British Journal of Pharmacology

British Journal of Pharmacology (2017) **174** 1750–1770 1750

Themed Section: Redox Biology and Oxidative Stress in Health and Disease

REVIEW ARTICLE

Redox-based therapeutics in neurodegenerative disease

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Received 26 August 2015; **Revised** 2 June 2016; **Accepted** 1 July 2016

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This review describes recent developments in the search for effective therapeutic agents that target redox homeostasis in neurodegenerative disease. The disruption to thiol redox homeostasis in Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis and multiple sclerosis is discussed, together with the experimental strategies that are aimed at preventing, or at least minimizing, oxidative damage in these diseases. Particular attention is given to the potential of increasing antioxidant capacity by targeting the Nrf2 pathway, the development of inhibitors of NADPH oxidases that are likely candidates for clinical use, together with strategies to reduce nitrosative stress and mitochondrial dysfunction. We describe the shortcomings of compounds that hinder their progression to the clinic and evaluate likely avenues for future research.

LINKED ARTICLES

This article is part of a themed section on Redox Biology and Oxidative Stress in Health and Disease. To view the other articles in this section visit http://onlinelibrary.wiley.com/doi/10.1111/bph.v174.12/issuetoc

Abbreviations

6(OH)DA, 6-hydroxydopamine; AD, Alzheimer's disease; ADME(T), absorption, distribution, metabolism and excretion/toxicity; ALS, amyotrophic lateral sclerosis; APP, amyloid precursor protein; ARE, antioxidant response element; ASSNAC, S-allylmercapto-N-acetyl cysteine; Aβ, amyloid-β; CA, carnosic acid; DMF, dimethylfumarate; EAE, experimental autoimmune encephalomyelitis; EpRE, electrophile response element; GCL, glutamate cysteine ligase; GR, glutathione reductase; Grx, glutaredoxin; GSK-3β, glycogen synthase kinase-3β; Keap1, Kelch-like ECH-associated protein-1; MMF, monomethylfumarate; MPTP, 1-methyl, 4-phenyl-1,2,3,6-tetrahydropyridine; MS, multiple sclerosis; MPO, myeloperoxidase; NAC, N-acetylcysteine; NACA, N-acetylcysteine amide; NOX, NADPH oxidase; NQO1, NAD(P)H quinone oxidoreductase-1; Nrf2, nuclear factor (erythroid-derived 2)-like 2; PD, Parkinson's disease; PS1, pre-senelin-1; RNS, reactive nitrogen species; SN, substantia nigra; Trx, thioredoxin; TrxR, thioredoxin reductase; xCT, functional subunit of the x_c^- exchanger

DOI:10.1111/bph.13551

Tables of Links

TARGETS	
Enzymes[a]	**Other proteins**[b]
Akt	Keap1, Kelch-like ECH-associated protein-1
GSK-3β, glycogen synthase kinase-3β	**Transporters**[c]
HO-1, haemoxygenase-1	xCT- cystine-glutamate exchanger, SLC7A11
Myeloperoxidase	**Ligand-gated ion channels**[d]
NO synthases	P2X7 purinergic receptor
P13K	

LIGANDS	
Bardoxolone	Levetiracetam
Chlorpromazine	Resveratrol
Curcumin	Sulfasalazine
DMF, dimethyl fumarate	Sulforaphane
DPI, diphenyleneiodonium chloride	Zonisamide

These Tables list key protein targets and ligands in this article that are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Southan *et al.*, 2016) and the Concise Guide to PHARMACOLOGY 2015/16 ([a,b,c,d]Alexander *et al.*, 2015a,b,c,d).

Disruption of cerebral redox homeostasis is a common occurrence in a range of human neurodegenerative disorders, and although much is understood of mechanistic dysfunction, the gap between knowledge and the availability of effective therapies remains wide. In this review, we focus on the potential for developing therapeutic agents that promote the availability of thiol redox antioxidants or boost the antioxidant capacity of cells via stimulation of the transcription factor, nuclear factor (erythroid-derived 2)-like 2 (Nrf2). In addition, we discuss options for reducing ROS production by inhibition of NADPH oxidases (NOXs), limiting nitrative stress or targeting mitochondrial dysfunction. Other potential strategies for limiting oxidative stress in neurodegenerative disease include nutrient and vitamin supplementation and metal chelators, which are excluded from the discussion. These approaches are the subject of several recent reviews, to which the reader is referred for more information (Bigford and Del Rossi, 2014; Casamenti *et al.*, 2015; Di Domenico *et al.*, 2015; Fernando *et al.*, 2015; Gess *et al.*, 2015; Harrison *et al.*, 2014; La Fata *et al.*, 2014).

