefore, we will discuss both Keap1-dependent and -independent Nrf2/ARE pathways.

2.1 Keap1 dependent Nrf2 pathway

2.1.1 Structural features of Keap1/ Nrf2 in the cytoplasm—Nrf2 belongs to the basic leucine zipper transcription factor family, which also contains NF-E2, Nrf1, Nrf3, Bach1, and Bach2 (Motohashi *et al.*, 2002). Nrf2 is composed of six functional domains known as Nrf2-ECH homologies (Neh) and designated as Neh1-6, respectively. Each Neh domain serves its own function and the details have been well summarized by Baird and colleagues (Baird and Dinkova-Kostova, 2011).

Keap1 (Kelch-like ECH associated protein 1) is composed of three functional domains: a bric-a-brac (BTB) domain, an intervening region (IVR), and a Kelch domain (also named DGR domain). Keap1 forms a homodimer and each dimer binds one molecule of Nrf2 via its two Kelch domains, with one weak affinity binding site (DLG motif, residues 24-31, latch) and one high affinity binding site (ETGF motif, residues 78-82, hinge). Both motifs are located in the Neh2 domain of Nrf2 (Tong *et al.*, 2007) (Figure 1). The ETGF motif ($K_D \approx 1000$ nmol/L) has a higher affinity for Keap1 than the DLG motif ($K_D \approx 5.3$ nmol/L) (Tong *et al.*, 2006). This is the so-called "hinge-and-latch" model. The Keap1-Nrf2 complex is linked to a functional E3 ubiquitin ligase complex (Rbx1) via an adaptor protein, Cullin3. Conjugating Nrf2 with Keap1 by the two DLG and ETGF motifs aligns the seven lysine residues of Nrf2 between the two motifs and facilitates Rbx1 mediated ubiquitination of Nrf2 (McMahon *et al.*, 2006). Ubiquitinated Nrf2 is subsequently degraded by the 26S proteasome. As a consequence, the binding of Keap1 homodimers to Nrf2 can be considered inhibitory to Nrf2 function because it effectively facilitates Nrf2 degradation.

2.1.2 Dissociation of Nrf2-Keap1 complex—One widely accepted mechanism of Nrf2/ARE activation involves the dissociation of the Nrf2-Keap1 complex in the cytoplasm. Endowed with high reactive cysteine residues, Keap1 can modulate Nrf2 dependent gene expression by serving as a sensor of various chemical signals. Specifically, two cysteine residues (Cys273, Cys288) in the IVR domain are essential for Keap1 to bind and suppress the activity of Nrf2, whereas C151 in the BTB domain exerts the opposite effect (Yamamoto *et al.*, 2008). The oxidation of these cysteine residues affects the conformation of Keap1 and thereby initiates the dissociation of Keap1 from the DLG motif of Nrf2. In this way, oxidative products enhance the stability of Nrf2 and increase the expression of phase II genes (McMahon *et al.*, 2006).

2.1.3 Nuclear translocation of Nrf2 and activation of Nrf2 pathway—As a transcription factor, it is essential that Nrf2 translocates to the nucleus in order to transactivate. Given its critical role in sensing stress, it is not surprising that this translocation is quite rapid. Indeed, Nrf2 accumulates in the nucleus within 15 min after *tert*-Butylhydroquinone (t-BHQ) treatment (Jain *et al.*, 2005). However, it is still not fully clear how Nrf2 translocates to nucleus. The key mediators that regulate nuclear import and export of transcription factors are the nuclear localization signals (NLS) and nuclear export sequences (NES). These sequences interact with the nuclear pore complex. A number of such nuclear shuttling signals have been identified on Nrf2, including three NLS motifs, named NLS1, NLS2 and NLS3, and two NES motifs, named NES1, NES2 and NES3 based on their location (Figure 2). In murine Nrf2, NLS1 contain residues 42–53 (RQKDYELEKQKK) in the Neh2 domain (Theodore *et al.*, 2008), NLS2 contains residues 494-511 (RRRGKQKVAANQCRKRK) in the Neh6 domain (Jain *et al.*, 2005) and NLS3 contains residues 587–593 (PKSKKPD) in the Neh3 domain (Theodore *et al.*, 2008). On the nuclear export side, NES1 contains residues 175-186 (LLSIPELQCLNI) in the Neh5 domain, which is redox sensitive as it has a cysteine residue (Cys183) (Li *et al.*, 2006), and NES2 contains residues 545-554 (LKRRLSTLYL) in Neh1 (Jain *et al.*, 2005). This may correspond to residues 537-546 (LKKQLSTLYL) in human Nrf2 (Li *et al.*, 2005).

The direction of Nrf2 movement seems to be determined by a homeostatic balance between import and export driving forces. It has been proposed that redox sensitive NES1 may play an important role in this equilibrium (Li and Kong, 2009; Li *et al.*, 2006). Under normal conditions, the import force is less than the export force while NES1 is functional, and Nrf2 stays in the cytosol; under oxidative or electrophilic stressors, Cys183 of NES1 is adducted and NES1 becomes inoperative. This import force then overwhelms the export force, leading to Nef2 nuclear translocation (Li and Kong, 2009). Additionally, Nrf2 phosphorylation may also contribute to Nrf2 nuclear transportation. Nrf2 is the substrate of

several protein kinases, including protein kinase C (PKC) (Huang *et al.*, 2002; Numazawa *et al.*, 2003), phosphatidylinositol 3-kinase (PI3K) (Lee *et al.*, 2001), glycogen synthase kinase-3 (GSK3! ! (Rada *et al.*, 2012), casein kinase 2 (CK2) (Apopa *et al.*, 2008), extracellular signal-regulated kinase (ERK) (Yu *et al.*, 1999), p38 (Yu *et al.*, 2000), PKR-like endoplasmic reticulum kinase (PERK) (Cullinan *et al.*, 2003) and Fyn (Jain and Jaiswal, 2006). Among them, the phosphorylation sites of PKC, GSK3! and Fyn have been identified. PKC phosphorylates Ser40 in Neh2 domain (Huang *et al.*, 2002) but its role in Nrf2 nuclear shuttling is controversial. It has been reported that the nuclear translocation of a Nrf2 S40A mutant protein was decreased compared to wild-type protein after electrophilic stress, indicating a role for Ser40 phosphorylation in nuclear import (Numazawa *et al.*, 2003). Another report showed that Ser40 was necessary for the release of Nrf2 from Keap1, although it was not required for Nrf2 accumulation in the nucleus (Bloom and Jaiswal, 2003). GSK3β phosphorylates Nrf2 at Ser342 and Ser347, mediating Nrf2 degradation via another E3 ligase, beta-transducin repeats-containing protein (TrCP) (Rada *et al.*, 2012). Fyn phosphorylates Tyr568 in the Neh3 domain, faciliating nuclear export of Nrf2 (Jain and Jaiswal, 2006; Rada *et al.*, 2011). The activity of Fyn itself is controlled by GSK3β (Jain and Jaiswal, 2007; Rojo *et al.*, 2008), suggesting an important role of GSK3β in the regulation of Nrf2 nuclear shuttling.

In summary, the Cul3-Keap1 complex sequesters Nrf2 in the cytoplasm and facilitates the ubiquitination and proteasomal degradation of Nrf2 under quiescent conditions (Kobayashi *et al.*, 2004). Indeed, the half-life of Nrf2 in non-stressed, physiological conditions is only about 20 min (Itoh *et al.*, 2003). When exposed to oxidants or electrophiles, Nrf2 is rapidly liberated from the Cul3-Keap1 complex and translocated into the nucleus, where it forms a heterodimer with a small musculo-aponeurotic fibrosarcoma (Maf) protein through its Neh1domain and subsequently binds ARE (Itoh *et al.*, 1997) (Figure 1). ARE is a *cis*-acting DNA response sequence located in the regulatory regions of phase II genes with the consensus of TGAG/CNNNGC (N represents any base) (Nguyen *et al.*, 2003). In this way, oxidants or electrophiles activate Nrf2 and up-regulate phase II genes in order to compensate against their damaging effects (Baird and Dinkova-Kostova, 2011).

2.2 Keap1 independent Nrf2 pathway

Recent studies have also proposed a Keap1 independent ubiquitination model of Nrf2 degradation (Rada *et al.*, 2011; Rojo *et al.*, 2012). In this model, GSK- 3β phosphorylates the Neh6 domain of Nrf2 at Ser342 and 347 (Rada *et al.*, 2012); phosphorylated Neh6 on Nrf2 can be recognized by and act as a bait for β-TrCP, an E3 ubiquitin ligase. β-TrCP is a scaffolding protein that directly links Nrf2 to the Cullin1/Rbx1 ubiquitination complex. Therefore, GSK-3β mediated phosphorylation of Neh6 causes the ubiquitination and degradation of Nrf2 via β-TrCP in place of Keap1. This model is supported by the stabilization of Nrf2 by GSK-3β inhibitors in Keap1 -/- mouse embryo fibroblasts (MEFs) (Rada *et al.*, 2011). Additionally, cancer-chemopreventive agent nordihydroguaiaretic acid can activate Nrf2 and increase HO-1 protein levels through inhibiting GSK-3β phosphorylation in Keap1-/- MEFs (Rojo *et al.*, 2012). However, this pathway needs to be better examined, as current studies are only limited to cultured cell lines. Efforts should be extended to physiological and pathological animal models to further evaluate this regulatory mechanism.

2.3 Inactivation of Nrf2 pathway

When challenged with oxidative products, the activated Nrf2/ARE pathway boosts the expression of phase II genes either by Keap1 dependent or independent means. Under these circumstances, cells also initiate endogenous regulatory mechanisms to quench this pathway to prevent excessive activation. Several contributors participate in this negative feedback

loop. First, AREs are located in the promoter region of Cul3, Rbx1 and Keap1 genes. Studies show that these inhibitory proteins are subject to transcriptional regulation by Nrf2. In other words, activation of Nrf2 enhances the expression of Rbx1-Cul3-Keap1 complex, which acts to sequester Nrf2 and mediate its rapid degradation (Kaspar and Jaiswal, 2011; Lee *et al.*, 2007). This negative feedback loop is known as an autoregulatory arm of the Nrf2/ARE pathway. Second, prothymosinα (ProTα), a Keap1 binding protein with a nuclear localization signal, can mediate the nuclear import of the Keap1-Cul3-Rbx1 complex. As a result, 10-15% of Keap1-Cul3-Rbx1 complex is localized in the nucleus. Once it enters the nucleus, the Keap1-Cul3-Rbx1 complex releases ProTα and binds Nrf2, leading to the ubiquitination and degradation of nuclear Nrf2 (Niture and Jaiswal, 2009). Third, oxidative stressors such as hydrogen peroxide promote the activation of GSK-3β by phosphorylating its tyrosine 216 residue. Activated GSK-3β subsequently phosphorylates Fyn (p-Fyn, a member of Src family) at a threonine residue(s), leading to the accumulation of p-Fyn in the nucleus and subsequent phosphorylation of Nrf2 at tyrosine 568. Ultimately, phosphorylated Nrf2 interacts with Crm1 or exportin 1 and is exported out of nucleus (Jain and Jaiswal, 2006, 2007) (Figure 1C). These compensatory factors all complement each other and guide the cell back towards homeostasis.

2.4 Proteins that directly regulate Nrf2/ARE signaling

Multiple proteins are involved in the regulation of the Nrf2/ARE signaling pathway. Some proteins exert their functions by directly modifying the Keap1-Nrf2 complex in the cytoplasm, others function in the nucleus. One example of cytosolic regulation involves $p21^{Cip1/WAF1}$, an essential protein that protects cells from oxidative stress. Recent findings suggest that $p21^{Cip1/WAF1}$ directly binds the DLG motif of Nrf2 through its C-terminal KRR motif. The DLG motif is also the binding site of Keap1. The competition between $p21^{Cip1/WAF1}$ and Keap1 for Nrf2 binding compromises Keap1 mediated ubiquitination of Nrf2 (Chen *et al.*, 2009b). This has been confirmed in p21 deficient mice, which demonstrate reduced expression of Nrf2 and Nrf2 target genes (Chen *et al.*, 2009b; Toledano, 2009). Separately from $p21^{Cip1/WAF1}$, another protein sequestosome-1(also known as p62) binds and inactivates Keap1 and thus also augments the expression of genes regulated by Nrf2 (Komatsu *et al.*, 2010). Specifically, the Ser349 of sequestosome-1 is the key residue in regulating binding activity of sequestosome-1 to Keap1 (Hancock *et al.*, 2011).

