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Nrf2—a therapeutic target for the treatment of neurodegenerative diseases

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Abstract

The brain is very sensitive to changes in redox status; thus maintaining redox homeostasis in the brain is critical for the prevention of accumulating oxidative damage. Aging is the primary risk factor for developing neurodegenerative diseases. In addition to age, genetic and environmental risk factors have also been associated with disease development. The primary reactive insults associated with the aging process are a result of oxidative stress (OS) and nitrosative stress (NS). Markers of increased oxidative stress, protein and DNA modification, inflammation, and dysfunctional proteostasis have all been implicated in contributing to the progression of neurodegeneration. The ability of the cell to combat OS/NS and maintain a clearance mechanism for misfolded aggregating proteins determines whether or not it will survive. A critical pathway in this regard is the Nrf2 (nuclear factor erythroid 2-related factor 2)- antioxidant response element (ARE) pathway. Nrf2 activation has been shown to mitigate a number of pathologic mechanisms associated with Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, Huntington's disease, and multiple sclerosis. This review will focus on the role of Nrf2 in these diseases and the potential for Nrf2 activation to mitigate disease progression.

Introduction

OS/NS in neurodegenerative diseases

The brain is very sensitive to changes in redox status; thus maintaining redox homeostasis in the brain is critical for the prevention of accumulating oxidative damage. Aging is the primary risk factor for developing neurodegenerative diseases. In addition to age, genetic and environmental risk factors have also been associated with disease development. The primary reactive insults associated with the aging process are a result of oxidative stress (OS) and nitrosative stress (NS). OS/NS are produced endogenously via enzymatic and spontaneous reactions through a variety of sources and normal physiological functions [1, p. 26; 2]. Downstream markers of OS/NS have been identified clearly in all neurodegenerative diseases. Signs of lipid peroxidation, such as aldehydes including 4-hydroxynonenol (4-HNE), are increased in Parkinson's disease (PD) [3–5], Alzheimer's disease (AD) [6–8], and amyotrophic lateral sclerosis (ALS) [9, 10]. There is also a correlation between the affected

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brain regions and 4-HNE adducted proteins [11–13]. Another major marker of OS production is protein carbonyls, representing protein oxidation. Carbonyls are present in the substantia nigra (SN) in PD [14, 15], AD [16], and ALS [17] and in affected brain regions in other diseases [18–21]. Oxidative damage to DNA/RNA has been evaluated by measuring 8-hydroxy-2-deoxyguanosine (8-OHDG) and is increased in central and peripheral nervous systems of patients with neurodegenerative diseases [22–30]. Post-translational modification proteins modify protein structure and function. Protein structure and function can be altered by phosphorylation, nitration, ubiquitination, and glycosylation. Such modifications have been observed in alpha-synuclein (SYN) in patients with synucleinopathies including PD and dementia with Lewy bodies (DLB) [31–33]. Nitration and phosphorylation of tau protein has been found in the hippocampus and neocortex of patients with AD and other tau pathologies [34, 35]. Nitrotyrosine-modified proteins are elevated eightfold in the hippocampus and neocortex of AD brains [36, 37]. These region-specific protein modifications correlate with areas of increased OS/NS in the brain. Furthermore, such modifications are thought to contribute to protein misfolding and subsequent aggregate/inclusion formation. Because most neurodegenerative diseases have characteristic misfolded protein aggregates, such as SYN in PD, beta-amyloid (Aβ) plaques and hyperphosphorylated tau neurofibrillary tangles (NFTs) in AD, huntingtin (Htt) in Huntington's disease (HD), and superoxide dismutase 1 (SOD1) in ALS, protein aggregation and regulation of misfolded protein clearance by the proteasome and autophagy appear to be vital to pathogenesis.

Keap1-Nrf2-ARE pathway

One of the primary endogenous sources of OS is the mitochondrial electron transport chain. Increased mitochondrial dysfunction associated with neurodegenerative diseases leads to increased OS generation and reduction in the production of ATP. In addition and associated with neuroinflammation, the enzymes of the NADPH oxidase system generate superoxide anions. The combination of superoxide anion with nitric oxide, produced by nitric oxide synthase, generates the highly reactive NS peroxynitrite. Combating OS/NS is dependent upon the cell's ability to maintain cellular redox homeostasis. A critical pathway in this regard is the Nrf2 (nuclear factor erythroid 2-related factor 2)- antioxidant response element (ARE) pathway. The ARE is an enhancer element having the consensus sequence RTGACnnnGC, which is located in the 5' flanking region of many phase II detoxifying and antioxidant genes [38, 39]. Nrf2 is a cytoplasmic protein sequestered by the actin-bound protein Keap1 (Kelch ECH associating protein) [40, 41]. Keap1, a Cul3-based E3 ligase, polyubiquitinates Nrf2, targeting it for subsequent proteasomal degradation [42, 43]. Oxidative stress or exposure to electrophilic agents that react with Keap1 stabilize Nrf2, leading to increased Nrf2 protein levels and nuclear accumulation of Nrf2. Once in the nucleus, Nrf2 dimerizes with small Maf proteins and binds to the ARE, transcriptionally driving expression of several detoxifying and antioxidant genes [44, 45].

Nrf2 contains six well-conserved Nrf2-ECH homologous (Neh) domains that support molecular functions. The CNC (cap 'n' collar) and DNA binding regions are located in the Neh1 domain, as is the Maf dimerization site. Neh4 and Neh5 are necessary for recruitment of transcription factors and other canonical proteins required for gene expression [46]. Molecular studies have determined that the Neh2 domain is required for the cytoplasmic

localization of Nrf2, because deletion of Neh2 leads to continuous nuclear translocation of Nrf2 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 [40]. 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 [44, 47]. The “hinge and latch” model proposes that two Keap1 molecules bind Nrf2 at high- and low-affinity sites located in the Neh2 domain [48]. 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 [49]. In addition to Keap1, Nrf2 turnover can be regulated by GSK3β/β-TrCP- and Hrd1-dependent mechanisms in different pathological states [50, 51]. GSK3β phosphorylates the Neh6 domain of Nrf2. Phosphorylation of Neh6 domain facilitates binding of the β-TrCP/Cul1 E3 ligase complex to Nrf2. Nrf2 is then ubiquitinated and degraded through β-TrCP-mediated proteasomal degradation. Hrd1 is another E3 ubiquitin ligase that resides in the endoplasmic reticulum membrane. Hrd1 directly interacts with Nrf2 at the Neh4–5 domains by binding to the cytoplasmic C-terminal region of Hrd1, leading to Nrf2 ubiquitination and degradation.

In addition, there are endogenous proteins that have been shown to interact with Keap1 and activate the Nrf2 pathway. An initial screen using Nrf2-dependent ARE-luciferase activity identified seven activating proteins: sequestosome 1 (SQSTM1 or p62), D-site of albumin promoter binding protein (DBP), dipeptidylpeptidase 3 (DPP3), BCL2-like the 1 (BCL2L1; longer isoform, Bcl-x_L), the kinesin family member 26B (KIF26B), cAMP-responsive element binding protein-regulated transcription coactivator 1 (TORC1), myeloid cell leukemia sequence 1 (MCL1; longer isoform, Mcl-1l), and the splicing factor arginine/serine-rich 10 (SFRS10) [52]. Subsequently, both SQSTM1 and DPP3 were shown to interact with Keap1 [53, 54]. The Wilms tumor gene on the X chromosome (WTX) and PALB2, a major BRCA2 binding partner, bind to Keap1, whereas p21 directly binds to Nrf2, leading to inhibition of Nrf2 ubiquitination and increased Nrf2-dependent gene expression [55–57]. This review will focus on the role of Nrf2 in neurodegeneration and the potential for Nrf2 activation to mitigate progression of neurological diseases.

Nrf2 in Alzheimer's disease

AD is an age-associated progressive neurodegenerative disorder characterized by memory loss and cognitive dysfunction and is the most common form of dementia. There are currently over 5 million AD patients in the United States and it is projected that, without the development of disease-modifying therapies, the number will increase to approximately 13.8 million by 2050 [58]. Pathological hallmarks of AD include brain atrophy due to neuronal and synapse loss, senile plaques predominantly consisting of fibrillar amyloid beta (Aβ)-peptide, and neurofibrillary tangles (NFT) of hyperphosphorylated tau, a cytoskeletal protein. Aβ is generated from the cleavage of amyloid precursor protein (APP) by β- and γ-

secretases, and it is believed that small oligomers of this self-aggregating peptide are responsible for the neuronal cell death in AD [59]. The major risk factor for developing AD is aging and most AD is sporadic; however, a small fraction of AD is familial in origin. Mutations in APP, presenilin 1 (PS1), and presenilin 2 are associated with early onset familial disease [60]. In addition to the plaques and NFT, the AD brain is characterized by mitochondrial dysfunction, reactive gliosis, and oxidative damage to lipids and proteins [36] (Marshak 1992, Smith 1997, Schipper 2006, Wang X 2007, Shaftel 2008).