We describe the current understanding of the basis for thiol redox imbalance in neurodegenerative disease and discuss the potential of thiol redox-based therapies for the treatment of disorders that include Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS) and multiple sclerosis (MS). In many instances, while the results from *in vitro* experiments are encouraging, the absence of good animal models that fully replicate the human condition hampers a thorough evaluation of antioxidant-based therapies. In other cases, the absence of early disease biomarkers adds to the difficulty in establishing successful therapeutic strategies. Moreover, many antioxidants showed no efficacy in clinical trials for reasons that include absence of specific targets, difficulty in gaining access to the brain or an inappropriate time-course of action that may not map to a late-onset and slowly progressing disease. Nonetheless, the search for potential therapies to combat disruption of thiol-redox homeostasis is ongoing, and we report on the most promising developments in this field.

The strategy of targeting Nrf2 in neurodegenerative disorders has much to offer. It is anticipated that modulation of Nrf2 activity may provide two advantages over direct antioxidants. First, the induction of NADPH, glutathione (GSH) and thioredoxin (Trx) metabolism is a natural system which may boost antioxidant activity in places where needed, whilst leaving physiological ROS signalling intact. Second, because proteins have a longer half-life than low MW activators, the effect on the antioxidant defence may be more prolonged. Many Nrf2 inducers are electrophilic compounds that react with the cysteine residues in Kelch-like ECH-associated protein-1 (Keap1). Nevertheless, it has been recently shown that several molecules are capable of inhibiting the protein–protein interaction between Keap1 and Nrf2.

The NOXs, as a key source of reactive oxygen species (ROS) in neurodegenerative diseases, are increasingly recognized as potential therapeutic targets. Recent developments in the design and synthesis of NOX subtype-specific ligands bring the likelihood of therapeutic application closer to reality.

Activation of glial cells (microglia and astrocytes) is a common characteristic of neurodegenerative diseases and is accompanied by an increased production of NO through upregulation of inducible nitric oxide synthase (iNOS). It is well established that NO release from activated glial cells has the capacity to cause extensive neurodegeneration, for example, via inhibition of mitochondrial cytochrome oxidase (Brown and Cooper, 1994) or release of glutamate causing hyperactivation of NMDA receptors (Bal-Price and Brown, 2001). The mechanisms of microglial cell activation are the subject of recent reviews and will not be discussed here (Rojo *et al.*, 2014; Vilhardt *et al.*, 2017). However, the oxidative effects of increased NO production are pertinent to this discussion and are viewed as a potential target for redox-based therapeutics.

There is increasing recognition that mitochondrial oxidative stress is a significant component in neurodegenerative disease (Ruszkiewicz and Albrecht, 2015). For example, one of the correlates of increased oxidant production in PD is mitochondrial dysfunction in dopaminergic neurons, leading

to neurodegeneration (Dranka *et al.*, 2012). Moreover, mitochondrial neuronal nitric oxide synthase (mtNOS) generates NO in immediate proximity to high concentrations of superoxide, thus favouring formation of peroxynitrite, leading to nitrative stress. With the emergence of molecules with improved membrane permeability, the possibility of selectively targeting mitochondrial respiration is closer to reality.