Other proteins affect Nrf2 activity in the nucleus. For instance, BACH1 has been identified as a repressor of Nrf2. Under basal conditions, BACH1 forms a heterodimer with the Maf protein and this complex subsequently occupies the ARE sequences and negatively regulates several phase II genes such as heme oxygenase 1 (HO-1). When cells are challenged by oxidative insults, BACH1 is phosphorylated and exported to the cytoplasm. Free Maf protein then forms a heterodimer with Nrf2 and triggers the expression of downstream genes (Dhakshinamoorthy *et al.*, 2005; Sun *et al.*, 2004).

2.5 Kinases involved in modulating Nrf2 transcriptional activity

Phosphorylation is another key mechanism that regulates Nrf2 dependent gene expression, and as discussed above, several protein kinases can phosphorylate Nrf2 (Figure 2). As will be discussed below, some kinases such as PKC and PI3K increase Nrf2/ARE transcription; other kinases such as the mitogen-activated protein kinase (MAPK) family play different roles in Nrf2/ARE activation depending on cell type.

It has been shown that the PKC activator phorbol 12-myristate 13-acetate (PMA) increases Nrf2 phosphorylation as well as ARE transcriptional activity, whereas the PKC inhibitor staurosporine down-regulates these effects in HepG2 cells (Huang *et al.*, 2000). PKC

phosphorylates Nrf2 at Ser40. Although this does not affect Nrf2 binding with ARE, it promotes its dissociation from Keap1 (Huang *et al.*, 2002). This phosphorylated residue is located in the Neh2 domain of Nrf2, which is critical for the interaction with Keap1. As a result, it has been suggested that a conformational change of Nrf2 secondary to its phosphorylation may lead to its dissociation from Keap1 (Bloom and Jaiswal, 2003). In addition to PKC, studies attempting to address the relationship between PI3K signaling and the Nrf2 pathway showed that PI3K lies upstream of Nrf2 and positively regulates its transcriptional activity in IMR-32 human neuroblastoma cells (Lee *et al.*, 2001) and primary cortical cultures (Kraft *et al.*, 2004), although the detailed mechanisms are not yet fully understood.

Because the MEK inhibitor PD98059 impairs ARE-dependent phase II gene expression in HepG2 cells, ERKs are also thought to upregulate phase II genes (Yu *et al.*, 1999). On the other hand, modulation of ERK1/2 activity does not affect Nrf2-dependent ARE activation in IMR-32 human neuroblastoma cells (Lee *et al.*, 2001). These discrepancies may be explained by the different cell types used in those studies. Another MAPK, p38, demonstrates opposite effects on Nrf2 activation depending on the model (Correa *et al.*, 2011). Although it functions as a negative regulator in human hepatoma HepG2 and murine hepatoma Hepa1c1c7 cells (Yu *et al.*, 2000), it acts as a positive regulator in MCF-7 mammary epithelial cells (Alam *et al.*, 2000). MAPK seems to regulate Nrf2-dependent gene transcription by post-translational modification of CBP (CREB-binding protein) and/or other unidentified transacting factors or co-activators that bind to the Nrf2 transcription machinery, in addition to directly phosphorylating Nrf2 (Shen *et al.*, 2004).

In aggregate, ubiquitination of Nrf2 either by Keap1 dependent or independent means is a fundamental mechanism to inhibit Nrf2. Activation of Nrf2 is initiated by the dissociation of Nrf2 from Keap1, preventing its ubiquitination, and causing its translocation into the nucleus. Through its exquisitely coordinated control by multiple kinases and proteins, Nrf2 ultimately binds with ARE and triggers phase II gene expression.

3 Inducers

As will be discussed below, the upregulation of phase II enzymes is protective against several models of neurological diseases. Therefore, it is important to identify and classify inducers of the Nrf2 pathway for subsequent translation of these compounds to the clinic. As described above, Keap1 is the major inhibitor of Nrf2 activation; variation in the structure of Keap1 or/and dissociation of Keap1 from Nrf2 results in the release and activation of Nrf2 (Figure 1 and 3A). This is the target mechanism of nearly all known Nrf2 inducers and might involve modulating the cysteine residues of Keap1 or blocking their binding sites.

The inducers of phase II enzymes can be classified into three ways based on their origin, chemical structure, and the reaction cascades that they ignite. Based on their origin, inducers can be divided into two classes, exogenous and endogenous. Exogenous inducers include xenobiotics, drugs, and heavy metals; endogenous inducers include lipid peroxidation products, nitric oxide and derivatives as well as prostaglandins and derivatives (Kobayashi *et al.*, 2009; McMahon *et al.*, 2010; Yu *et al.*, 2006). The second method to classify the inducers is based on chemical structure. Holtzclaw and co-workers divide them into 10 groups, which include oxidizable phenols, Michael reaction acceptors, isothiocyanates, thiocarbamates, trivalent arsenicals, 1,2-dithiole-3-thiones, hydroperoxides, vicinal dimercaptans, heavy metals, and polyenes (Holtzclaw *et al.*, 2004).

Based on their effects on the Keap1-Nrf2 complex, Nrf2 inducers have also been divided into 6 classes as suggested by Kobayashi and colleagues (Kobayashi *et al.*, 2009). This is a useful way to group Nrf2 inducers as it combines both structural and mechanistic aspects.

However, with advances in this field, this taxonomy can be further improved. We propose a four-class categorization, based on the Keap1 domains that the inducers react with (Table 2).

3.1 Inducers that act on the BTB domain

The BTB domain of Keap1 plays two important roles. It is thought to serve as a dimerization domain, maintaining the dimer structure of Keap1 because mutation of Ser140 in BTB leads to the dedimerization of Keap1 and subsequent release of Nrf2 (Zipper and Mulcahy, 2002). Furthermore, the BTB domain is also the binding site for Cul3 (Eggler *et al.*, 2009).

A critical amino acid residue of Keap1 is Cys151. As a nucleophile, Cys151 is sensitive to many inducers and is therefore considered to be a stress sensor (McMahon *et al.*, 2010; Zhang and Hannink, 2003). Modulation of Cys151 leads to structural changes in Keap1, separating it from Cul3 (Eggler *et al.*, 2009). As a result, Nrf2 ubiquitination is prevented, and isolated Nrf2 is then free to translocate to the nucleus and induce phase II genes (Figure 3B).

Inducers that act on the BTB domain include tBHQ, 1,2-dithiole-3-thiones (D3T), Michael reaction acceptors such as 4-HNE and ebselen, oxidizable phenols such diethylmaleate (DEM) and naphthoquinone (1,2-NQ), and isothiocyanates such as sulforaphane (SFN) (Kobayashi *et al.*, 2009; McMahon *et al.*, 2010). Additionally, some small molecular inducers, such as H_2O_2, NO, and HOCl, all demonstrate C151-dependent inhibition of Keap1 (Fourquet *et al.*, 2010). Co^{2+} can replace Zn^{2+} in the BTB domain and also reconfigure Keap1 (Dinkova-Kostova *et al.*, 2005). Dihydro-CDDO-trifluoroethyl amide (CDDO-TFEA), a newly identified potent inducer, also belongs to this group (Ichikawa *et al.*, 2009).

3.2 Inducers that act on the IVR domain

The IVR domain is enriched with cysteines. It is therefore a potential site for modulation by inducers. Two cysteines, Cys273 and Cys288 are indispensable for Keap1 activity (McMahon *et al.*, 2010; Yamamoto *et al.*, 2008; Zhang and Hannink, 2003). Oxidation of these cysteines changes the structure of Keap1 and reduces its affinity for Nrf2 (Wakabayashi *et al.*, 2004) (Figure 3C). Additionally, Keap1 structural changes caused by oxidization of these two cysteines may also dissociate Cul3 from Keap1, as the N-terminal of the IVR domain is also a Cul3 binding site (Kobayashi *et al.*, 2006) (Figure 3D).

Most inducers in this group are Michael reaction acceptors, including 5d-PGJ2, PGA2 (Kobayashi *et al.*, 2004), and alkenals such as 4-HNE and acrolein (McMahon *et al.*, 2010). They represent two kinds of endogenous inducers and all of them are derived from polyunsaturated fatty acids.

3.3 Inducers that act on the DGR domain

The DGR domain is composed of 6 Kelch repeats. It binds the Neh2 domain of Nrf2 and also associates with cytoskeletal actin. Keap1 with a mutated DGR domain fails to bind either Nrf2 or actin (Kang *et al.*, 2004).

Inducers in this group interrupt the binding of Nrf2 to Keap1. Two members, both of which are proteins, have been identified. One is p62 (Komatsu *et al.*, 2010) and another is p21 (Chen *et al.*, 2009b). P62 binds the DGR motif of Keap1, leading to the separation of Nrf2 and Keap1 (Komatsu *et al.*, 2010). Eight amino acids of the DGR motif, especially Y334, S363, N382, and S602, are important for the interaction of Keap1 with p62 (Komatsu *et al.*, 2010). Mechanistically, p21 competes with Keap1 to directly bind the DLG and ETGE motifs of Nrf2 (Chen *et al.*, 2009b). Additionally, some kinases can also be considered

inducers in this group. For example, MAPK, PI3K, PKC can phosphorylate Nrf2 amino acids and change its conformation to prevent the association with Keap1 (Taguchi *et al.*, 2011) (Figure 3). It is not surprising that the PI3K and MAP kinase families of proteins lie upstream of Nrf2 activation in some models, because these proteins are highly sensitive to cellular stress and often determine the balance of pro-survival or apoptotic signaling cascades. By collaring Nrf2 and phase II enzymes into the cellular response to stress, these proteins can help determine cellular fate under conditions of injury.

3.4 Inducers acting on multiple domains

Heavy metal such as Hg^{2+}, Cd^{2+}, Zn^{2+}, As^{3+}, and Se^{4+} can also activate Nrf2 (McMahon *et al.*, 2010; Prestera *et al.*, 1993). A recent study showed that at least three amino acids from two domains are simultaneously necessary to sense the presence of heavy metals. These include the His225/Cys226 dyad from the IVR domain and Cys613 from the DGR domain (McMahon *et al.*, 2010).

To summarize, Nrf2 inducers mainly target Keap1 or target its binding with Nrf2. They can be divided into 4 groups based on the Keap1 domains that they react with. However, this taxonomy is not perfect because some inducers can be classified into more than one group, such as 4-HNE and 15d-PGJ2 (Eggler *et al.*, 2009; McMahon *et al.*, 2010). Thus, the classification of inducers needs to be further explored.

4 Effectors

Currently, over two hundred Nrf2/ARE driven genes are exploited for detoxification and antioxidant defense. Obviously, a description of the roles of each of these effectors is beyond the scope of this review. Phase II genes initially caught the interest of scientists for their preventative action against carcinogens (Zhang *et al.*, 1992). Furthermore, their function has recently been expanded to neuroprotection (Kraft *et al.*, 2006; Shih *et al.*, 2005). Below, we will focus our discussion on the phase II enzymes that participate in neuroprotection against models of common neurological diseases.