Nrf2 and Nrf2-dependent genes in AD brains

Initial studies demonstrated that NAD(P)H:quinone oxidoreductase 1 (NQO1) is increased in AD compared to control tissues [61–63]. The same was found for glutathione reductase, glutathione peroxidase, and heme oxygenase 1 (HO-1) [64–66]. In addition, p62, HO-1, and glutamate cysteine ligase modifier subunit (GCLM) were increased in AD brains [67]. Tanji and colleagues [67] also demonstrated that Keap1 and p62 interact with each other and are present in both the soluble and insoluble protein fractions in AD brain, as well as showing co-localization of Keap1 with NFT. A study that examined Nrf2 via immunohistochemistry found that Nrf2 is present in both the nucleus and cytoplasm of neurons in normal hippocampi, with greater intensity in the nucleus; however, in AD, Nrf2 was confined to the cytoplasm [68]. In contrast to the previous studies, this suggests that there is reduced Nrf2 activation in the AD brain. Indeed, studies have shown that some of the same Nrf2-dependent genes discussed above are reduced or do not change in AD [69–71]. These contradictory findings are not surprising and could be very dependent on the stage of disease at the time of tissue collection. In addition, our laboratory has unpublished and published data indicating that the vast majority of Nrf2-dependent genes and proteins, as well as glutathione (GSH) levels in the brain and spinal cord, are not decreased in Nrf2−/− mice [72, 73].

Nrf2 and animal models of AD

Multiple papers have demonstrated that Nrf2 activation can protect against Aβ-mediated toxicity in vitro, so the following discussion will focus primarily on the in vivo data generated that support a role Nrf2 in modulating AD. The first study to examine Nrf2 in a mouse model of AD (APP/PS1; APP_{Swe}/PS1ΔE9) demonstrated a decrease in mRNA levels of GCLM, glutamate cysteine ligase catalytic subunit (GCLC), and NQO1 at 6 months of age, as well as a reduction in Nrf2 at 16 months, using immunohistochemistry [74]. However, recent work in 7-month-old APP/PS1 mice did not see any significant changes in mRNA levels of these same genes [73]. At the protein level, there was a modest but significant increase of GCLC with no change in Keap1, NQO1, or GCLM in the APP/PS1 mice. Studies using the 3xTG model of AD also found a significant increase in NQO1 protein in hippocampus and cortex at 2 months of age, but by 6 months of age, NQO1 levels were actually significantly reduced in hippocampus [75]. Finally, nuclear Nrf2 levels were increased at 3 months and decreased at 15 months in the male cortex but unchanged in the female cortex of 3xTG mice [76]. In spite of these observations, there was no increase in GCLC and there were decreases in SOD1 (superoxide dismutase 1), HO-1, and Prdx-1 (peroxiredoxin-1) mRNA in the 3-month-old male 3xTG cortex. Overall, it is hard to argue

that there are consistent changes in protein or mRNA in mouse models of AD, which is similar to the observations in human tissue.

Nrf2−/− mice crossed with APP/PS1 mice showed significant increases in intracellular Aβ, APP fragments, and full-length APP in CA1 neurons in APP/PS1/Nrf2−/− compared with APP/PS1 mice, without a clear change in plaque load [73]. Furthermore, levels of Aβ, APP fragments, and full-length APP in the insoluble protein fraction from hippocampus were increased in APP/PS1/Nrf2−/− mice. It has been suggested that Nrf2 is involved in regulation of autophagy via increasing p62 [77–79]. Interestingly, the same APP/PS1/Nrf2−/− had punctate staining patterns for p62 versus a more diffuse staining in APP/PS1 mice, implying that the lack of Nrf2 in APP/PS1 mice drives p62 into more insoluble aggregates. There was an increase in the ratio of phospho-mTOR/total mTOR, as well as an increase in the ratio of phospho-p70S6k/total p70S6k in hippocampus of APP/PS1/Nrf2−/− mice compared with littermate controls, indicative of autophagy inhibition. More evidence of autophagy inhibition in APP/PS1/Nrf2−/− mice was the increase in multivesicular bodies, endosomes, and lysosomes in CA1 neuron compared with APP/PS1 mice. Jo and co-workers [80] published that tau and phosphorylated tau increase spontaneously in 6-month-old Nrf2 knockout mice [80]. Although there was no change in tau kinase or phosphatase activities in Nrf2−/− mice, there was a significant reduction in NDP52 (an autophagy adaptor protein). NDP52 contains ARE sequences and is induced by Nrf2 activating compounds that lead to a reduction of phosphorylated tau. In contrast to these data, Joshi and co-workers found no increase in tau or phosphorylated tau in either the 7-month-old Nrf2−/− mice or APP/PS1/Nrf2−/− (unpublished data).

An antisense oligonucleotide approach targeting GSK3β has been done in the SAMP8 AD model [81]. As mentioned earlier, Nrf2 turnover can be regulated by the GSK3β/β-TrCP pathway, and inhibition of GSK3β-mediated phosphorylation of Nrf2 would prevent the binding of β-TrCP and subsequent Nrf2 degradation via this pathway. The treatment increased nuclear Nrf2 and glutathione s-transferase (GST) levels. This correlated with improved behavior, reduced nuclear and cytoplasmic GSK3β, reduced protein carbonyl and protein bound 4-hydroxynonenal (HNE), and reduced phosphor-tau. Lentivirus (LV) was used to deliver Nrf2 intrahippocampally [82]. LV-Nrf2 was stereotactically injected bilaterally into the hippocampus of 9-month-old transgenic APP/PS1 mice. Spatial learning deficits of aged APP/PS1 mice in a Morris water maze were significantly improved by LV-Nrf2 compared with LV-GFP. There was also a reduction in astrocytic but not microglial activation and increased neuronal HO-1 up to 6 months postinjection. Finally, earlier studies in our laboratory had demonstrated that Nrf2 activation in astrocytes could confer protection from oxidative-stress-induced death on neurons [83, 84]. Based on these observations and to further address the potential role of astrocytic Nrf2 activation providing neuroprotection in neurodegenerative diseases, transgenic mice were generated that selectively overexpressed Nrf2 in astrocytes [85]. The glial fibrillary acidic protein (GFAP) promoter was used to drive Nrf2 expression in astrocytes. These GFAP–Nrf2 mice have been crossed with APP/PS1 and mutant tau mice to determine if astrocytic Nrf2 activation can mitigate the AD-like pathogenesis in these mouse models.

Many compounds and natural products have been identified as Nrf2 activators. CDDO-methylamide (CDDO-MA) improved memory and decreased plaques, Aβ(1-42), and markers of oxidative stress in the Tg19959 mice [86]. Puerarin, a phytoestrogen with antioxidant properties, was shown to improve the phenotype of APP/PS1 mice [87]. The effect was attributed to preventing the activation of GSK3β, thus preventing proteasomal degradation of Nrf2 through the β-TrCP pathway. Gracilins A and C, *Spongionella*-derived compounds shown to activate Nrf2 and have antioxidant properties, have shown some benefit in 3xTG mice [88]. A number of recent papers have used what they refer to as an acute model of AD by directly injecting Aβ(1–40) or Aβ(1-42) into the hippocampus. When compounds were administered prior to the injection of Aβ, beneficial effects were noted for SuHeXiang essential oil [89], sulforaphane [90, 91], β-hydoxybutyrate [92], orientin [93], and baicalin [94]. A major caveat to all the studies discussed in this section is that none of them were done in Nrf2−/− or knockdown mice to clearly demonstrate that these same compounds being tested require Nrf2 for their therapeutic effects.