Thiol-based redox therapies in neurodegenerative disease

GSH is the principal thiol-based antioxidant in the brain and is present in millimolar concentrations in both neurons and glial cells. GSH readily oxidizes to GSSG, but is maintained in the reduced form due to transfer of electrons from NADPH in a reaction catalysed by glutathione reductase (GR). Astrocytes and microglial cells are responsible for *de novo* synthesis of GSH, whereas neurons rely on precursors that are supplied by astrocytes (Dringen *et al.*, 1999). Cysteine and glutamate are co-substrates of the rate-limiting step of GSH synthesis that is catalysed by γ-glutamate cysteine ligase (GCL). In astrocytes, transulphuration of methionine to cysteine makes a minor contribution to GSH synthesis (Vitvitsky *et al.*, 2006; Kandil *et al.*, 2010; McBean, 2012), but in these cells and microglia, the majority of cysteine comes from uptake of its oxidized form, cystine, from the extracellular space. The inward transport of cystine is mediated by the x_c^- cystine-glutamate exchanger located in the plasma membrane that was first characterized in the 1980s (Bannai, 1984). The exchanger is comprised of two subunits, 4F2hc, which stabilizes the protein to the membrane and xCT that confers substrate selectivity. The two subunits are joined by a disulphide bridge (Sato *et al.*, 1999). One of the most interesting characteristics of the x_c^- exchanger is that the export of glutamate provides the driving force for cystine uptake (Bannai, 1984). This occurs as a 1:1 exchange, and it is increasingly recognized that extracellular glutamate derived from the exchanger has both physiological and pathological functions (Lewerenz *et al.*, 2013). Glutamate released from the exchanger is re-cycled into astrocytes by the high affinity glutamate transporters (Piani and Fontana, 1994; Sato *et al.*, 1999). Changes in expression and function of the x_c^- exchanger in response to disease have a significant bearing on thiol redox balance and antioxidant status. Moreover, the inextricable association between the need to import cystine for GSH and the concurrent release of glutamate via the exchanger gives rise to a potentially excitotoxic concentration of glutamate (see McBean *et al.*, 2015). Thus, while such a change in extracellular glutamate does not directly involve redox homeostasis, it is part of the bigger picture and cannot be entirely excluded from this discussion.

In PD, a deficiency in complex 1 of the electron transport chain is regarded as one of the contributing factors in the development of the disease (Papa and De Rasmo, 2013). On the one hand, it increases O_2^- production, but, on the other, it decreases ATP. In turn, a fall in ATP leads to a decline in the GSH/GSSG redox potential. Brain tissue taken *post mortem* from PD patients shows a 40% reduction in GSH in the substantia nigra (SN), compared with normal tissue, but no change in the rest of the brain (Perry and Yong, 1986). Additionally, GSH in the SN of other neurodegenerative diseases affecting this region, such as multiple system atrophy and supranuclear palsy, is unchanged. Expression of the functional subunit (xCT) of the x_c^- exchanger in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD is increased in the striatum, but reduced in the SN. However, MPTP-associated depletion of dopamine in the SN is unaffected by loss of the exchanger (Bentea *et al.*, 2015a).

Neurodegenerative disorders that entail activation of microglial cells commonly display enhanced activity of the x_c^- exchanger and elevated levels of extracellular glutamate. In AD, senile plaques contain reactive microglial cells that contribute to neurotoxicity by releasing glutamate via the x_c^- exchanger (Qin *et al.*, 2006). Similarly, injection of amyloid-β (Aβ) into the hippocampus of adult mice promotes expression of the exchanger subunit xCT in activated microglia (Qin *et al.*, 2006). Increased expression of xCT and elevated extracellular glutamate in the cortex in 18 month old AβPP23 mice has also been identified (Schallier *et al.*, 2011b). In ALS, the x_c^- exchanger is expressed in spinal cord samples taken *post mortem* from ALS patients that correlate positively with the extent of inflammation (Mesci *et al.*, 2015). Similarly, in the SOD1-G93 A mouse model of ALS, there is increased protein expression of the x_c^- exchanger in spinal cord and isolated microglial cells. In acute spinal cord slices of 70 day old SOD1 G93 A mice, x_c^--mediated cystine uptake was increased, but was unchanged at 50, 100 or 130 days (Albano *et al.*, 2013).

In MS, the elevated ROS originate from activated microglia and invading macrophages, due to increased expression of NOXs, myeloperoxidases (MPOs) and inducible nitric oxide synthase (iNOS) (Carvalho *et al.*, 2014). In addition, thiol redox homeostasis is disrupted: GSH levels, as measured by magnetic resonance imaging, are reduced in the CSF of MS patients, whereas blood GSH levels are increased, possibly due to heightened activity of GR, compared with normal controls (Carvalho *et al.*, 2014). Increased expression of the x_c^- exchanger has been reported in activated human monocytes that are accompanied by enhanced release of glutamate. Expression of the x_c^- exchanger is likewise elevated in the experimental autoimmune encephalomyelitis (EAE) mouse model of MS, as well as in CNS and peripheral blood samples from patients with the disease (Pampliega *et al.*, 2011). Accordingly, elevated concentrations of glutamate have been recorded in CSF and plasma of MS patients.