4.1 Thioredoxin enzyme system

Thioredoxin (Trx) enzyme systems are fundamental guards in defense against oxidative stressors. They reduce disulfide bridges of various proteins and remove H_2O_2 or peroxides by utilizing NADPH as the electron donor (Patenaude *et al.*, 2005). This system is mainly comprised of Trx, Trx reductase (TrxR), peroxiredoxin (Prx), and sulfiredoxin (Srxn). The central core of this system is Trx, while other members act to keep Trx functional. The graphic depiction of this cascade is illustrated in Figure 4. Molecules subjected to regulation by Nrf2 are highlighted in red.

4.1.1 Thioredoxins(Trxs)—Trxs are 12-kDa enzymes initially reported in 1964 as hydrogen donors for ribonucleotide reductase in Escherichia coli (Laurent *et al.*, 1964). Subsequent research demonstrated that mammalian Trxs contain a conserved Cys-X-X-Cys motif in their active center, and established their role in reducing oxidized proteins via the exchange between cysteine thiol and protein disulfides (Powis and Montfort, 2001). Currently, two isoforms of Trxs have been identified – Trx1 and Trx2. Trx1 localizes to the cytosol and Trx2 localizes to the inner membrane of mitochondria (Rybnikova *et al.*, 2000). Both of them are widely distributed in the central nervous system, including piriform cortex, the dentate gyrus, the CA3/CA4 region of the hippocampal formation, the locus coeruleus, as well as the paraventricular hypothalamic nucleus and the nucleus of the solitary tract in rat brain (Lippoldt *et al.*, 1995). This distribution pattern is probably related to the high metabolic activity of these brain regions and consequent oxidative stress.

Evidence shows that the induction of Trxs by heme or tBHQ is dependent on the activation of Nrf2/ARE in cultured cells (Kim *et al.*, 2003; Nakaso *et al.*, 2003). Trx1 is subject to the regulation of other transcriptional elements such as SP1, GCF, AP-1 (Masutani *et al.*, 1996). In contrast, the transcriptional regulation of Trx2 is remains to be elucidated.

Trxs have been shown to confer neuroprotection *in vitro* and *in vivo*. Trxs in the submicromolar range prevent the apoptosis of neuronal SH-SY5Y cells invoked by serum deprivation or by MPP^+(Andoh *et al.*, 2002; Chen *et al.*, 2006). Overexpression of either Trx1 or Trx2 significantly reduces retinal ganglion cell death in rat models of glaucoma (Caprioli *et al.*, 2009; Munemasa *et al.*, 2009). Transgenic mice over-expressing Trxs have attenuated focal ischemic brain damage (Takagi *et al.*, 1999). Promising findings of translational relevance reveal that intravenously infused human Trx (rhTrx) permeates the blood-brain barrier in the ischemic hemisphere and exerts neuroprotective effects in the mouse middle cerebral artery occlusion (MCAO) model (Hattori *et al.*, 2004).

In addition to their direct roles in attenuating oxidative damage, Trxs also perform several other biological functions which may contribute to their neuroprotective effects. For example, reduced Trxs form a complex with apoptosis signal-regulating kinase 1 (ASK1) and negatively regulate the ASK1/JNK/p38 apoptotic pathway (Hu *et al.*, 2011). Trx1 can be translocated into the nucleus and enhance the biosynthesis of Mn-superoxide dismutase (Mn-SOD) and mitochondrial anti-apoptotic Bcl-2 (Andoh *et al.*, 2002). Moreover, recent data demonstrated that Trx1 and Trx2 are able to facilitate ischemia-induced angiogenesis, which may promote recovery from ischemic brain injury by increasing reperfusion (Dunn *et al.*, 2010; Takagi *et al.*, 2011).

4.1.2 Thioredoxin reductases (TrxRs)—TrxRs are a group of selenocysteine-containing enzymes that reduce oxidized Trxs by consuming NADPH (Mustacich and Powis, 2000; Patenaude *et al.*, 2005) (Figure 4). A remarkable upregulation of Trxs and TrxRs is detected in the rat retina following exposure to bright-cyclic-light-reared stimulation. This is coupled to increased nuclear translocation of Nrf2 and binding with the ARE sequence. Conversely, TrxR-deficient animals showed increased accumulation of nuclear Nrf2 protein, which may be due to a compensatory antioxidative effect (Suvorova *et al.*, 2009). Furthermore, Nrf2 dependent expression of Trxs and TrxRs is enhanced by sublethal doses of HNE (5uM), which protect 661W cells (Tanito *et al.*, 2007). Collectively, these studies support the concept that TrxRs are subject to regulation by Nrf2/ARE and are indispensable in neuroprotection.

4.1.3 Peroxiredoxins (Prxs)—Prxs, also known as thioredoxin peroxidases, catalyze the reduction of peroxides by utilizing Trxs (Figure 4). Six isoforms of Prxs (Prx 1-6) exist in the CNS and can be classified into two groups: 2-Cys Prxs (Prx1-Prx5) and 1-Cys Prx (Prx1) (Rhee and Woo, 2011). Nrf2 was identified as a critical transcription factor for induction of Prx 6 in primary murine bone marrow-derived macrophages (Erttmann *et al.*, 2011) as well as in a human lung-derived cell line (Chowdhury *et al.*, 2009). Similarly, induction of Prx1 is also subject to Nrf2 regulation in other models (Ishii *et al.*, 2000). However, Nrf2 has not been implicated in the induction of the other isoforms. Several reports suggest that Prxs are highly neuroprotective. For example, recent studies showed that enhancement of Prx1 expression by icariin confers neuroprotection against H_2O_2 in primary cortical neurons (Zhang *et al.*, 2010b) and that transgenic overexpression of Prx2 protects brain against ischemic injury (Gan *et al.*) and models of Parkinson's disease (Hu *et al.*, 2011). Prx2 also contributes to the protective effects of probucol and atorvastatin against stroke (Du *et al.*, 2012).

4.1.4 Sulfiredoxin (Srxn)—Srxn is an enzyme that works upstream of 2-Cys Prxs (Woo *et al.*, 2005). Srxn reduces the sulfinic acid phosphoric ester on oxidized Prxs in an ATP-dependent manner, thereby reactivating Prxs (Jonsson *et al.*, 2008; Rhee and Woo, 2011). In other words, Srxn is a partner of Prxs. Since Trxs cannot reduce Prx-SO3, Srxns are very important in restoring Prx-SO_3 back to the thioredoxin cycle and preventing permanent oxidative inactivation of Prxs after exposure to strong oxidation (Figure 4) (Jonsson *et al.*, 2008). Both D3T and SFN up-regulate Srxn in primary cortical neurons and glia, and Nrf2 directly regulates Srxn1 expression via a *cis*-acting ARE, as reported in a *Srxn*-Luc reporter gene study (Bae *et al.*, 2009; Soriano *et al.*, 2008). Furthermore, a recent study show that ischemic preconditioning increases the transcription of Srxn, suggesting a neuroprotective role for Srxn (Bell *et al.*, 2011)

4.2 Glutathione system (GSH)

GSH is composed of three animo acids- glutamic acid, cysteine and glycine, and is well known for its anti-oxidant role in the CNS. In fact, GSH is so important to redox homeostasis that it is present in the millimolar range in many cell types. GSH scavenges multiple oxidative species such as superoxide, NO, hydroxyl radical, and ONOO (Aoyama *et al.*, 2008); it also serves as a reservoir for cysteine to protect against toxicity secondary to high cysteine concentrations (Janaky *et al.*, 2000). In addition to GSH itself, the GSH redox system contains three groups of enzymes that catalyze its biosynthesis, transfer GSH to its substrates, and catalyze the reduction of oxidized GSH. Nrf2 governs the expression of these GSH-related enzymes. Because GSH is so fundamental for cellular self-defense, Nrf2 plays an important protective role in the maintenance of cellular redox state.

The synthesis of GSH requires the sequential action of two enzymes. The first enzyme, γ-glutamylcysteine synthetase (GCL) ligases glutamic acid to cysteine, the rate-limiting step in GSH biosynthesis. The second enzyme, glutathione synthetase, adds glycine to form the final GSH product. In primary glial and neuronal cultures, overexpressing Nrf2 via adenovirus increases GSH synthesis as well as the expression of GSH synthetase (Shih *et al.*, 2003). Nrf2 also controls the expression of some membrane transporters that transport the raw materials required for GSH synthesis. One example is the excitatory amino acid carrier 1 (EAAC1), which transfers cysteine, the rate limiting substrate for GSH biosynthesis, into neurons. The transcription of EAAC1 is subject to regulation by Nrf2 both in vitro and in vivo (Escartin *et al.*, 2011). Furthermore, disruption of either EAAC1 or Nrf2 perturbs the synthesis of neuronal GSH (Escartin *et al.*, 2011).

The transfer of GSH to its substrates is mediated by several enzymes. Two specific enzymes that catalyze this process are glutathione peroxidase (Gpx) (Cho *et al.*, 2005; Singh *et al.*, 2006) and glutathione S-transferases (GST) (Shih *et al.*, 2003). There are eight isoenzymes of Gpx, known as Gpx1 to Gpx 8; they play a role in reducing levels of hydrogen peroxide or oxidized lipids. GST is an abundant protein that has a number of isoenzymes; their role is to conjugate GSH to electrophiles and xenobiotics (Raza, 2011). Some of these GST isoenzymes participate in neuroprotection. For example, glutathione-S-transferase pi 1 (GSTP1), the most abundant member of the GST family, has been identified as a negative regulator of cyclin dependent kinase-5, which is implicated in many neurological disorders (Sun *et al.*, 2011). Other studies have proposed that GSTP1 suppresses pro-apoptotic c-Jun N-terminal kinases (JNK) activation by stress (Elsby *et al.*, 2003). Glutathione reductase (GR) plays an important role in recycling GSH by converting oxidized GSH back to reduced GSH. This recycling process consumes NADPH. The protective role and expression regulation of GR is not fully understood. In short, many enzymes that utilize GSH to scavenge ROS are subjected to regulation by Nrf2 and are depicted in red in Figure 5, which illustrates GSH synthesis and utilization.

Glia are well known for their ability to support redox homeostasis in neurons. For example, neurons are somewhat dependent on glia for GSH synthesis. Specifically, astrocytes provide neurons directly with glutathione (Anderson *et al.*, 2003; Dringen *et al.*, 2000), or provide the precursors for GSH synthesis, such as Cys-Gly and γGlu-Cys from the hydrolysis of GSH (Dringen *et al.* , 1999; Qin *et al.*, 2006). Nrf2-overexpressing glia release GSH and can protect neurons from glutamate toxicity in neuron-glia co-cultures (Shih *et al.*, 2003). The collaboration between neurons and neighboring astrocytes in GSH homeostasis is described in Figure 5.

4.3 Transferases

Transferases are a group of enzymes that transfer various functional groups to the polar groups of their acceptors. Based on the functional groups they transfer, the transferases can be divided into several subtypes, including 1) glutathione S-transferase, which transfers a GSH, methyltransferase that, in turn, transfers a methyl group, 2) N-acetyltransferase, which transfers an acetyl group, 3) sulfotransferase, which transfers a sulfate group, and 4) UDP-glucuronosyltransferase (UGT), which transfers a glycosyl group (Nakata *et al.*, 2006; Xu *et al.*, 2005). Except for GST, the neuroprotective roles of other transferases are relatively unexplored, either because they are not protective or because their function has not yet been investigated. The major function of the transferases is to conjugate drug metabolites, making them more hydrophilic and excretable. As a result, they need specific substrates, which may or may not be present in cells. In addition, transferases possess little, if any, of the anti-oxidative function that characterizes other phase 2 enzymes and that is critical for neuroprotection. Despite these observations, their roles still need to be further investigated for a better understanding of their evolutionary roles.

4.3 Detoxifying enzymes

Heme and quinone both transfer electrons and are therefore direct sources of free radicals and ROS. As a result, overall oxidative stress is effectively reduced by the degradation of heme and quinone.