Nrf2 in Parkinson's disease

PD is the most common neurodegenerative movement disorder, characterized by motor symptoms such as tremor, bradykinesia, posture instability, and rigidity and a number of nonmotor neuropsychiatric problems [95]. One pathological hallmark of PD is the loss of dopaminergic neurons in the substantia nigra pars compacta (SN), resulting in reduced levels of dopamine nerve terminals projecting to the striatum. A second hallmark is the formation of intracellular inclusions made up primarily of alpha-synuclein (SYN) bound to ubiquitin, also referred to as Lewy bodies (LB) [96–98]. As with AD, the majority of PD cases are sporadic, with familial cases accounting for about 15% of PD [99]. Numerous genes are associated with familial PD, including α-SYN, parkin, leucine-rich repeat kinase 2 (LRRK2), PTEN-induced putative kinase 1 (PINK1), and DJ1 [100]. Similarly to sporadic and familial cases of AD, the pathologic hallmarks for PD are present in both sporadic and most cases of familial cases of PD. Thus, the familial cases of PD and AD are, for the most part, an accelerated early onset version of sporadic disease.

Nrf2 and Nrf2-dependent genes in PD brains

It has been noted that NQO1 is up-regulated in astrocytes, endothelial cells, and dopaminergic neurons in human PD brain [101]. A similar pattern is observed with HO-1 and peroxiredoxin in SN of PD patients [102–104]. Immunostaining for Nrf2 demonstrated nuclear localization of Nrf2 in dopaminergic neurons of PD brains [68]. Oxidative stress has been implicated in PD along with mitochondrial dysfunction, protein carbonyls, and oxidative DNA damage in the SN [4, 14, 22]. GSH levels are also reduced in PD brain [105, 106]. In addition, Keap1 is co-localized with SYN and p62 in LB of PD brain [67]. A recent study in induced pluripotent stem cells (iPSC)-derived from PD and control patients also supports Nrf2 involvement in PD. Neurons differentiated from the PD iPSC had reduced GSH, increased oxidative stress, increased Nrf2 activation, and increased NQO1 levels [107].

Nrf2 and animal models of PD

In the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model, Chen and colleagues determined changes in mRNA and measured NQO1 and hPAP (human placental alkaline phosphatase) activity [108]. The ARE-hPAP reporter mice have a 51-base-pair segment of rat NQO1 promoter containing the core ARE that was inserted into a TATA-Inr minimal promoter:hPAP reporter gene construct [109]. The SN had increased hPAP and NQO1 activity, as well as increased mRNA levels of Nrf2, NQO1, GCLC, GCLM, and HO-1 following MPTP [108]. In contrast, hPAP and NQO1 activity were reduced in striatum, along with decreased mRNA for Nrf2 and NQO1. GCLC, GCLM, and HO-1 remained unchanged. Another study found an increase in striatal protein levels of Nrf2 and increased mRNA amounts for GCLC, GCLM, and HO-1 [110]. Finally, Innamorato et al. showed increases in NQO1, GCLM, GCLC, and HO-1 protein, as well as HO-1 mRNA level in striatum [111]. These discrepancies may be due to the method of MPTP administration. In the first study, MPTP (30 mg/kg) was administered once a day for five days, with tissues being harvested seven days after the last dose [108]. The second study administered MPTP (4 mg/kg) for 20 days [110] and the third at 30 mg/kg, once a day, for five days with tissue being harvested immediately after the last dose [111]. Others have directly injected MPP+ into the brain and shown decreased SN levels of Nrf2 and GCLC protein [112]. Using the same MPP+ method, others found decreased Nrf2 DNA binding, no change in Nrf2 or GCLC, and increased HO-1 protein levels in SN [113]. This group also determined that there was no change in Nrf2 DNA binding, decreased Nrf2 and GCLC protein, but increased HO-1 protein in striatum. In the 6-hydroxydopamine (6-OHDA) model of PD, only a few studies could be identified. One study using ARE-hPAP mice demonstrated increased Nrf2 activity in striatum seven days following injection [114]. A second study determined that protein levels of both Keap1 and Nrf2 were decreased in striatum [115]. Obviously, the data are not consistent and more studies need to be performed to sort out how the Nrf2 pathway is responding to the toxin models of PD.

In transgenic mouse models of PD, only two papers could be found that directly investigated how familial protein overexpression affected the Nrf2 pathway [116, 117]. Gan and co-workers used the Thy1-hSYN^{A53T} PD mouse model and evaluated changes at two and six months of age (symptomatic and nonsymptomatic). Since the synucleinopathy in these mice presents in spinal cord with motor neuron loss, only the spinal cords were examined. In nonsymptomatic mice, significant but modest changes were noted at two months (15% increase in GCLM), with only NQO1 increasing (1.5-fold mRNA; 2.6-fold protein) and a 10% increase in GSH at six months. In symptomatic six-month-old mice, a trend of reduced GSH that correlated with a 20–30% reduction in GCLM and GCLC mRNA levels was observed [116]. These same mice were also crossed with the ARE-hPAP reporter mice, which demonstrated increased hPAP staining in the ventral horn of the spinal cord. The second study used mice with the tyrosine hydroxylase (TH) promoter driving hSYN^{WT} for targeted expression in the dopaminergic neurons [117]. At one month of age NQO1, GCLM, GCLC, and HO-1 were increased ranging from 1.6- to 2.0-fold in the SN and 2.1- to 2.5-fold in striatum. However, by the age of six months, all changes returned to control levels.

Early experiments utilized Nrf2−/− mice to show increased sensitivity to the dopaminergic toxins MPTP and 6-OHDA [118, 119]. The increased sensitivity of the Nrf2−/− mice to MPTP has been repeated in multiple laboratories [108, 111, 120–122]. In general, there is a greater loss of TH and reduction of dopamine (DA), as well as the dopamine metabolites 3,4-dihydroxyphenylaceticacid (DOPAC) and homovanillic acid (HVA). There is increased neuroinflammation associated with greater astrocyte (GFAP) and microglial (Iba-1; ionized calcium binding adaptor molecule 1) activation in Nrf2−/− mice. It is also important to note that the basal level of mRNA expression and protein content of Nrf2-dependent genes remained unchanged [108, 121] or slightly reduced [122] in striatum and SN (ventral midbrain) of the Nrf2−/− compared to control mice. This strongly suggests that the primary role for Nrf2 in the central nervous system is to respond to insults by activation of gene expression. Thus, the lack of an Nrf2 response in Nrf2−/− mice leads to greater damage in the mouse brain. Interestingly, it is hypothesized that Nrf2 responsiveness is attenuated with age, reducing the endogenous Nrf2 response to oxidative stress similarly to what is most likely occurring in the Nrf2−/− mice. Adeno-associated virus (AAV) has been used to deliver $hSYN^{WT}$ (AAV-$hSYN^{WT}$) to Nrf2+/+ and Nrf2−/− mice [123]. AAV-$hSYN^{WT}$ was stereotactically injected into the ventral midbrain and tissues were harvested 2 months after injection. There was a small but significant loss of TH-positive neurons in both Nrf2+/+ and Nrf2−/−, with slightly more loss (23% more) in the Nrf2−/− mice. However, there were no changes in DA, DOPAC, or HVA. The authors speculate that the Nrf2 pathway may be involved in SYN degradation through the ubiquitin–proteasome system (UPS) but the mRNA levels of the proteasome subunits PSMB7, PSMC3, and PSMC4 in the ventral midbrain were not significantly different between Nrf2+/+ and Nrf2−/− mice.

Immunohistochemical analysis for PSMB7 allowed evaluation of the dopaminergic neurons, which stained positive for $hSYN^{WT}$ and PSMB7. These data did suggest that there are differences in neuronal PSMB7 in Nrf2+/+ and Nrf2−/− mice. There were also changes in astrocytic and microglial activation based on cellular morphology without biochemical validation. Based on early studies showing the importance of Nrf2 activation in astrocytes [83, 84], Jakel and co-workers transduced astrocytes in vitro with adenovirus containing Nrf2 (Ad-Nrf2) or GFP (Ad-GFP) and then transplanted these Nrf2-overexpressing and control GFP astrocytes into the ipsilateral and contralateral striatum, respectively, by stereotactic injection [119]. Five weeks later, bilateral injection 6-OHDA toxicity revealed a significantly reduced lesion volume in the Nrf2 astrocyte striatum. To better understand if astrocytic Nrf2 activation can modulate PD, GFAP-Nrf2 mice were treated with MPTP. Dramatic protection of the dopaminergic neurons was observed in the GFAP-Nrf2 mice following MPTP administration, which was sustained when the GFAP-Nrf2 mice were on an Nrf2−/− background [108]. GFAP-Nrf2 mice were then crossed with the Thy1-$hSYN^{A53T}$ to evaluate if Nrf2 overexpression in astrocytes could modulate $hSYN^{A53T}$ aggregation and proteostasis [116]. The median survival of the $hSYN^{A53T}$ mice was dramatically extended by Nrf2 overexpression in astrocytes from 206.5 days in $hSYN^{A53T}$mice to 272.0 days in $hSYN^{A53T}$/GFAP-Nrf2 double transgenic mice. During pathogenesis, $hSYN^{A53T}$ shifted from the soluble to the insoluble/aggregated protein fraction. Six-month-old symptomatic $hSYN^{A53T}$ mice had a significant increase in insoluble/aggregated $hSyn^{A53T}$ that was completely reversed by overexpression of Nrf2 in