GSH precursors, analogues and conjugates

Several attempts have been made to potentiate the antioxidant capacity of brain tissue in animal models of neurodegenerative disease. For example, strategies to reduce neurodegeneration in PD include both direct and indirect approaches to restore GSH levels, on the premise that such action would boost the antioxidant capacity and therefore, reduce the rate of neurodegeneration (Johnson *et al.*, 2012; Smeyne and Smeyne, 2013; Johnson *et al.*, 2015a). Efforts to supply GSH directly have failed, due to limits of solubility, absorption, stability and the fact that GSH has a short half-life in human plasma. For example, Zeevalk and colleagues observed a dose-dependent increase in GSH using a monoethylester of GSH (GEE) in rat mesencephalic cultures

(Zeevalk *et al.*, 2007). However, peripheral administration of GEE to rats (0.1–50 mg·kg^{-1}·day^{-1} for 28 days) failed to produce a significant elevation in brain GSH, whereas direct intracerebral delivery was effective.

The recent emergence of GSH derivatives may provide an opportunity for development of more effective therapeutic agents. Other approaches to assess delivery of GSH as a therapeutic option in PD include liposomes or nanoparticles, co-drugs (GSH: dopamine or GSH:L-dopamine decarboxylase conjugates) and GSH analogues or other hybrid compounds (Zeevalk *et al.*, 2007; Zeevalk *et al.*, 2010; Cacciatore *et al.*, 2012; Smeyne and Smeyne, 2013). Overall, the results are variable and minimal improvement in intracellular GSH levels has been observed. Ultimately, nanoparticle-associated GSH delivery may prove to be the best option, as this technique permits longer-term drug delivery and better brain penetration than comparable approaches.

In a comparative study on the effects of antioxidants on the activity of cerebrocortical Mn superoxide dismutase (MnSOD) in mitochondria isolated from *post mortem* human AD brain, GSH was ineffective, whereas synthetic GSH analogues (for example, 4-methoxy-L-tyrosinyl-L-γ-glutamyl-L-cysteinylglycine) effectively increased MnSOD activity (Kairane *et al.*, 2014). However, further work is required to determine whether off-target effects of GSH analogues limit their potential as therapeutic agents.

Direct administration of cysteine to increase GSH is not a viable option, because of poor absorption and the toxicity of high doses (Johnson *et al.*, 2012). In experimental systems, N-acetylcysteine (NAC) can be substituted for cysteine as a GSH precursor and has been used many times to boost GSH levels (Figures 1 and 2). NAC is readily taken up *in vitro* and potentiates intracellular GSH synthesis, promotes GSH-mediated detoxification mechanisms and scavenges ROS. *In vivo*, the picture is less clear; NAC is well absorbed by the intestine, and dietary supplementation is effective for increasing GSH (Atkuri *et al.*, 2007). However, NAC is only effective in situations in which GSH is depleted, and it does not work independently of GSH as an antioxidant (Schmitt *et al.*, 2015). Moreover, dietary NAC may be metabolized to hepatic GSH before reaching the brain (Schmitt *et al.*, 2015). N-acetylcysteine amide (NACA) has greater lipophilicity, membrane permeability and antioxidant capacity, compared with NAC. The blood brain barrier (BBB) permeability of NACA has been demonstrated using cell lines and rodent models of PD and AD (Sunitha *et al.*, 2013). NACA has recently been shown to prevent neuronal degeneration following focal penetrating traumatic brain injury in rats (Günther *et al.*, 2015) and may ultimately prove to be a more effective therapy than NAC for neurodegenerative disorders.

Another option is S-allylmercapto-N-acetylcysteine (ASSNAC) (Savion *et al.*, 2014). In the model of MS, mice with experimental autoimmune encephalomyelitis (EAE), spinal cord and brain GSH increased by 54 and 47%, respectively, following administration of ASSNAC (200 mg·kg^{-1}·day^{-1}) and the clinical symptoms showed greater improvement than with NAC. ASSNAC activates Nrf2 and increases the expression of phase II detoxifying enzymes that include the x_c^- exchanger and GCL (Savion *et al.*, 2014).

In summary, the effectiveness of compounds that increase GSH production through provision of cysteine in the brain may be limited by hepatic metabolism, and/or the fact that they can only restore depletion of GSH to normal levels. It is possible that, in the future, a stimulation of GSH beyond normal levels may be achieved by coupling GSH precursors with Nrf2 activators to upregulate enzymes that control GSH synthesis.