4.4.1 Heme oxygenase 1 (HO-1)—Heme oxygenase catalyzes the first and rate-limiting step of heme catabolism, the breakdown of heme to carbon monoxide, biliverdin and iron (Ferrandiz and Devesa, 2008; Yoshida and Kikuchi, 1974). There are two isoforms of active HO, HO-1 and HO-2. Whereas HO-2 is expressed constitutively, HO-1 is only expressed in an inducible manner and belongs to the phase II enzymes. The human HO-1 gene (HMOX1) is mapped at 22q12, spanning five exons and four introns (Kuwano *et al.*, 1994). The molecular weight of HO-1 is around 32 kDa. Because of its characteristic induction by stress, it is also called heat shock protein 32 (HSP32). HO-1 has a short half-life, about 3 hr for messenger RNA and 15-21 hr for protein (Dwyer *et al.*, 1992; Leautaud and Demple, 2007; Schipper *et al.*, 2009). The importance of HO-1 in neuroprotection is two-fold: the breakdown of heme and the generation of antioxidants.

Three major classes of proteins contain heme: hemoglobin, oxidase, and peroxidase. The heme in hemoglobin is essential for oxygen transport, whereas the hemes in oxidases and peroxidases play key roles in superoxide generation and electron transfer (Chrissobolis and Faraci, 2008; Everse and Coates, 2009). Heme-containing oxidases include non-mitochondrial NADPH oxidase and cyclooxygenases, as well as mitochondrial succinate dehydrogenase (Complex II) and cytochrome c oxidase (Complex IV). All of these molecules are major sources of superoxide and ROS (Chrissobolis and Faraci, 2008). On the other hand, heme-containing peroxidases catalyze the reactions between hydrogen peroxide and large biomolecules, leading to their damage (Everse and Coates, 2009). By breaking

down heme, HO-1 can thus protect cells through a net reduction in superoxide and other ROS.

In addition, the breakdown products of heme possess protective properties. For example, biliverdin and bilirubin are both strong antioxidants (Deguchi *et al.*, 2008; Stocker *et al.*, 1987) that can protect the brain from ischemic injury (Deguchi *et al.*, 2008). CO is similar to NO but much more stable and can activate guanylate cyclase, generating the secondary messenger cyclic 3',5'-monophosphate (cGMP) (Verma *et al.*, 1993). In turn, cGMP activates protein kinase G, which then decreases intracellular calcium levels, leading to cytoprotection and vasodilation (D'Ascenzo *et al.*, 2002; Lincoln *et al.*, 2006; Takuma *et al.*, 2001). In a positive feedback loop, CO plays an anti-apoptotic role by inducing the expression of HO-1 under conditions of endoplasmic reticulum stress (Kim *et al.*, 2007; Wang *et al.*, 2007b).

4.4.2 NAD(P)H: quinone oxidoreductase 1 (NQO1)—NQO1, also named DT-diaphorase, was first identified by Ernster and his colleagues in 1958 (Smith, 1999). Using either NADPH or NADH as the hydride donor, NQO1 catalyzes the two-electron reduction of quinone to the redox-stable hydroquinone, preventing free radical formation from quinone derivatives (Talalay et al. 1995). The hydroquinone generated from NQO1 reduction can be subsequently converted into glucuronide and sulfate conjugates and ultimately expelled.

NQO1 plays an important role in neuroprotection through its anti-oxidative properties (Lim *et al.*, 2008). For example, in cultured dopaminergic CATH.a cells, the induction of NQO1 by BHA dramatically and dose-dependently blocked METH-induced cytotoxicity by scavenging quino proteins (Miyazaki *et al.*, 2006). Furthermore, deprenyl can protect PC12 cells against MPP^+ induced oxidative stress via the upregulation of NQO1 (Xiao *et al.*, 2011). Upregulation of NQO1 by 4-hydroxybenzyl alcohol also reduces cerebral infarct size and improves neurological functions in rats (Yu *et al.*, 2011).

5 Protection against neurological diseases

As will be discussed below, the Nrf2/ARE pathway confers neuroprotection in various models of neurological diseases by the regulation of multiple downstream genes

5.1 Acute Neurological Diseases

5.1.1 Traumatic brain injury (TBI)—TBI is a serious public health problem affecting millions of people annually and remains a leading cause of death and disability (Feeser and Loria, 2011). Oxidative stress plays an integral role in neuronal injury after TBI. Therefore, activating the Nrf2 pathway to battle TBI has generated recent interest.

TBI significantly increases the level of Nrf2 as well as phase II enzymes such as NQO1 and HO-1 (Yan *et al.*, 2009; Yan *et al.*, 2008), suggesting that the Nrf2/ARE pathway is an endogenous compensatory adaptation against TBI. It has also been reported that intraperitoneal administration of SFN is capable of reducing neuronal death, contusion volume, and neurological dysfunction 7d after TBI in rats (Hong *et al.*, 2010). In line with this result, Nrf2-/-mice exhibit exacerbated deficits in neurologic function and oxidative damage. Furthermore, the neuroprotective capacity of SFN is blunted in Nrf2-/- mice. Histone deacetylase inhibitors (Wang *et al.*, 2012a) and tBHQ (Hatic *et al.*, 2011) can also protect against traumatic neuronal injury by activating Nrf2. These results all demonstrate that activation of Nrf2 enhances recovery from TBI.

Nrf2 activation also protects the blood brain barrier (BBB) during TBI. TBI causes a biphasic opening of the BBB. The first opening happens within hours (acute phase) after TBI and the other peaks 1-3 days (secondary phase) after injury. The latter opening is associated with a loss of endothelial cells and tight junction proteins (Zhao *et al.*, 2007a). Enhanced Nrf2 staining can be detected in the blood brain barrier following TBI (Yan *et al.*, 2009). SFN reduces Evans Blue extravasation in the acute phase when applied before injury and also reduces the secondary phase of BBB permeability when administrated 6 h after TBI (Zhao *et al.*, 2007a). This protective effect was abolished in Nrf2-/- mice or in rats pretreated with decoy ARE oligonucleotides containing the binding site of Nrf2 (Zhao *et al.*, 2007a). Independent studies from Jin and his coworkers also reported that mice with Nrf2 disruption exhibit increased severity of brain edema at 24h after TBI (Jin *et al.*, 2009). Further investigations are needed to address which cell type or which tight junction proteins contribute to these conspicuous changes.

Jin's group has also shown that the neuroprotective mechanism of Nrf2 in TBI may involve anti-inflammatory effects. Increased mRNA and protein expression of inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), intercellular adhesion molecule-1 (ICAM-1) and interleukin-6 (IL-6) have all been detected in Nrf2-/- mice after TBI. The inhibitory role of Nrf2 on cerebral NF-κB activity likely contributes to the pro-inflammatory cytokines changes in Nrf2-/- mice (Jin *et al.*, 2008b). Jin's lab also exploited the systemic effect of TBI on inflammation and detected exacerbated inflammatory responses in the lung (Jin *et al.*, 2008d) as well as the intestines (Jin *et al.*, 2008a; Jin *et al.*, 2008c) in Nrf2 deficient mice. These studies underscore the systemic effects of TBI and support both central and peripheral effects of Nrf2 on inflammatory changes after TBI

5.1.2 Ischemic Stroke—Strokes are the leading cause of disability and the third leading cause of mortality in the world. Ischemic stroke is the most common type of stroke. Multiple pathological processes are involved in the progression of stroke, including excitotoxicity, oxidative stress, inflammation, mitochondrial dysfunction, etc. Oxidative stress represents a potential target for treatment because it is one of the most critical insults in stroke. Similar to the situation in TBI, Nrf2 may play a role as an endogenous compensatory adaptation against stroke. For example, Keap1 is decreased 2 hours after reperfusion following MCAO. The fall in Keap1, not surprisingly, is paralleled by a rise in Nrf2 that starts at 2 hours and peaks at 8 hours after reperfusion. Furthermore, antioxidative proteins that are downstream of Nrf2, including Trxs, GSH, and HO-1 all showed significant increases 24–72 hours after MCAO in the peri-infarct region (Tanaka *et al.*).

Administration of tBHQ or CDDO through either intracerebroventricular or intraperitoneal routes reduces sensorimotor deficits and infarct size after ischemia in the rodent MCAO model (Shih *et al.*, 2005; Zhang *et al.*, 2012). Consistent with this study, $Nrf2^{-/-}$mice subjected to 90-min MCAO followed by 24 h reperfusion exhibit worse neurological deficits and larger infarct sizes (Shah *et al.*, 2007). Furthermore, $Nrf2^{-/-}$ mice display an exacerbated outcome 7 days after injury in a focal ischemia model combined with permanent distal middle cerebral artery occlusion (Shih *et al.*, 2005).

Activation of the Nrf2 pathway is critical for scavenging ROS, which contributes to neuroprotection against ischemic brain injury. For example, $Nrf2^{-/-}$ mice produce more ROS species in brain injury (Zhao *et al.*, 2007b). In order to identify the cell type which elicits Nrf2-dependent anti-oxidative effects, Shih and colleagues transfected neuron-glia co-cultures with an adenovirus-based Nrf2 over-expression vector and found that Nrf2-overexpressing astrocytes exhibited more efficient antioxidant properties than neurons. The underlying mechanism may involve GSH homeostasis because Nrf2 overexpressing

astrocytes release more GSH to protect neurons against H_2O_2 (Shih *et al.*, 2003). However, an independent study from Wang and colleagues proposed that the severe outcome in Nrf2$^{-/-}$ mice is caused by ROS released from neutrophils (Wang *et al.*, 2007a). It is quite likely that upregulation of GSH redox systems and suppression of inflammatory mediators both underlie the protection elicited by Nrf2.

It is of interest that tBHQ can not only protect primary cultured neurons from free radicals but also from excitotoxic insults such as NMDA or glutamate (Shah *et al.*, 2007). One might therefore speculate that Nrf2 may also regulate the transcription of glutamate transporters or receptors. Future studies to examine this possibility are warranted.

5.1.3 Hemorrhagic strokes—Depending on where the bleeding occurs and blood accumulates, hemorrhagic strokes can be divided into intracerebral hemorrhage (ICH) when there is bleeding into brain parenchyma and subarachnoid hemorrhage (SAH) when there is bleeding into the subarachnoid space. With hypertension as the major cause, ICH is associated with high mortality due to its acute onset and mass effects of hematoma and edema, which lead to intracranial hypertension and brain herniation (Keep *et al.*, 2012). Subsequent ischemic and oxidative stress also contribute to the brain injury after ICH. The Nrf2 pathway is activated in the brain following ICH, as indicated by increased HO-1 expression. In mice, HO-1 upregulation begins at 24 hr after ICH, peaks at day 5 and subsides on day 8 (Chen and Regan, 2007). HO-1 is predominantly expressed in microglia/macrophage and endothelial cells, modestly in astrocytes and rarely in neurons (Wang and Doré, 2007).

It has been reported that Nrf2-deficient mice demonstrate more severe neurologic deficits and ICH-mediated damage after ICH (Wang *et al.*, 2007a ; Zhao *et al.*, 2007b), suggesting a neuroprotective role of Nrf2 against ICH. In support of this notion, administration of SFN (Zhao *et al.*, 2007b) or curcumin (Sun *et al.*, 2011) protected against ICH by activating Nrf2 and reducing oxidative stress, brain edema and neuroinflammation. Though Nrf2 activation is protective, the responsible enzymes in this model have not been clarified. HO-1 is the only characterized enzyme in the setting of ICH. However, HO-1 is detrimental to ICH, because HO-1 knockout mice exhibit a decreased injury volume after ICH (Wang and Doré, 2007). An increase in free iron may lie behind this observation, as HO-1 degrades heme from the hematoma and generates large amount of free iron, leading to oxidative stress and neuroinflammation (Wang and Doré, 2007). It will be interesting to investigate whether concomitant administration of deferoxamine would reduce free iron overload and thereby protect the brain (Okauchi *et al.*, 2009).