astrocytes. The insoluble hSYN^{A53T} was also highly phosphorylated (Ser129) (p-hSYN^{A53T}) and immunohistochemically co-localized with hSYN^{A53T} aggregates and p62. Again, these observations were completely reversed by GFAP-Nrf2. Autophagic dysfunction was also observed in the in hSYN^{A53T} mice. Reduced macroautophagy, as well as impaired chaperone-mediated autophagy, was noted in the hSYN^{A53T} mice. Astrocytic Nrf2 activation prevented neuronal autophagic dysfunction and correlated with increased lifespan and reduced hSYN^{A53T} aggregation in the hSYN^{A53T}/GFAP-Nrf2 mice. These data suggest that Nrf2 activation in astrocytes stabilizes proteostasis and reduces oxidative stress in neurons overexpressing hSYN^{A53T}. The question of how this works mechanistically remains to be answered.

Numerous Nrf2-activating compounds have been tested in animal models of PD (Table 1). In general, the data show different degrees of protection of dopaminergic neurons in rats or mice. Three papers used Nrf2−/− mice to show that the protective effects of the chemical being tested were Nrf2-dependent [118, 121, 122]. Burton and colleagues demonstrated that activation of the Nrf2 pathway by oral administration of 3H-1,2-dithiole-3-thione (D3T) to wild-type mice partially protected them from MPTP-induced neurotoxicity. This protective effect was not observed in Nrf2−/− mice. Sulforaphane treatment also showed partial protection in Nrf2+/+ mice that was not observed in Nrf2−/− mice [121]. In addition, of the verified Nrf2-dependent compounds, CDDO-EA (CDDO-EA) and CDDO-trifluoroethylamide (CDDO-TFEA) had significant effects across all endpoints measured in an MPTP study. A more recent study did not examine a compound but evaluated whether treadmill exercise could regulate Nrf2 and modulate MPP$^+$ pathology in rats [113]. Exercised rats were run 60 min/day, 5 days a week, for 4 weeks. Sedentary rats were placed on the treadmill for 60 min/day without running. MPP$^+$ was injected into the striatum and the rats were harvested 1 week after injection. The loss of TH-positive neurons and TH protein levels was completely reversed by exercise. LV-shNrf2 striatal injection 1 week prior to MPP$^+$ injection reversed the protective effects of exercise.

Nrf2 in amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is the most common adult-onset motor neuron disease, caused by the progressive degeneration of motor neurons in the spinal cord, brain stem, and motor cortex [[124]. Motor neurons contain ubiquitin-positive hyaline and skein-like inclusions [125]. These inclusions contain SOD1 that co-localizes with p62 [126]. TDP-43 (TAR DNA-binding protein)- and FUS-positive inclusions have also been identified in ALS [127, 128]. As with AD and PD, the majority of ALS cases are sporadic (sALS), with approximately 10–15% of cases associated with familial mutations (fALS). The first mutant gene identified in fALS was Cu/Zn-superoxide dismutase 1 (SOD1) and the mutation caused disease through a toxic gain of function [129]. Over 150 mutations have been identified in SOD1. Rodents overexpressing mutated forms of hSOD1 generally develop an ALS-like phenotype [130, 131]. Although the molecular mechanism underlying this toxic gain of function remains unknown, toxicity to motor neurons requires mutant SOD1 expression in non-neuronal cells as well as in motor neurons [132]. In recent years, 24 additional genes have been associated with sALS and fALS [133] including p62 [134], which has a direct link to Keap1/Nrf2 and autophagy.

Nrf2 and Nrf2-dependent genes in ALS brains

Nrf2 and Keap1 have been examined in the primary motor cortex and spinal cord of ALS patients [135]. There was a reduction in Nrf2 mRNA and protein in ALS patient tissue relative to Nrf2 levels in control tissues, but Keap1 mRNA and protein levels did not change. Nrf2-dependent genes were not evaluated. In addition, Keap1 has been shown to co-localize with intracellular inclusions in motor neurons of postmortem ALS spinal cord [67].

Nrf2 in animal models of ALS

Initial studies in the hSOD1^{G93A} rat model of ALS demonstrated that both the mRNA and protein levels of HO-1 and Nrf2 were increased in spinal cord compared to those in nontransgenic littermate controls [136]. Interestingly, both Nrf2 and HO-1 co-localized with reactive astrocytes in the degenerating spinal cord of hSOD1^{G93A} rats, leading to speculation that Nrf2 activation in astrocytes may be involved in determining motor neuron fate in ALS. This concept was tested in vitro by demonstrating that Nrf2 activation in primary astrocytes increased survival of co-plated purified primary motor neurons and protected the motor neurons from a variety of insults causing motor neuron death [85, 136–138]. In the hSOD1^{G93A} mouse, protein levels of Nrf2, thioredoxin, HSP-70, HO-1, NQO1, GCLC, and GCLM increased with disease progression in lumbar spinal cord but not cortex [139, 140]. Finally, the hSOD1^{G93A} and hSOD1$^{H46R/H48Q}$ ALS models were both crossed with the ARE-hPAP reporter mice [141]. The earliest and most significant activation of this genetic sensor of Nrf2 activation occurred in the distal muscles of both mutant SOD mice. Subsequently, Nrf2 activation proceeded pathology in a retrograde fashion along the motor pathway. In the muscles of hSOD1^{G93A} mice, gastrocnemius was the first muscle to show activation at 30 days, followed by the triceps at 60 days, and intercostal muscles at 110 days. Interestingly, Nrf2 activation was isolated to type I fibers, which are known to be more resistant to degeneration than type II fiber in ALS patients. Significantly increased hPAP staining was also observed in the motor neurons and astrocytes in the ventral horn of the spinal cord. Thus, it appears that Nrf2 activation in the muscle is in response to denervation and occurs prior to Nrf2 activation in spinal cord.

Nrf2 knockout mice have been crossed with the hSOD1^{G93A} and hSOD1^{G85R} models of ALS [140, 142]. The first study found a modest decrease in lifespan by six days in male and seven day in female hSOD1^{G93A} mice on an Nrf2−/− background [140]. In the second study, Nrf2 knockout had no effect on lifespan or onset in the hSOD1^{G93A} and hSOD1^{G85R} mice [142]. Viral-mediated therapy was performed using intramuscular injection of AAV6-Nrf2. The virus can transduce axons at the neuromuscular junction, followed by axonal retrograde transport and expression of Nrf2 in motor neuron cell bodies [143]. The hSOD1^{G93A} mice were injected with either AAV6-Nrf2 or AAV6-GFP at 30 days of age in multiple muscles (facial, tongue, intercostal, diaphragm, and hind limbs). No effect on lifespan, onset, motor neuron survival, or rotarod performance was observed. The negative results were attributed to low viral transduction efficiency of motor neurons. Although this may be the case, another study using transgenic mice to drive Nrf2 overexpression in neurons also resulted in a negative outcome [142]. Here, these investigators crossed the Thy1-cre, hSOD1^{G93A}, and caNrf2 mice. The caNrf2 mice carry an Nrf2 transgene that lacks the N-terminal Neh2 domain responsible for binding to Keap1 under control of the

chicken β-actin promoter. Between the promoter and caNrf2 lies a transcription/translation STOP cassette flanked by loxP sites. In the presence of cre recombinase the STOP is removed and caNrf2 is expressed. Thy1 promoter is a neuronal selective promoter driving the expression of cre recombinase, which allowed the overexpression of caNrf2 in neurons. Although neuronal overexpression of caNrf2 in hSOD1^{G93A} led to a significant seven-day delay in onset, there was no significant effect on survival. Based on the ARE-hPAP expression profile in hSOD1^{G93A} mice discussed earlier, overexpression of Nrf2 in the degenerating type II fiber was hypothesized. Transgenic mice were generated using the MLC1 (myosin light chain 1) promoter driving expression of Nrf2 selectively in type II fiber of skeletal muscle [142]. Similarly to the neuronal overexpression of Nrf2, there was a significant eight-day delay in onset, but no significant effect on survival. The most compelling data come from experiments using transgenic GFAP-Nrf2 mice that overexpress Nrf2 only in astrocytes [85]. Crossing these mice with the hSOD1^{G93A} and hSOD1$^{H46R/H48Q}$ mice significantly delayed onset by 17 days and extended lifespan by 20.5 days. Furthermore, a delay in astrogliosis, but more importantly a significant attenuation of microglial activation, was observed. Finally, there was reduced neuronal loss and muscle denervation in the GFAP-Nrf2/hSOD1^{G93A} mice.