SAH is caused by ruptured arterial aneurysms or arteriovenous malformation (AVM). The major pathophysiological processes of SAH are vasospasm, secondary ischemia and subsequent early brain injury (Zhou *et al.*, 2011). Following experimental SAH in rats, Nrf2 was activated in both endothelial and smooth muscle cells of the basilar artery, as indicated by increased nuclear Nrf2 levels and DNA binding (Wang *et al.*, 2010). HO-1 was also upregulated in cerebral arteries after SAH (Ono *et al.*, 2000), as well as in microglia and astrocytes (Matz *et al.*, 1996).

It has been reported that Nrf2 activation plays a protective role against SAH. For example, the administration of curcumin reduced vascular inflammation and cerebral vasospasm in mice after ICH (Wakade *et al.*, 2009), and decreased both oxidative stress and mortality in rats (Kuo *et al.*, 2011). SFN also activated Nrf2, upregulated downstream enzymes such as HO-1 and NQO-1, and reduced cortical apoptosis, brain edema and BBB impairment (Chen *et al.*, 2011). In addition to these classic Nrf2 inducers, two hormones have also been reported to protect the brain from SAH by activating the Nrf2 pathway - erythropoietin

(Zhang *et al.*, 2010a) and melatonin (Wang *et al.*, 2012b). These hormones upregulated phase 2 enzymes such as HO-1 and NQO-1, reduced early brain injury such as cortical apoptosis and protected the BBB (Wang *et al.*, 2012b ; Zhang *et al.*, 2010a). Unlike the detrimental role of HO-1 in ICH, the upregulation of HO-1 is thought to be beneficial in SAH. It has been reported that fusion of HO-1 with an eleven-arginine transduction domain can facilitate HO-1 crossing of the cell membrane (Ogawa *et al.*, 2011). When fused HO-1 was injected into the cisternal space, an increase of HO-1 level and activity was detected in the basilar artery, which attenuated cerebral vasospasm following SAH in rats (Ogawa *et al.*, 2011). Similarly, adenovirus-mediated HO-1 expression also reduced cerebral vasospasm after experimental SAH, indicating a protective role of HO-1 against SAH (Ono *et al.*, 2002).

5.2 Neurodegenerative Diseases

5.2.1 Parkinson's Disease (PD)—PD is an incurable movement disorder characterized by progressive loss of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and deposition of Lewy bodies across many regions of the brain (Tufekci *et al.*, 2011). Postmortem data from five PD patients reveal that Nrf2 is expressed at higher levels in the nucleus of substantia nigra neurons (Ramsey *et al.*, 2007). This observation is reminiscent of TBI and stroke (see above) and the upregulation of Nrf2 in PD may also be a compensatory attempt to enhance antioxidant defenses in response to oxidative toxicity.

In an acute Parkinson's model induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), a greater loss of dopamine transporters was observed in the striatum of $Nrf2^{-/-}$ mice at all MPTP doses used, ranging from 20 to 60 mg/kg. In addition, oral administration of the Nrf2 inducer D3T to wild-type mice is protective against MPTP (Burton *et al.*, 2006). In a subacute model, $Nrf2^{-/-}$ mice display fewer TH positive cells than WT mice (Chen *et al.*, 2009a). In addition to the MPTP studies, cortical neurons are more vulnerable to another neurotoxin, 6-hydroxydopamine (6-OHDA) in $Nrf2^{-/-}$ mice (Jakel *et al.*, 2007). Furthermore, overexpressing Nrf2 or its DNA-binding dimerization partner-Maf, or down-regulating Keap1 can all restore locomotor activity in genetic models of familial PD (Barone *et al.*, 2011).

A protective role of Nrf2 in PD is further supported by the clinical use of Deprenyl (selegiline), a B-type monoamine oxidase inhibitor. Recent studies show that the effect of Deprenyl is dependent on its activation of Nrf2 (Xiao *et al.*, 2011). Nrf2 may also be involved in the neuroprotective effects of DJ-1/PARK7, a gene implicated in 1-2% of early onset familial PD. DJ-1 has 189 amino acids and belongs to the Thi/PfpI protein superfamily. It decreases the ubiquitination of Nrf2 by preventing its association with Keap1. Furthermore, tBHQ can no longer induce the nuclear translocation of Nrf2 in DJ-1 knockout mice (Clements *et al.*, 2006; Gan *et al.*, 2010). DJ-1-deficient patients exhibit reduced expression of Nrf2-dependent genes coupled with increased oxidative stress (Mosser and Edwards, 2008; Zhou and Freed, 2005). Thus, Nrf2 dysfunction may be involved in the pathogenesis of early onset familial PD linked to DJ-1 mutations.

Similar to ischemic stroke, astrocytic Nrf2, rather than neuronal Nrf2, is thought to play a dominant role in neuroprotection against PD models. Using transgenic mice with Nrf2 under control of an astrocyte-specific promoter on both Nrf2+/+ and Nrf2-/- backgrounds, Peichun and colleagues showed that only astrocytes with Nrf2 expression can abolish the neurotoxicity of MPTP (Chen *et al.*, 2009a). Indeed, early studies in rodent primary neuronal cultures already showed that basal expression and activation of the ARE occurred predominantly in astrocytes (Ahlgren-Beckendorf *et al.*, 1999; Eftekharpour *et al.*, 2000). Furthermore, the majority of the protective genes induced by tBHQ are expressed heavily in astrocytes (Kraft *et al.*, 2004). Astrocytes indirectly support neuronal activity through GSH,

as described in Section 4. As in the animal models of PD, decreased levels of GSH in astrocytes are considered a hallmark of PD, potentially leading to neuron death (Dawson and Dawson, 2003). In addition to astrocytic expression, Nrf2 expression in meningeal cells can also protect against neurotoxicity from excessive dopamine (Shih *et al.*, 2007).

The mechanism underlying the sensitization of Nrf2 deficient mice to MPTP or 6-OHDA may be two-fold. The first mechanism may involve toxin detoxification. It has been reported that Nrf2 deficient mice demonstrated more severe neuronal loss than HO-1 deficient mice in response to MPTP (Innamorato *et al.*, 2010). This may not be surprising because Nrf2 also upregulates other phase II enzymes in addition to HO-1 that might detoxify MPTP or MPP^+. The second mechanism involves the anti-inflammatory effects of Nrf2. Microglia have at least two different phenotypes: the classical activation phenotype (CA-MU), which participates in inflammatory stress and neuronal death under pathological conditions and the alternative activation phenotype (AA-MU), which contributes to the resolution of inflammation and wound healing. Following exposure to MPTP, both wild type and Nrf2 deficient mice show increased levels of COX-2 and iNOS, two markers of classical microglial activation. However, only Nrf2 deficient mice also demonstrate decreased levels of FIZZ-1, Arginase-1 and IL-4, all of which are markers of alternative microglial activation (Rojo *et al.*, 2010). These studies indicate that Nrf2 may contribute to the resolution of inflammation and wound healing via AA-MU and suggest an important role for Nrf2 in the modulation of microglial dynamics.

5.2.2 Alzheimer's Disease (AD)—AD is a neurodegenerative disorder manifested by a pathological loss of synapses and neurons and the formation of intracellular neurofibrillary tangles and extracellular deposits of amyloid-beta (Aβ). AD patients exhibit a dramatic reduction in nuclear Nrf2 within hippocampal neurons (Ramsey *et al.*, 2007). In addition, the widely used APP/PS1 transgenic mice with significant amounts of Aβ deposits demonstrate a decline in Nrf2/ARE targeted proteins (Kanninen *et al.*, 2008). Because oxidative stress is integral to the pathogenesis of AD and can activate the Nrf2 pathway, it is not clear if the decreases in Nrf2 nuclear translocation and phase II enzymes cause pathology in AD or are the result of Aβ-induced neuron death. To attempt to address this, Nrf2 expression and activation in early stages of AD must be examined. Nevertheless, these findings suggest that the Nrf2/ARE pathway is impaired in AD and may form at least part of the pathology. This appears fundamentally different from the situation in TBI, stroke, and PD, where there are compensatory *rises* in Nrf2, not a fall.

Recent studies have investigated the role of the Nrf2/ARE pathway and its potential therapeutic value in AD models. Boosting Nrf2 activity by tBHQ or over-expressing Nrf2 through adenovirus-mediated gene delivery confers protection against $A\beta_{1\text{-}42}$ induced neuronal death of cultured hippocampus (Kanninen *et al.*, 2008). In vivo, delivering lentiviral vectors encoding human Nrf2 bilaterally into the hippocampus of APP/PS1 mice alleviates the spatial learning deficits and robustly reduces the infiltration of astrocytes but not microglia. A promising study reported that feeding Aβ-injected rats with tBHQ reduced Aβ accumulation and Aβ induced cell apoptosis (Nouhi *et al.*, 2011). These data confirm a protective role of Nrf2 activation in AD models. Thus, loss of Nrf2 in the human disease may exacerbate amyloid-related pathology.

The Nrf2/ARE pathway may also protect against vascular dementia. In hypobaric hypoxia - induced dementia, ALCAR (acetyl-L-carnitine) was documented to increase TrkA expression and ERK phosphorylation. Phosphorylated ERK then increased translocation of Nrf2 into the nucleus, which in turn ameliorated memory impairment induced by hypobaric hypoxia by combating oxidative stress (Barhwal *et al.*, 2009).

5.2.3 Multiple sclerosis (MS)—Multiple sclerosis is an autoimmune and inflammatory disease with lesions typically located in the white matters of the brain and spinal cord. The precise etiology of MS has not been identified. However, it is generally accepted that the proliferation of CNS-infiltrated immune cells, such as T cells, damage oligodendrocytes and axons via neuroinflammation and oxidative stress (Frohman *et al.*, 2006; Linker *et al.*, 2011). MS may be initiated by the abnormal activation of CD4+ T cells exposed to myelin-like antigenic peptides in the periphery. Subsequently, these sensitized CD4+ T cells cross the blood brain barrier and result in a series of toxic effects (Benedict and Zivadinov, 2011).

CD4+ T cell infiltration leads to excessive activation of macrophages, microglia and astrocytes, generating ROS and directly damaging normal tissues. Interestingly, Nrf2 can modulate autoimmune neuroinflammatory responses in MS models. Experimental autoimmune encephalomyelitis (EAE) is a widely accepted MS animal model. Evidence suggests an enhanced immune cell infiltration (CD4+ T cells, CD19+ B cells) and glial cell activation (astrocytes, microglia) in Nrf2 knockout mice suffering from EAE (Johnson *et al.*, 2010). Furthermore, Nrf2 deficient mice with EAE exhibit increased expression of inflammatory enzymes (iNOS, phox-47, gp91-phox, and phox-67), cytokines (IFN-γ, IL1-β, TNF-α, and IL-12), and chemokines (BLC and MIG) (Johnson *et al.*, 2010). Furthermore, Nrf2 knockout mice are highly sensitive to the neuroinflammation induced by LPS and exhibit increased microglia infiltration and inflammatory mediator expression. These features can be reversed by SFN (Innamorato *et al.*, 2008).

Elegant pathological studies of MS patients' postmortem tissue demonstrate that Nrf2-mediated transcription occurs mostly in MHC class II-positive infiltrating macrophages and to a lesser extent in reactive astrocytes. In patients with chronic-progressive MS, alpha-motor neurons express higher Nrf2 compared to controls (Linker *et al.*, 2011). Surprisingly, Nrf2 is undetectable in oligodendrocytes in either control white matter or MS brain tissue (van Horssen *et al.*, 2010).

Nrf2 knockout mice suffering from EAE exhibit more severe behavioral dysfunctions and enhanced leukocyte infiltration as well as glial activation in the spinal cord (Hubbs *et al.*, 2007; Johnson *et al.*, 2010). Consistent with these findings, Johnson and co-workers reported that Nrf2 knockout mice displayed pronounced demyelination and axonal loss in the brain (Johnson *et al.*, 2010).