The published data on Nrf2-activating compounds has been more limited in ALS than in PD. This may be a reflection of either a lack of experimental studies using Nrf2-activating compounds or the failure of these compounds, because of which no studies have been published. For example, sulforaphane has been used and failed without publication (personal communication). Some of the same compounds used in PD models, CDDO-EA and CDDO-TFEA, have been used in ALS models [144]. These compounds were fed in the diet and feeding was initiated at 30 and at 75 days of age (onset) to hSOD1^{G93A} mice. The former treatment protocol resulted in an extension of lifespan by 20.6 days for CDDO-EA and 17.6 days for CDDO-TFEA. The latter treatment protocol resulted in an extension of lifespan by 17.5 days for CDDO-EA and 16 days for CDDO-TFEA. Feng and colleagues tested DL-3-n-butylphthalide in hSOD1^{G93A} mice [145]. The compound was administered by gavage once a day starting at onset, and there was a significant extension of lifespan at the highest dose of approximately 18 days. This was accompanied by increased neuronal survival and suppression of neuroinflammation (astrocytic and microglial activation). S-[+]-apomorphine was identified as an Nrf2 activator in a compound screen and used to treat hSOD1^{G93A} mice [146]. Drug treatment was started at 21 days until end stage. Although, there was some delay in onset and behavioral outcome measures, there was no effect on lifespan. CPN-9 was identified through an in silico drug screen to identify compounds that suppress oxidative stress-induced cell death and was subsequently shown to be an Nrf2-activating compound [147]. hSOD1^{H46R} mice were treated with CPN-9 by gavage at the day of onset, demonstrating a small, but significant, six-day extension of lifespan at the highest dose of CPN-9. Finally, the same group produced a novel acylaminoimidazole derivative, WN1316, based on the structure of CPN-9 that retained Nrf2 activity [148]. The hope was to develop a compound with better pharmacokinetics for the treatment of ALS. Again, treatment was initiated at onset in both the hSOD1^{H46R} and hSOD1^{G93A} mice and a significant extension of survival of hSOD1^{H46R} by nine days and hSOD1^{G93A} by eight days was seem at the

highest dose of WN1316. In addition, a delay in neuronal loss, as well as astrocytic and microglial activation, was observed.

Nrf2 in Huntington's disease

HD is an autosomal dominant neurodegenerative disease that is associated with expansion of a CAG repeat in the gene encoding the Htt protein [149]. This results in a stretch of N-terminal glutamine residues, and the severity and onset of disease correlates with the length of this polyglutamine repeat. Furthermore, a conformational change in the mutant Htt increases self–aggregation associated with intracellular inclusion formation [150]. HD is pathologically characterized by degeneration in neostriatal (caudate and putamen) and cerebral cortex that is believed to be the underlying contributor to motor impairment, cognitive decline, and psychiatric symptoms, which worsen as the disease progresses. Oxidative stress due to mitochondrial dysfunction has been implicated in human patients [151]. Defects in mitochondrial complex II, III, and IV were observed in striatum of postmortem HD brain [152, 153].

Nrf2 and Nrf2-dependent genes in HD brains

Surprisingly little work has been done on HD tissues and Nrf2. There has been only one study examining Nrf2 or Nrf2-dependent genes in postmortem HD brains [154], in which HO-1 immunohistochemical staining was increased in HD brain.

Nrf2 in animal models of HD

Malonate and 3-nitropropionic acid (3NP), mitochondrial complex II inhibitors, produce striatal medium spiny neuron degeneration, a characteristic feature observed in HD [155, 156]. Malonate-treated ARE-hPAP reporter mice show significant increases in hPAP activity in astrocytes that form the border of the penumbra [157, 158]. Malonate also increased Nrf2 and HO-1 mRNA levels in mouse striatum seven days after injection into striatum [158]. In the 3NP model, HO-1 and NQO1 protein levels were increased in rat striatum [159]. However, another study using 3NP did not demonstrate changes in Nrf2 protein or mRNA levels of NQO1, HO-1, GCLM, or glutathione-S-transferase P1 [160]. The Nrf2-depdendent subunit of system x_c^- (a cystine transport system), xCT, is reduced in R6/2 striatum, suggesting the possible disruption of Nrf2 activity in the mouse model [161]. Expanding on the role of Nrf2 in the malonate and 3NP HD models. Nrf2−/− mice exposed to these agents had more significant lesion volumes at doses that had little or no effect in wild type mice [157, 162, 163].

In addition, Calkins and colleagues demonstrated that Ad-Nrf2-, in contrast to Ad-GFP-transduced primary astrocyte and mouse neural progenitor cells transplanted into the mouse striatum had significant protection form malonate toxicity [157, 158]. Four to five weeks after transplantation of both cell types, the mice were injected with malonate and Ad-Nrf2 transplants had an 80–90% reduction in lesion volume. Direct injection of Ad-Nrf2 into the striatum also conferred significant but less protection against 3NP toxicity [162]. Malonate was also injected into the striatum of GFAP-Nrf2 mice, and similarly, the GFAP-Nrf2 mice were more resistant to malonate [158]. Interestingly, there was no difference in baseline

levels of Nrf2-dependent genes in GFAP-Nrf2 striatum. In addition, astrocytic and microglial activation were not attenuated. However, there was dramatic acceleration and robust Nrf2 activation that occurred within 24 h of malonate dosing in the GFAP-Nrf2 mice. In contrast, the wild type mice had virtually no increased Nrf2, suggesting that activation of the Nrf2 pathway after a toxic insult could be beneficial.

Similarly to PD and ALS models, the CDDO-MA has been tested in the 3NP model, whereas CDDO-EA and CDDO-TFEA have been tested using the N171-82Q mouse model of HD [164, 165]. In all cases, significant protection was observed. The N171-28Q mice were started on diets containing the CDDO compounds at 30 days of age. Lifespan was increased 34 and 29 days for CDDO-EA and CDDO-TFEA, respectively. However, Nrf2−/− mice were not used in these studies. Another group fed tert-butylhydroquinone (tBHQ), a well-known Nrf2-activating compound, to Nrf2+/− and Nrf2−/− mice followed by 3NP administration [162]. tBHQ was started 1 week prior to starting the 3NP, and the data showed protection with tBHQ in Nrf2+/− but not Nrf2−/− mice. A similar experiment gave cystamine once daily for seven days to Nrf2+/+ and Nrf2−/− mice prior to starting 3NP dosing [163]. The Nrf2+/+ mice given cystamine did not have any striatal lesions, and in the Nrf2−/−, cystamine actually significantly increased lesion volume. The R6/2 and YAC128 models of HD were treated with dimethylfumarate (DMF), another known Nrf2-activating compound [166]. DMF dosing twice daily via gavage began at 4 weeks of age in the R6/2 mice. Survival was extended from 94 to 100.5 days and was associated with preserved muscle function and body weight, as well as increased number of intact neurons. Formation of intracellular ubiquitin-positive Htt aggregates, however, was not affected by DMF treatment. DMF treatment of YAC128 mice resulted in reduced clasping score and neuronal loss in the striatum and cortex at 1 year of age. Two additional compounds, naringin, a flavonone from grapefruit, and protopanaxtriol, a compound isolated from ginseng, protect against 3NP-induced neurodegeneration [159, 160]. Much more work is required in the HD and genetic HD models to better understand how Nrf2 can modulate HD pathology. Current work in our laboratory is examining the protective effects of neuronal versus astrocytic Nrf2 overexpression in R6/2 transgenic and zQ175 knockin mice.

Nrf2 in multiple sclerosis

It is estimated that as many as 400,000 people in the United States and more than 2.3 million people worldwide are affected by multiple sclerosis (MS). MS is a chronic neuroinflammatory disease onset by activation of peripheral $CD4^+$ T cells that traverse the blood–brain barrier and mount a damaging autoimmune attack on myelin and oligodendrocytes in the central nervous system (CNS) [167]. Neurological deficits manifest in heterogenic conditions that include but are not limited to vision loss, dyscoordination, weakness, sensory loss, and cognitive changes, as well as bladder and bowel dysfunction. Pathology of MS is attributed to the pathogenic attack on myelin sheathing and oligodendrocytes by autoreactive $CD4^+$ Th1 and Th17 cells, as well as infiltrating macrophages and resident microglia, resulting in axonal retraction and astrogliosis. The reactive microglia and astrocytes, in turn, secrete inflammatory mediators that not only include cytokines and chemokines, but also produce reactive oxygen and nitrogen species that contribute to oxidative stress, exacerbating the already deleterious effects of the

neuroinflammatory-associated tissue damage contributing to the disease progression [168, 169]. Today, there are currently eight therapies approved by the FDA for the treatment of relapsing remitting MS, the most common (~87% of MS patients) of the four clinical forms of this disease, which focus primarily on preventing the penetration of immune cells across the blood–brain barrier. However, the extreme complexity and heterogeneity of the disease, therapy resistance developed over time, and the more current dogma that oxidative stress contributes to the disease, warrants further investigation to improve and/or contribute to existing treatments [170].

Nrf2 and Nrf2-dependent genes in MS brain lesions

Severe oxidative damage has been observed in lesions from MS brain tissue and attributed to the inflammatory-mediated generation of reactive oxygen and nitrogen species. Expression of antioxidant proteins and enzymes, as well as Nrf2 protein, coincided with cellular stress markers in active lesions when compared with normal-appearing white matter (NAWM) and white matter tissue from non-neurological control brains [171–173]. Thus, activation of an Nrf2-mediated antioxidant response may reflect protective cellular defense mechanisms against the oxidative stress-mediated damage associated with persistent inflammation. Initial studies identified NQO1, a well-known Nrf2-regulated enzyme, as highly up-regulated in active and highly active MS lesions, particularly in hypertrophic astrocytes and myelin-laden macrophages. Oligodendrocytes sporadically displayed NQO1 immunnoreactivity, while neuronal NQO1 staining was rarely observed [171]. Subsequent studies identified active MS lesions as being immunoreactive for the oxidative stress markers 4-HNE, 8-hydroxydeoxyguanosine, and nitrotyrosine, with these same active lesions coexpressing the antioxidant enzymes HO-1, SOD1, SOD2, and catalase. Similarly to the expression of NQO1, this staining was morphologically and histochemically identified as being present in hypertrophic astrocytes and myelin-laden macrophages with modest or no staining in NAWM. Consistent with the expression of antioxidants, up-regulation of Nrf2 in MS lesions was localized to both the nucleus and cytoplasm of infiltrating macrophages and reactive astrocytes, with weak expression in oligodendrocytes bordering active lesions [173].

Nrf2 and animal models of MS

Studies using mouse models of multiple sclerosis indicate the importance of Nrf2 in the progress and/or prevention of this demyelinating neuroinflammatory disease. For instance, Nrf2 knockout mice immunized with myelin oligodendrocytic protein (MOG 35–55) to induce an acute autoimmune experimental autoimmune encephalomyelitis (EAE), a humanized model of MS in mice, demonstrated a more severe course of disease, a more rapid onset, and a greater percentage of Nrf2 knockout mice with EAE. In spines from diseased mice, there were more severe demyelination and increased immune cell infiltration and glia cell activation, as well as increased mRNA expression of inflammatory enzymes (*iNOS, phox-47, gp91-phox, and phox-67*), cytokines (*IFN-gamma, IL1-b, and IL-12*) and chemokines (*BLC* and *MIG*), in Nrf2-deficient mice than in WT mice, further suggesting that Nrf2 can modulate autoimmune neuroinflammatory responses [174].

More recently, compound- or natural product-mediated activation of the Nrf2 signaling pathway has been found to ameliorate oxidative stress and neuroinflammation in EAE and MS [175–178], supporting the notion that induction of Nrf2 in both mouse and human can prevent or alleviate the pathogenesis associated with MS and EAE. Remarkable examples are the fumaric acid esters (FAE), methyl hydrogen fumarate (MHF), and dimethyl fumarate (DMF). These were previously used for effective treatment of the autoimmune skin disorder psoriasis via immunomodulation and immunosuppression. Treatment with DMF also had a significant therapeutic effect on clinical scores of disease and reduced macrophage inflammation in spinal cords of EAE mice versus control treatments [179]. Interestingly, in early studies using murine primary astrocytes, DMF, a strong glutathione-depleting agent, had been shown to activate the Nrf2-ARE pathway [180]. Both DMF and MMF (a primary metabolite of DMF) have been shown to react with the Keap1 cysteine 151 residue (cys151) in studies using Keap1-C151S mutant macrophages and mass spectrometric analysis of astrocytes, respectively [175, 181]. It was further demonstrated that the FAE indeed had neuroprotective effects in EAE via activation of the Nrf2 pathway. Preventative or therapeutic (after disease was initiated) application of DMF attenuated disease severity and improved preservation of myelin, axons, and neurons, which did not occur in Nrf2−/− mice. Immunoreactivity for protein nitrosylated tyrosines, indicative of oxidative stress, was reduced in spinal cord sections from mice with EAE by treatment with DMF. Furthermore, induction of Nrf2 in neurons of the motor cortex and brainstem, astrocytes of spinal cord gray matter, and oligodendrocytes from spinal cord white matter was observed [175]. Treatments of animals or primary cultures of CNS cells with DMF or MMF resulted in increased levels of Nrf2 nuclear localization, as well as increased levels of canonical antioxidant target genes that were not observed in Nrf2 knockout mice [182]. DMF (BG-12) has proved effective in Phase III clinical trials for the treatment of relapsing MS and was approved for use by the FDA in March of 2013. Importantly, biopsies from a patient with MS, as well as three psoriasis patients with progressive multifocal leukoencephalopathy (single cases in connection with FAE treatments), identified astrocytes as staining positive for both nuclear and cytoplasmic Nrf2, whereas oligodendrocytes had mostly cytoplasmic staining and only on single occasions nuclear Nrf2 staining [183]. Thus, although FAE indeed modulate immune function in MS, activation of the Nrf2 signaling pathway may also be an important mechanism of action for the prevention of oxidative-stress-induced neuronal toxicity.

In addition to the FAE, several more recent studies using natural compounds such as sulforaphane, an organosulfur compound found in cruciferous vegetables, and matrine, a quinolizidine alkaloid derived from the herb *Sophora flavescens*, as well as CDDO-TFEA, a derivative of the natural triterpene oleanolic acid, have all been identified as attenuating EAE through Nrf2-modulatory, as well as immunomodulatory mechanisms [176–178]. More specifically, sulforaphane was shown to ameliorate EAE by preserving the blood–brain barrier, reducing the levels of the autoimmune T cell, Th17, in addition to antagonizing oxidative stress via activation of Nrf2, as evidenced by increased expression of NQO1 and HO-1 in brain tissues of treated mice [177]. Matrine treatments also were effective in EAE, by which tissue from spinal cord immunohistochemically identified increased protein levels of Nrf2 and HO-1 in treated mice [178]. Last, CDDO-TFEA

completely suppressed EAE disease in mice, presumably through suppression of inflammatory cytokines, specifically IL-17, and induction of Nrf2-dependent antioxidant gene expression in both CNS and peripheral lymphocytes. In addition, CDDO-TFEA enhanced myelin repair in a lysophosphatidylcholine-induced model of demyelination [176]. Alleviation of neuroinflammatory-induced oxidative stress through activation of Nrf2 and induction of antioxidant is thus an important therapeutic target for prevention of oxidative stress associated with MS, other autoimmune disorders, as well as neuroinflammation associated with other neurodegenerative diseases.

Nrf2 and neuroinflammation

A pathological hallmark feature of neurodegenerative disease is neuroinflammation, mediated primarily by activated resident innate immune cells, the microglia. When activated, microglia secrete cytotoxic reactive oxygen and nitrogen species, contributing to the deleterious affects on neurons, especially in cases of microglial overactivation and dysregulation. Furthermore, components of dead or damaged neurons also activate microglia (reactive microgliosis) via pattern recognition receptors (e.g., toll-like receptors), resulting in a perpetuating cycle of neuronal cell death [184, 185]. In an adult human brain, cell densities of microglia are brain-region-dependent and range from 0.5 to 16.6%. Under normal conditions, they reside predominantly in a resting ramified-like morphology in the gray matter, with the highest concentrations found in hippocampus, olfactory telencephalon, basal ganglia, and substantia nigra [185, 186]. Thus, it comes as no surprise that these brain regions may be more susceptible to reactive microgliosis, as observed in several neurodegenerative diseases, such as AD, PD, HD, and ALS [187].