Efforts have been made to treat MS by activating the Nrf2 pathway. This is warranted based on findings that dimethyl fumarate (DMF), a promising drug in clinical trials for MS, can promote Nrf2 activation through direct modification of Keap1 at cysteine residue 151 (Kappos *et al.*, 2008; Linker *et al.*, 2011). Fumarate compounds further upregulate GSH and HO-1 and protect against MS models? or is it really human MS? (Lin *et al.*, 2011; Scannevin *et al.*, 2012). Additionally, CDDO-TFEA, a strong inducer of Nrf2, suppresses neuroinflammation in EAE (Pareek *et al.*, 2011), suggesting anti-oxidative and anti-inflammatory roles of Nrf2 against MS.

5.2.4 Huntington's Disease (HD)—HD is an autosomal dominantly inherited neurodegenerative disease. HD is caused by excessive trinucleotide CAG repeat expansion in the HD gene coding huntingtin (HTT), resulting in an expanded N-terminal polyglutamine tract. It is characterized by abnormal body movements called chorea, cognitive impairments, and personality changes (Kumar *et al.*, 2010).

Several lines of transgenic HD mice have been generated based on the length of HTT N-terminal fragments. Some knock in mice only over-express part of N-terminal fragment. For instance, mice expressing a 90 amino acid N-terminal fragment are designated as R6/2 mice

and mice expressing a 171 amino acid N-terminal fragment are named N171-82Q mice. The mice that over-express full-length HTT are named BAC or YAC, which are more valuable for studies (Ross and Tabrizi). HD transgenic R6/1 mice show increased activity of Cu/Zn SOD, an Nrf2 dependent enzyme, at the age of 19 weeks. However, when these mice reach 35 weeks, the activity of Cu/Zn SOD diminished (Santamaria *et al.*, 2001). In the more severe N171-82Q transgenic HD model, basal levels of striatal Nrf2 were significantly reduced (Chaturvedi *et al.*, 2010). These findings were confirmed in a PC12 HD model (van Roon-Mom *et al.*, 2008).

It has been reported that DMF increases neuronal Nrf2 and promotes recovery in R6/2/YAC128 mice, a well known Huntington's model in which the HD gene is expressed with 141–157 CAG repeats (Ellrichmann *et al.*, 2011). One notable feature of this study is that Nrf2 is expressed within neurons but not glia (Ellrichmann *et al.*, 2011). In addition, oral administration of triterpenoids upregulates Nrf2/ARE induced genes and reduces striatal atrophy in N171-82Q mice (Stack *et al.*, 2010). Mitochondrial complex II inhibition with 3-nitropropionic acid (3-NP) or malonate leads to a type of striatal degeneration that resembles Huntington's disease. As expected, Nrf2 -/- mice are more sensitive to the mitochondrial complex II inhibitors 3-NP and malonate (Calkins *et al.*, 2005). Strikingly, transplantation of primary astrocytes infected with Ad-Nrf2-GFP into the striatum protects neurons from the malonate-induced lesions (Calkins *et al.*, 2005). Collectively, these findings show that targeting the Nrf2/ARE pathway shows promise for the treatment of HD.

5.2.5 Amyotrophic lateral sclerosis (ALS)—ALS is an adult-onset motor neuron disease caused by progressive degeneration of upper and lower motor neurons in the spinal cord, brain stem, and motor cortex. Epidemiological studies show that ALS is sporadic in 90–95% of cases and familial in 5–10% of cases, and that approximately 10%–20% of familial ALS cases are caused by the mutations in the ubiquitously expressed Cu/Zn SOD antioxidant protein (Rosen, 1993). The most widely used animal model of ALS exploits the mutation of a glycine to an alanine at position 93 of human SOD1 (SODG93A mice) (Gurney *et al.*, 1994).

Motor neurons over-expressing the SOD1 (G93A) mutation display decreased levels of Nrf2 and the enzymes involved in GSH biosynthesis (Pehar *et al.*, 2007). Similarly, Nrf2 transcriptional genes are also repressed in NSC34 cells exposed to mutant SOD1, as revealed by microarray analysis (Kirby *et al.*, 2005). Studies on human primary motor cortex and the spinal cord of postmortem tissue samples from five ALS patients confirm that mRNA and protein levels of Nrf2 were reduced in ALS motor cortices as well as spinal cord. In addition, there was a trend toward higher Keap1 mRNA signal intensity in the motor cortex of ALS (Sarlette *et al.*, 2008). This rise in mRNA did not, however, translate into higher protein levels. Nonetheless, the situation in ALS thus appears more similar to AD than PD, stroke, and TBI, because in the latter three conditions, Nrf2 levels were raised by the disease process instead of lowered.

Two potent Nrf2 activators - 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO) ethylamide and CDDO-TFEA can up-regulate Nrf2 and its downstream enzymes, not only in NSC-34 cells transfected with mutant G93A SOD1 but also in the spinal cord of G93A SOD1 mutant mice (Neymotin *et al.*, 2011). Both compounds significantly attenuate the progression of ALS and extend the survival of G93A SOD1 mice when administrated either at a presymptomatic age or a symptomatic age (Neymotin *et al.*, 2011). Consistent with other neurodegenerative studies, ALS researchers also propose that Nrf2 activation within astrocytes protects the motor neurons against mutant SOD1 toxicity. It is possible that the GSH secreted by astrocytes helps neuronal defense against oxidative insults, because Mrp1-

siRNA or MK-571, inhibitors of GSH secretion from astrocytes, abolish the neuroprotection of Nrf2 (Vargas *et al.*, 2008).

6 Conclusions and perspectives

It is evident that significant progress has been made in our understanding of pathways converging on and diverging away from Nrf2. Nrf2 levels can rapidly fluctuate in response to dynamic changes in the environmental milieu, revealing an elaborate and sophisticated machinery designed to preserve homeostasis in mammalian cells. The exquisite sensitivity of Nrf2 to stressors and its upregulation in various neurodegenerative conditions speaks to the fundamental importance of this molecule in intrinsic or endogenous protective responses to injury. However, in some conditions, such as AD and ALS, a drop in Nrf2 may actually contribute to the pathology. The up- or down-regulation of Nrf2 thus appears to closely depend on the nature of the stress or type of disease and is likely to involve its elegant positive and negative feedback loops.

Collectively, Nrf2 and its downstream phase II genes are promising targets for the treatment of neurological diseases (Table 3) owing to their potent ability to detoxify harmful compounds, combat ROS and directly or indirectly modulate the inflammatory response, immunological system, and BBB permeability. Many Nrf2 inducers have been identified and proven effective in animal models of common neurological diseases. However, there is still a dearth of clinical trials of these inducers. This may be attributed to several concerns about Nrf2 modulators that preclude their rapid translation to the clinic. First, some reports have proposed that Nrf2 activation arms the cell with extensive protection against inherently fluctuating microenvironments. This imbalance may affect normal cell growth and apoptosis (Hayes and McMahon, 2009; Shibata *et al.*, 2008). Additional studies to interpret this effect are warranted. Second, current pharmacological inducers of the Nrf2 pathway do not cross the blood brain barrier to a significant degree with the exception of luteolin (Wruck *et al.*, 2007). Further studies directed towards these concerns will help to develop Nrf-2 based strategies for neurological diseases.

Acknowledgments

This work was supported by funds from the National Institutes of Health (NS36736, NS43802 and NS45048 to J.C.), the VA Merit Review Grant (to J.C.) and the American Heart Association (10SDG2560122 to F.Z.). We thank Pat Strickler for secretarial support.

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Abbreviations

Aβ	amyloid-beta
AA-MU	alternative activation phenotype
AD	Alzheimer's disease
AhR	aryl hydrocarbon receptor
ALCAR	acetyl-L-carnitine
ALS	amyotrophic lateral sclerosis
ARE	antioxidant response elements
AP-1	activator protein-1
ASK1	apoptosis signal-regulating kinase
BBB	blood brain barrier
BTB	bric-a-brac
β-TrCP	beta-transducin repeats-containing proteins
CA-MU	classical activation phenotype
CBP	CREB-binding protein
CDDO	2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid
cGMP	cyclic 3',5'-monophosphate
CHD6	chromo-ATPase/helicase DNA binding protein family member 6
CK2	casein kinase 2
CNS	central nervous system

Cul3	Cullin3
D3T	3H-1,2-dithiole-3-thione
DMEs	drug metabolizing enzymes
DMF	dimethyl fumarate
EAE	experimental autoimmune encephalomyelitis
ERKs	extracellular signal-regulated kinases
EAAC1	excitatory amino acid carrier 1
GCL	γ-glutamylcysteine synthetase
Gpx	glutathione peroxidase
GR	glutathione reductase
GST	glutathione *s*-transferases
GSTP1	glutathione-S-transferase pi 1
GSK-3β	glycogen synthase kinase-3
6-OHDA	6-hydroxydopamine
HD	Huntington's Disease
HTT	Huntingtin
HSP32	heat shock protein 32
ICH	intracerebral hemorrhage
MAPK	mitogen-activated protein kinase
MCAO	middle cerebral artery occlusion
MEFs	mouse embryo fibroblasts
MS	multiple sclerosis
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
3-NP	3-nitropropionic acid
NES	nuclear export sequence
NLS	nuclear localization signal
Nrf2	nuclear factor erythroid 2 related factor 2
Keap1	Kelch-like ECH associated protein 1
PD	Parkinson's disease
PERK	PKR-like endoplasmic reticulum kinase
ProTα	prothymosinα
Prx	peroxiredoxin
PKC	protein kinase C
PI3K	phosphatidylinositol 3-kinase
Rbx1	E3 ubiquitin ligase complex
SAH	subarachnoid hemorrhage

SNpc	substantia nigra pars compacta
Srxn	sulfiredoxin
SFN	sulforaphane
tBHQ	*tert*-butylhydroquinone
TBI	traumatic brain injury
Trx	thioredoxin
TrxR	Trx reductase
XRE	xenobiotic response element

Highlights

- Phase II metabolic enzymes detoxify xenobiotics by increasing their hydrophilicity and enhancing their disposal.
- The Nrf2/ARE pathway is the main regulator of phase II genes
- Several phase II genes demonstrate neuroprotective properties in vivo and in vitro
- Upregulation of Nrf2/ARE, either by genetic or chemical approaches, confers neuroprotection in neurological disease models

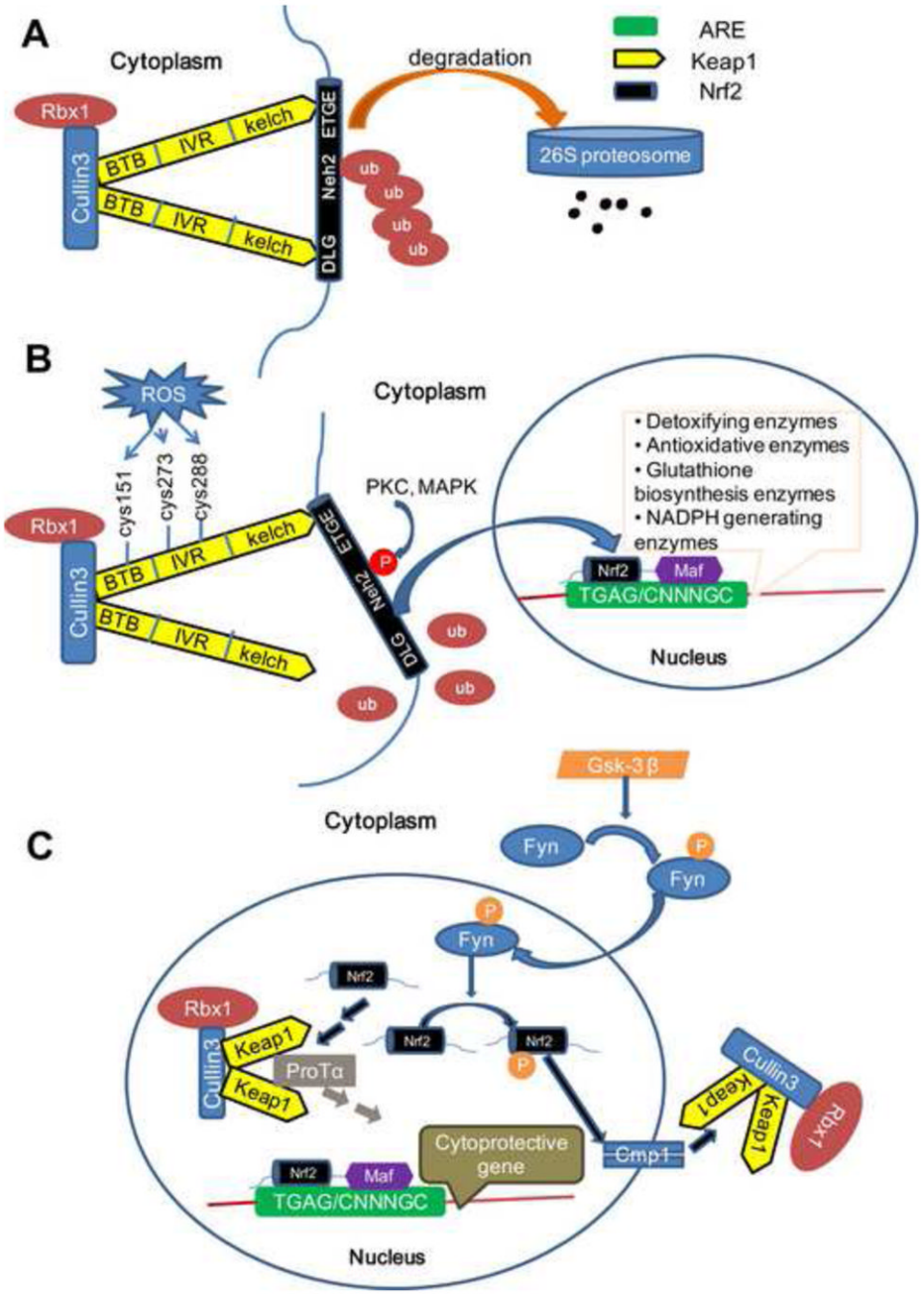

Figure 1. Classic model of Keap1/Nrf2/ARE signaling

(A) Under basal conditions, the Cul3-Keap1 complex sequesters Nrf2 in the cytosol by binding its ETGF and DLG motifs. This facilitates the ubiquitination and proteasomal degradation of Nrf2. (B) The DLG motif of Nrf2 is loosened from the Cul3-Keap1 complex when cells are exposed to ROS which blocks the ubiquitination and degradation of Nrf2. Following an intricate series of phosphorylations by several kinases, Nrf2 translocates into the nucleus and subsequently binds to the ARE elements by forming a heterodimer with Maf protein and initiating the transcription of phase II genes. (C) Nuclear Nrf2 can be phosphorylated by Fyn and be extruded back to the cytoplasm through the Cmp1 system. On the other hand, nuclear Nrf2 may also be sequestered by several Cul3-Keap1 complexes in

the nucleus that are imported by ProTα. Both of these mechanisms help cells return back to basal conditions.

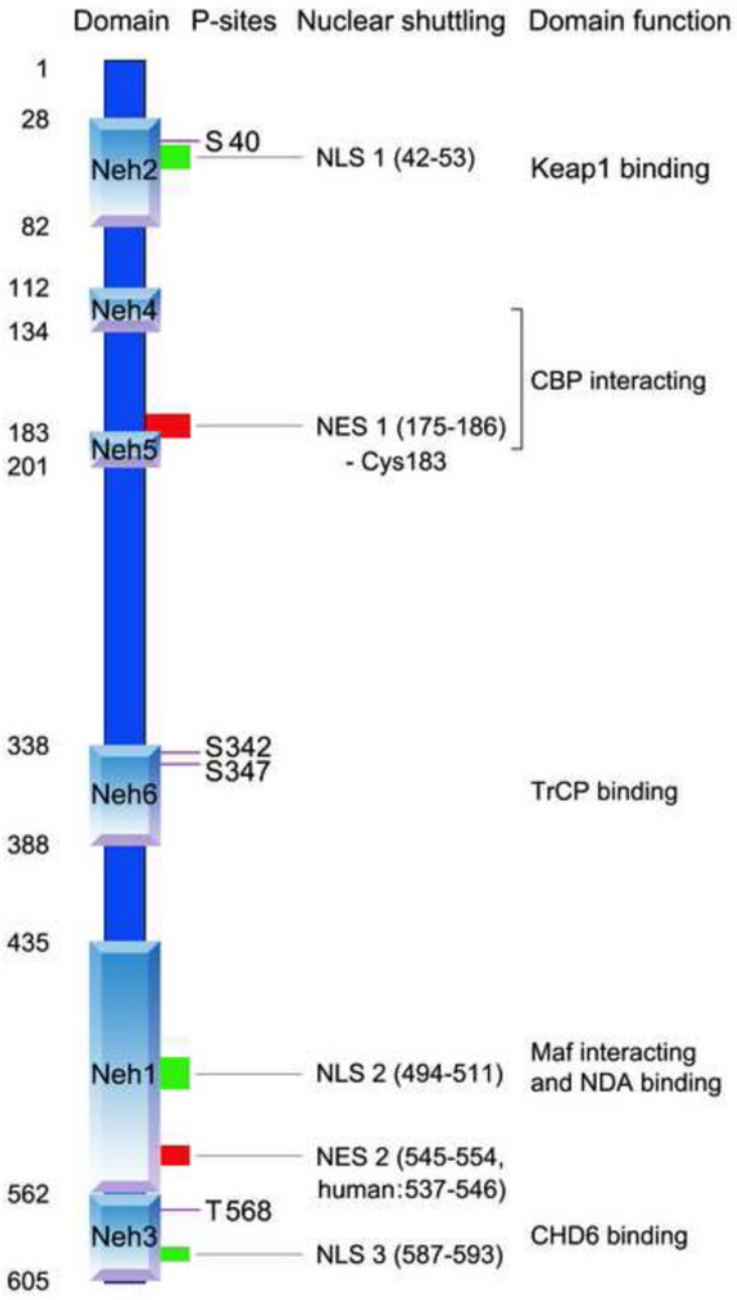

Figure 2. Structure of Nrf2: domain, phosphorylation sites and nuclear shuttling signals

Nrf2 has six domains. From N-terminus to C-terminus, these include Neh2, Neh4, Neh5, Neh6, Neh1 to Neh3. Four phosphorylation sites have been pinpointed - Ser40 in Heh2 domain by PKC, Ser342/Ser347 in Neh6 by GSK3! and Tyr 568 in Neh3 by Fyn. Three nuclear localization signals (NLS) have been identified - NLS1 located in Neh2, NLS2 located in Neh1, and NLS3 located in Neh 3. Two nuclear export sequences (NES) have also been identified - NES1 partially overlapped with Heh5, which contains a cysteine (Cys183) and NES2 located in Neh1. Purple line: phosphorylation sites; green box: NLS; red box: NES. CBP: CREB-binding protein; CHD6: chromo-ATPase/helicase DNA binding protein family member 6 (both CREB? and CHD6 are transcriptional co-activators); Maf: musculo-aponeurotic fibrosarcoma; TrCP: beta-transducin repeats-containing proteins, an E3 ligase.

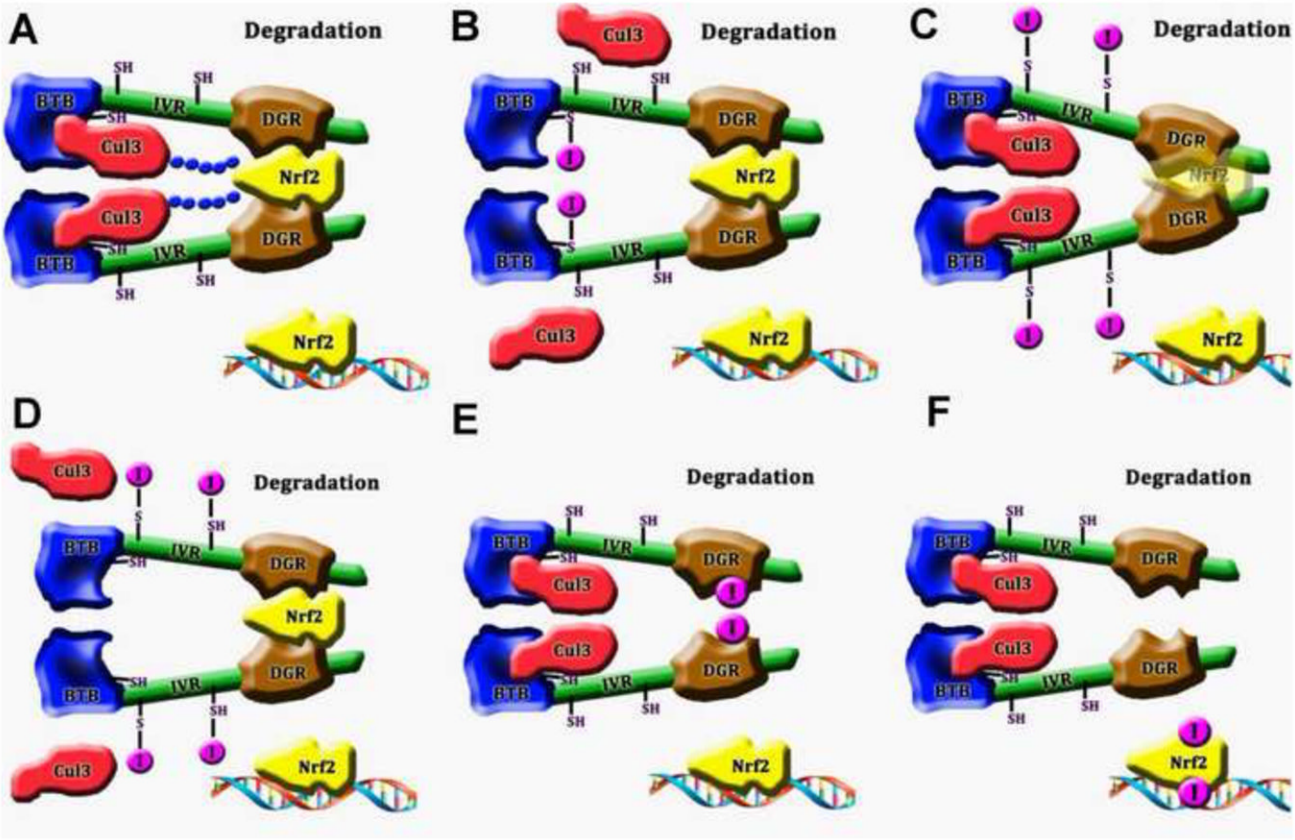

Figure 3.

Mechanisms of Nrf2 activation by different classes of inducers (

: Inducers

: Ubiquitin)

(A) Without inducers, Keap1 binds Nrf2 and facilitates the degradation of the Nrf2-Keap1 complex via Cul3. Under these circumstances, Nrf2 remains bound to Keap1 and is not free to translocate to the nucleus. (B) Inducers that act on the BTB domain. These interactions lead to structural changes of Keap1, blocking the binding of Cul3 to Keap1. Nrf2 is thereby protected from ubiquitinization. Consequently, the available pool of Nrf2 increases in size. (C) Inducers that act on IVR and interrupt the association of Keap1 with Nrf2. Translucent Nrf2 indicates that it cannot bind to Keap1. (D) Inducers that also act on IVR but interrupt the association of Keap1 with Cul3. (E) Inducers that act on the DGR domain and block the binding site of Nrf2. (F) Inducers that phosphorylate Nrf2.