The most abundant source of reactive species produced by microglia is initiation of the NADPH oxidase (NOX) system generating superoxide anions ($\bullet O_2^-$). Upon activation by various stimuli, including pathogen-associated molecular patterns (PAMPs), inflammatory cytokines, and/or neurotoxins, the membrane-bound NOX enzyme complex innately promotes microbial killing, as well as changes in morphology, cellular migration, and regulation of proinflammatory gene expression. However, persistent activation of NOX results in peroxidation of lipids and other macromolecules and activation of apoptosis, thus promoting neuronal cell death and degeneration. Similarly, inducible nitric oxide synthase (iNOS) enzyme is induced and its activation results in generation of nitric oxide (NO•), which like $\bullet O_2^-$ is neurotoxic at continuously high levels. High concentrations of NO• unbalance redox homeostasis, and NO• can also react with $\bullet O_2^-$, resulting in a more toxic reactive species, peroxynitrite ($ONNO^-$) [188].

Under resting conditions, microglia continuously survey the surrounding environment in search of pathogens, sick cells, or cellular debris, as well as participating in synaptic remodeling and/or maintenance. However, upon activation by hazardous components, microglia become active and in turn secrete proinflammatory mediators such as TNF, interleukin (IL)-1, IL-6, IL-12, and IL-23. This phase of microglia activation is commonly known as the classical proinflammatory phase and termed M1. Following the M1 phase is a longer-lasting anti-inflammatory or alternative phase, M2. Microglia in this phase are activated by anti-inflammatory cytokines, which include IL-4, IL-10, and IL-13.

Subsequently, these same cytokines are produced, in addition to increased expression of genes involved in wound healing, such as arginase-1 (ARG1), mannose receptors (MMC), found in inflammatory zone 1 (FIZZ1), and chitinase-3-like-1 (Ym1 in rodents). Under normal conditions however, the microglia antioxidant machinery maintains cellular homeostasis and thus microglia are maintained in steady state surveillance mode [185].

The master regulator of the inflammatory responses to brain infections and environmental and cellular damage is the transcription factor NF-kappaB (NF-kß), and it is the redox state of microglia that controls regulatory kinases upstream of NF-kß, as well as NF-kß nuclear levels (recently reviewed in [185]). Microglia function normally when basal levels of OS are managed by antioxidant machinery; however, persistent OS or NS continuously activates the NF-kß signaling pathway, amplifying the proinflammatory function of microglia and thus contributing to microglial overactivation and neurotoxic consequences. Thus, the transcription factor Nrf2, associated with activation of antioxidant genes and redox homeostasis, is the master regulator of microglia fate, protecting microglia from oxidative stress and overactivation of the M1 phenotype. More specifically, activation of Nrf2 drives expression levels of genes involved in (1) OS clearance-SOD3, GPx, and Prx; (2) reducing factor producing enzymes GCLM/C or NADPH (via glucose-6-phosphate dehydrogenase and/or phosphogluconate dehydrogenase); (3) enzymes involved in regeneration of reduced cofactors and proteins, GR, Trx-TrxR, Prx/sulfiredoxin, or redox transporters, xCT; 4) metal chelators, MT1/2 or ferritin; and 5) antioxidant enzymes, HO-1, NQO1, and Trx, and inhibition of the expression of the Trx inhibitor, TXNIP [185].

Several studies have described Nrf2 as a therapeutic target for the alleviation of brain inflammation associated with neurodegeneration and subsequent neuronal protection. In one study, Nrf2−/− mice injected with lipopolysaccharide (LPS), a common inflammogen, were hypersensitive to LPS-induced neuroinflammation in hippocampal tissue. Protein and mRNA for the cellular microglia marker F4/80 were increased in hippocampi of Nrf2−/− mice compared with WT mice, as were the inflammatory markers iNOS, IL-6, and TNF-α. Additionally, mice treated with SFN displayed a two- to threefold increase in HO-1, a reduced abundance of microglia, and an attenuated production of these same inflammatory markers in response to LPS [189]. SFN-treated rats subjected to spinal cord injury had significantly increased levels of Nrf2 and GCL and decreased levels of the inflammatory cytokines, IL-1ß and TNF-α, resulting in reduced contusion volume and improved coordination [190].

In a mouse model of PD, inoculation of MPTP in Nrf2−/− mice demonstrated hypersensitivity to OS, with basal ganglia of these mice exhibiting more severe dopaminergic dysfunction when compared with WT littermate controls. Although Cd11b-positive/CD45 highly stained cells, indicative of peripheral macrophage infiltration, were not significantly different between WT and Nrf2−/− mice, the latter mice exhibited more astro- and microgliosis, as determined by increased expression levels of message and protein for GFAP and F4/80 [120]. This validated earlier studies showing increased neuroinflammation associated with astrocytic (GFAP) and microglia (Iba-1) activation in Nrf2−/− mice exposed to MPTP [108]. Furthermore, inflammatory markers for classically activated microglia (M1), COX2, iNOS, IL-6, and TNF-α were also increased, while

markers for alternatively activated microglia (M2), FIZZ-1, YM-1, arginase-1, and IL-4, were decreased. These results demonstrate a critical role of Nrf2 in the modulation of neuroinflammation secondary to a neurotoxic insult [120].

In hippocampus of mice that were stereotactically injected with an adeno-associated viral vector expressing TAU^{P301L} (inducing a mouse model of tauopathy), Nrf2−/− mice showed increased microgliosis and astrogliosis. This was verified by mRNA levels for TNF and IL-6 in hippocampus as well as immunohistochemical staining for Iba-1 and GFAP. In contrast to WT mice, hippocampal mRNA levels for HO-1 and GCLC did not increase in the Nrf2−/− nor did cellular staining for HO-1. This suggests that the Nrf2 signaling pathway was activated by TAU^{P301L}-induced pathology in an attempt to modulate the inflammatory response. Based on these observations, as well as studies demonstrating that neuronal expression of the chemokine fractalkine (CX3CL1) modulates TAU-mediated neuroinflammation, these same authors demonstrated that CX3CL1 secreted by injured neurons acted on CX3CR1 receptors (exclusive to microglia), activating the Nrf2-ARE signaling pathway and attenuating TAU^{P301L} -induced microgliosis. The latter studies were supported by observations of increased hippocampal microgliosis in CX3CR1-deficient mice in response to TAU^{P301L}, when compared with WT controls, in addition to the complete loss of the Nrf2-dependent HO-1 immunohistochemical staining in this same cohort. Thus, the release of the chemokine CX3CL1 by injured neurons in turn communicates to microglia via the CX3CR1 receptor, subsequently activating the Nrf2-ARE pathway, to dampen the neuroinflammatory response to injury [191].

In another study using mouse primary neuronal microglia-conditioned media and co-culture experiments, the release of the milk fat globule-EGF factor 8 (MFG-E8) from microglia in response to signals from degenerating neurons exposed to neurotoxic oligomeric amyloid ß (oAß) enhanced the phagocytic activity of microglia. Additionally, microglia treated with MFG-E8 activated the Nrf2 signaling pathway and HO-1. However, in neuronal-microglia co-cultures, the neuroprotection afforded by MFG-E8 was reversed with the HO-1 inhibitor, Tin (IV)-mesoporphyrin IX dichloride (SnMP) treatment, suggesting the importance of HO-1 increase in microglia and subsequent protection of neurons from toxicity [192]. These studies suggest that cellular communication between neurons and microglia and activation of the Nrf2 signaling pathway may be a novel therapeutic approach in the protection of neurons, particularly in neurodegenerative diseases.