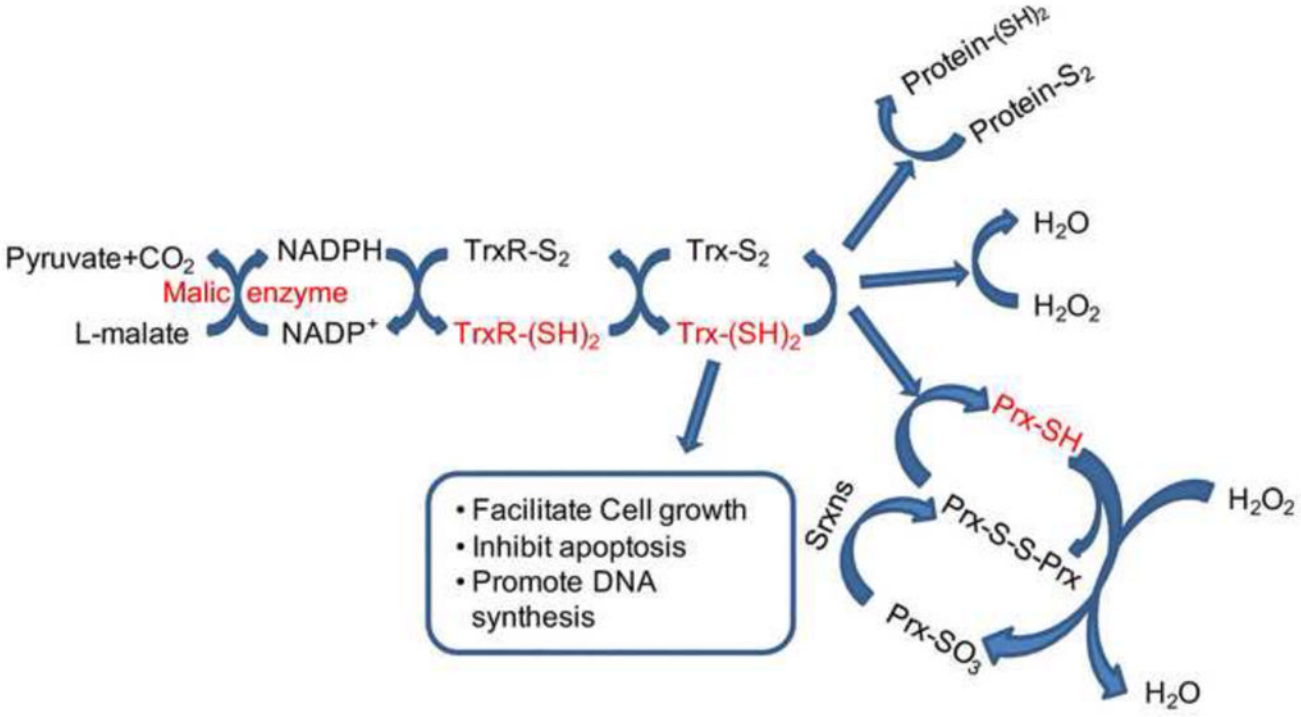

Figure 4. The impact of Nrf2 on the antioxidant activities of Trx system

Trx can be oxidized to Trx-S_2. This helps to reduce Protein-S_2, H_2O_2 and Prx-S-S-Prx. Because Prx-SO_3 cannot be reduced by Trx, Sfx restores Prx-SO_3 back into the Trx cycle. Trx-S_2 is reduced by TrxRs, with NADPH as the electron donor. The hydrogen and electron necessary for NADPH restoration come from pyruvate and the L-malate reaction cycle, which is catalyzed by the malic enzyme. The molecules that are subject to the control of Nrf2 are highlighted in red and include malic enzyme, TrxR-$(SH)_2$,Trx-$(SH)_2$, Prx-SH, Srxns. Abbreviations: GSH: glutathione; Prx: peroxiredoxin; Srxn: sulfiredoxin; Trx: thioredoxins; TrxR: thioredoxin reductase.

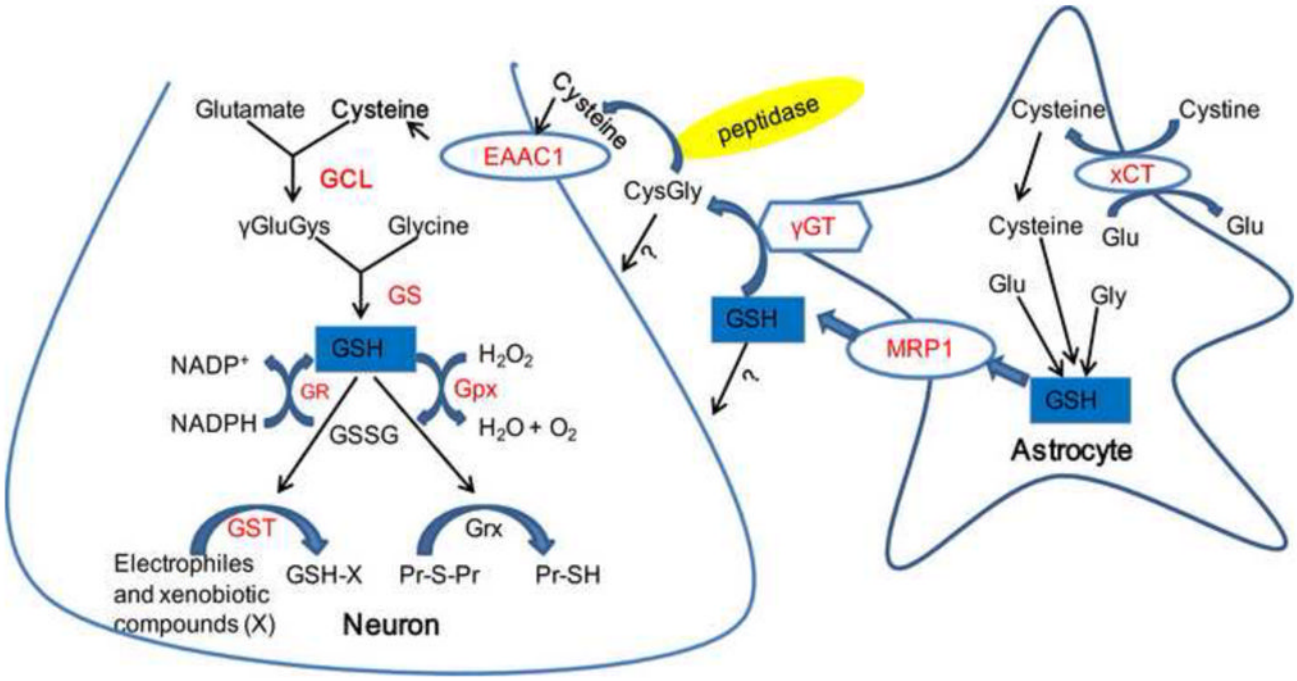

Figure 5. The impact of Nrf2 on the collaboration between neurons and astrocytes in GSH synthesis and function

GSH is synthesized from glutamate, cysteine and glycine, a reaction which is catalyzed by GCL and GS. Cysteine is the rate limiting substrate and GCL is the rate limiting biosynthetic enzyme in neurons. Cystine imported from xCT participates in astrocytic GSH synthesis. Synthesized GSH is then extruded by MRP1 and further cleaved by γGT and peptidases to generate free cysteine in the extracellular space. Cysteine then enters neurons through EAAC1 and facilitates neuronal GSH production and function. GSH exerts antioxidant effects by detoxifying H_2O_2, endogenous toxic and xenobiotic compounds and Pr-S-Pr. The molecules controlled by Nrf2 are highlighted in red. Abbreviations: Glu: glutamate; Cys: cysteine; Gly: glycine; γGluCys: γ-glutamylcysteine; CysGly: cysteinylglycine; GSSG: glutathione disulfide; GCL: γ-glutamylcysteine ligase; GS: glutathione synthase; Gpx: glutathione peroxidase; GR: glutathione reductase; GST: glutathione-S-transferase; γGT: γ-glutamyltransferase; Grx: glutaredoxin; Pr: protein; MRP1: multidrug resistance protein 1.

Table 1

Summary of drug metabolizing enzymes

Categories	Enzymes	Locations	Functions
Phase I	Aldo-keto reductase (AKR), Carboxylesterases (CES), Cytochrome P450 monooxygenase (CYP), Epoxide hydrolase.	Liver, lung, GI tract, and kidney	Oxidize, reduce or hydrolyze xenobiotics and drugs.
Phase II	γ-glutamylcysteine synthetase (GCL) Glutathione peroxidase (GPX) Glutathione S-transferase (GST), Heme oxygenase 1 (HO-1), Menadione reductase (NMO), N-acetyltransferase (NAT), NADPH quinine oxidoreductase 1 (NQO-1), Peroxiredoxin (PRX), Sulfiredoxin (SRXN), Sulfotransferase (SULT), Thioredoxin (Trx), Thioredoxin reductase (TrxR), UDP-glucuronosyltransferase (UGT).	Varies dependingon the specific gene and its subfamily.	Conjugate drug metabolites or endobiotics via acetylation, glucuronidation, glutathionylation, methylation, and sulphation, making them more hydrophilic; or degrade heme or quinone.
Phase III	Multidrug resistance associated protein (MRP), Organic anion transporting polypeptide 2 (OATP2), P-glycoprotein (P-gp), Transporters.	Brain, liver, intestine, and kidney.	Transport or excrete drug metabolites out of cells.

Table 2

Classification of Nrf2 inducers

Class	**I**	**II**	**III**	**IV**
Target domain	BTB	IVR	DGR	IVR &DGR
Target amino acids	C151	C273, C288	Y334, N382, S363, S602	H225, C226, C613
Chemical characteristics	Multiple: NO and small molecules with a sulfydryl	Alkenals, prostaglandin and derivatives	Proteins	Heavy metal
Sources	Mostly exogenesis	Endogenesis	Endogenesis	Exogenesis
Mechanism	Free from Cul3	Free from Cul3 or Keap1	Free from Keap1	Unknown

Table 3

Nrf2 and neurological diseases *in vivo*

Diseases	Ways to regulate Nrf2	Animal models	Results	References
TBI	Administration of SFN	TBI in rats	Protective	Hong, 2011; Zhao, 2007
	Nrf2 KO	Nrf2-/- mice	Exacerbated neurologic deficit	Zhao, 2007
Spinal cord injury	Nrf2 KO	Nrf2-/- mice	Exacerbated motor dysfunction and neuronal death	Mao, 2011
Ischemia	tBHQ injection (ICV or IP)	MCAO in rats	Protective	Shi, 2003;
	Nrf2KO	MCAO in Nrf2-/- mice	Increased infarct size and neurologic deficits	Shi, 2005; Shi, 2007
	IP injection of Curcumin	MCAO in rats	Protective	Yang, 2009
	Systemic administration of SFN	MCAO in rats	Protective	Zhao, 2006
	Oral administration of D3T	MPTP in mice	Protective	Burton, 2006
PD	Nrf2KO	6-OHDA in Nrf- /- mice	Exacerbated neurologic function	Jakel, 2007
	Oral selegiline	PD patients	Protective	Xiao, 2011
AD	Intrahippocampal injection of a lenti-Nrf2	APP/PS1 mice	Protective	Kanninen, 2009
	Oral tBHQ	Amyloid beta in rats	Protective	Nouhi, 2011
	Oral ALCAR	Hypobaric hypoxia - induced dementia in rats	Protective	Barhwal, 2009
MS	Nrf2 KO	EAE in Nrf2-/- mice	Impairedbehavior and enhanced pathology	Johnson, 2010 Hubbs, 2007
HD	Oral DMF	R6/2 and YAC128 HD transgenic mice	Protective	Ellrichmann, 2011
	Nrf2 -/- mice	3-NP or malonate in Nrf2-/- mice	Exacerbated neurologic function	Calkins, 2005
	Intrastriatal transplantation of Nrf2- overexpressing astrocytes	Malonate induced HD in mice	Protective	Calkins, 2005
ALS	Oral CDDO-EA CDDO-TFEA	G93A SOD1 mutated mice	Protective	Vargas, 2008