NFE2L2 and *KEAP1* associated haplotypes and neurodegenerative diseases

Based on substantial evidence (as discussed in this review) that neurodegenerative effects of OS/NS in diseases such as PD, AD, and ALS can be modulated and/or prevented by up-regulation of Nrf2, genomic studies have been undertaken to ascertain if common genetic variations exist in the genes encoding Nrf2 and its repressor protein Keap1, *NFE2L2* and *KEAP1*, thus contributing to the onset or risk of these diseases. Interestingly, based on compilation of publicly available SNPs and other genetic variations, it is estimated that the mutagenic frequency of human *NFE2L2* is 1 per 72 bp, suggesting that several polymorphisms of this gene may exist [193]. In initial case-controlled studies of patients

with PD, von Otter et al. identified a protective *NFE2L2* haplotype, G**AGC**AAAA, which included a fully functional variant of the Nrf2 promoter associated with high transcriptional activity in Swedish and Polish material [194]. In the Swedish case study, the GAAAA haplotype allele was associated with an estimated increased age at onset (AAO) of PD of approximately five years per haplotype allele ($p = 0.001$); while in the Polish study, the full protective haplotype, G**AGC**AAAA, showed a decrease in risk of PD with odds ratios of 0.4 and 0.2 for heterozygous and homozygous carriers, respectively ($p = 2 \times 10^{-6}$). Additionally, the Swedish material identified the haplotypes GAGGG and GAAAG in association with increased risk of PD with odds of 2.4 and 3.7 per allele, respectively. Interestingly, the promoter haplotype AGA showed a tendency toward increased risk of PD and full risk with the full promoter haplotype G**AGA**AGGG (odds ratio of 2.8 per haplotype allele). However, the full haplotype allele, G**AGC**AAAA, was estimated to delay PD AAO per haplotype allele. No individual SNPs alone, however, significantly affected risk of PD in either Swedish or Polish material. In addition, genetic variations of *KEAP1* were also analyzed; however, no associations to AAO or risk of PD were detected [194].

A more recent meta-analysis of genetic associations of Nrf2-encoding *NFE2L2* variants in PD using the above data in addition to four new independent European patient-control materials (from Malta, Germany, Italy, and a second Swedish study) also demonstrated an association of haplotype G**AGC**AAAA (including the fully functional promoter haplotype AGC) with decreased risk (odds ratio (OR) of 0.8 per allele, $p = 0.012$) and delayed onset of (+1.1 years per allele, $p = 0.048$) of PD [195]. These results supported the initial haplotype study that associated variations in the *NFE2L2* gene with pathogenesis of PD. The latter study also described four single nucleic acid polymorphisms (SNPs) included in the haplotypes that were associated with AAO of PD (three SNPs that promoted onset included: rs7557529 G>A, −1.0 years per allele, $p = 0.042$; rs35652124 A>G, −1.1 years per allele, $p = 0.045$; rs2886161 A>G, −1.2 years per allele, $p = 0.021$; and delayed onset SNP: rs1806649 G>A, +1.2 years per allele). Of note, SNP rs35652124 is fully functional and located in the *NFE2L2* promoter [195].

In contrast with the above PD studies, genetic analysis of the *NFE2L2* promoter did not show variation in a Taiwanese patient controlled PD study. Three SNPs located in the *NFE2L2* promoter, rs35652124, rs6706649, and rs6721961, were analyzed independently or with haplotype variants that were associated with PD susceptibility, yet did not correlate with PD susceptibility. The authors concluded that the disparity between the Taiwanese and European studies might be attributed to genomic differences in ethnicity and environmental factors in different geographical regions [196].

Similar studies using AD Swedish case-controlled material did not support *NFE2L2* or *KEAP1* single SNPs or haplotypes as risk susceptibility genes for AD [197]. However, haplotype analysis of age at AD onset showed a significant association of haplotype GAAAA with 2 years earlier age at AD onset ($p = 0.013$), suggesting that common variants of the *NFE2L2* gene may affect AD progression thus altering clinically recognized disease onset. Gene variants of *NFE2L2* or *KEAP1* were not associated with results of mini-mental state examination (MMSE) or CSF biomarker levels of total tau protein and amyloid ß 1-42 ($Aß_{42}$) in these same AD case-control studies [197].

Swedish case-control studies of *NFE2L2* and *KEAP1* in ALS disease identified one *NFE2L2* haplotype (GGGAC) associated with decreased risk of sporadic ALS (OR = 0.62 per allele, $p = 0.015$) and one haplotype in *KEAP1* (CGC) correlated with delayed sporadic ALS onset (+3.4 years per allele, $p = 0.015$). In addition, further stratification of ALS into subgroups identified one subgroup having an *NFE2L2*-associated haplotype of GAGCAGA, which included three functional promoter SNPs associated with high levels of Nrf2 protein expression, with 4.0 years delayed onset of ALS per allele ($p = 0.008$) [198]. In one other case-controlled study in Italian ALS patients, three *NFE2L2* promoter polymorphisms (SNP -653 A/G, -651 G/A, and -617 C/A), no significant differences were observed between ALA cases and controls. The -653 A/G promoter polymorphism was slightly higher in patients than in controls, but it did not attain significance. Furthermore, oxidative stress biomarkers assessed in this same study did not associate with and were independent from the *NFE2L2* promoter polymorphisms surveyed [193]. Similar studies have not yet been conducted on HD or MS patients.

Conclusions

Based on the information provided herein, the Nrf2-ARE pathway is a high-value therapeutic target for neurodegenerative diseases. The existing data are strongest for PD, ALS, and MS, but ongoing experiments in AD and HD should provide more insight into how important Nrf2 is in these diseases as well. Numerous cell-based and in silico high-throughput screens have identified novel Nrf2-activating compounds [199–205]. The most promising approach may be to find compounds that noncovalently disrupt the DLG or ETGE motif interactions with Keap1 [206, 207]. Currently, DMF (BG-12) from Biogen Idec has been FDA-approved for treating MS. As discussed above, DMF has also been tested in preclinical models of HD; however, only the CDDO compounds have been tested in preclinical models of the diseases discussed. Reata Pharmaceuticals moved the first CDDO compound (Bardoxolone methyl; CDDO methyl ester) into a Phase III clinical trial for the treatment of chronic kidney disease. Unfortunately, the trial was stopped in 2012 due to a higher rate of cardiovascular events observed in the bardoxolone methyl group compared to placebo [208]. A new CDDO derivative (RTA 408) is already developed and being tested in clinical trials for a variety of non-neurodegenerative diseases, [209–212]. More recently, a clinical trial using RTA 408 was initiated for Friedreich's ataxia, a neurodegenerative condition responsible for cerebellar ataxia due to impaired production of the protein frataxin leading to profound deficiencies in mitochondrial respiration (ClinicalTrials.gov Identifier NCT02255435). Movement of these next generation CDDO compounds into the major neurodegenerative diseases discussed in this review could be an important step forward, based on the preclinical data from earlier generation CDDO compounds.

An interesting new twist to therapeutic approaches targeting this pathway is that one cell type may be better than another at protecting neurons from degeneration. The lack of protection in vivo by overexpression of Nrf2 in neurons or muscle, but protection by overexpression in astrocytes in ALS models, as well as PD models, highlights this concept that not only the pharmacological target, but also the cell type targeted may be relevant when a Nrf2-dependent therapeutic approach is considered.

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Highlights

- Oxidative stress in neurodegenerative diseases
- The Keap1-Nrf2-ARE pathway in the brain
- Changes in Nrf2 and Nrf2-dependent genes in diseased brain tissue
- Transgenic, viral, and chemical-mediated activation of Nrf2 in animal models
- Effect of Nrf2 activation on progression of neurodegeneration in animal models

Table 1

Chemical activators of Nrf2 that have been tested in animal models of PD.

Compound	PD model	Species	Nrf2−/−	Reference
3H-1,2-Dithiole-3-thione	MPTP	Mouse	Yes	[118]
CDDO-MA	MPTP	Mouse	No	[164]
Sulforaphane	MPTP	Mouse	Yes	[121])
S-Allyl cysteine	6-OHDA	Rat	No	[213]
S-Allyl cysteine	MPTP	Mouse	No	[214]
Gly-Pro-Glu bound to L-dopa	MPTP	Mouse	No	[110]
siKeap1	MPTP	Mouse	No	[215]
Puerarin	6-OHDA	Rat	No	[115]
CDDO-EA	MPTP	Mouse	Yes	[122]
CDDO-TFEA	MPTP	Mouse	Yes	[122]
(R_S)-Glucoraphanin	MPTP	Mouse	No	[216]
Phloroglucinol	6-OHDA	Rat	No	[217]
Tetramethylpyrazine	MPP^+	Rat	No	[11]
Naringenin	6-OHDA	Mouse	No	[218]
Gastrodin	MPTP	Mouse	No	[219]
Vinyl sulfones	MPTP	Mouse	No	[220]
VSC2	MPTP	Mouse	No	[221]
Dimethyl fumarate	6-OHDA	Mouse	No	[222]
KMS04014	MPTP	Mouse	No	[223]
Protocatechuic acid + Chrysin	6-OHDA	Mouse	No	[224]
Fasudil	MPTP	Mouse	No	[225]