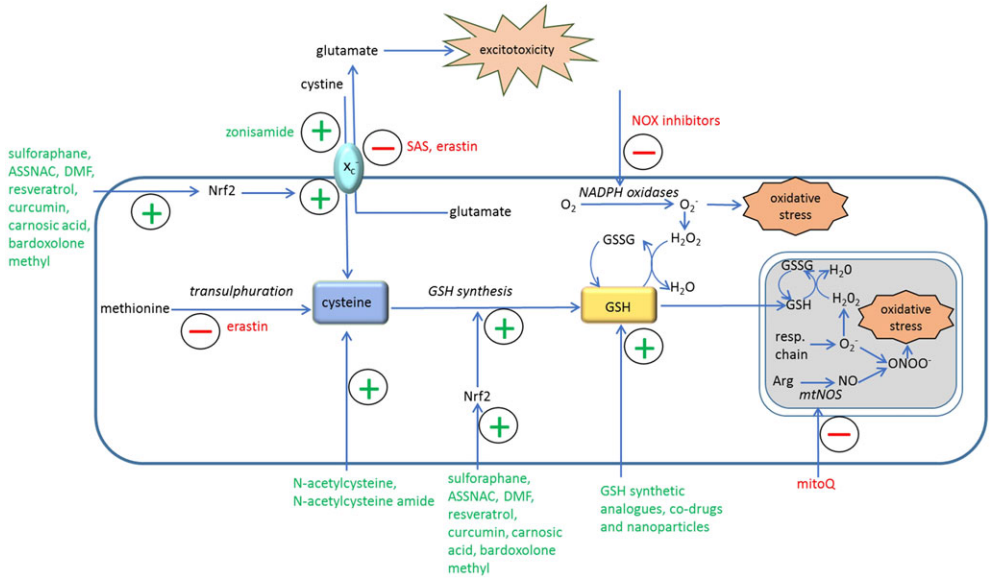

Figure 1

Schematic diagram of redox-based therapeutic strategies in neurodegenerative disease models. Arg, L-arginine; ASSNAC, S-allyl-mercapto-N-acetyl cysteine; GSSG, oxidised glutathione; DMF, dimethylfumarate; mtNOS, mitochondrial nitric oxide synthase; resp.chain, mitochondrial respiratory chain; SAS, sulfasalazine; x_c^-, cystine-glutamate exchanger.

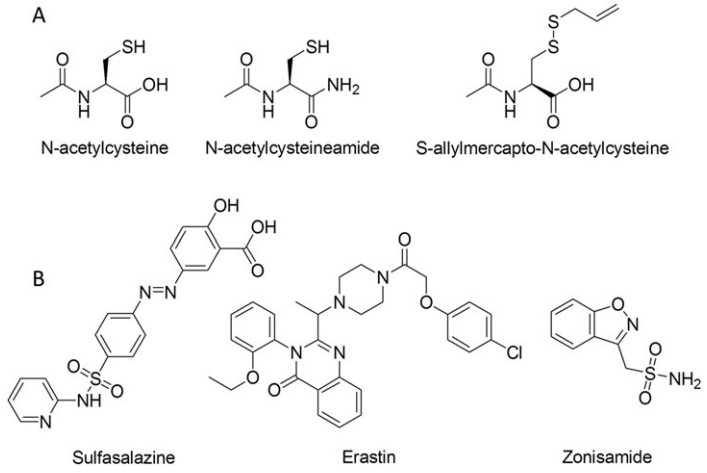

Figure 2

A, chemical structures of low MW thiols that increase cysteine or GSH and B, compounds that target the x_c^- cystine glutamate exchanger.

The x_c^- cystine-glutamate exchanger

The x_c^- exchanger is viewed by many as a potential therapeutic target, particularly in instances wherein limitation of cysteine availability and GSH synthesis is deemed advantageous for patient survival, such as in the treatment of brain tumours (McBean, 2012). Considerable effort has been devoted to the search for selective inhibitors of the x_c^- exchanger (De Groot and Sontheimer, 2011; Bridges *et al.*, 2012; Sontheimer and Bridges, 2012). Sulfasalazine (2-hydroxy-5-[(E)-2-{4-[(pyridin-2-yl)sulfamoyl]phenyl}diazen-1-yl] benzoic acid) is one of the most promising, but *in vivo* use (tested for treatment of glioma patients) has been of limited value. This is due to lack of selectivity and a high rate of metabolism by intestinal bacteria (Gout *et al.*, 2001). More recently, erastin (2-[1-[4-[2-(4-chlorophenoxy)acetyl]-1-piperazinyl]ethyl]-3-(2-ethoxyphenyl)-4(3H)-quinazolinone) has been shown to inhibit both the x_c^- exchanger and cystathionine-γ-lyase, regulatory enzyme of the transsulphuration pathway, in glioma cells (Chen *et al.*, 2015) (Figure 2). Its effectiveness in models of neurodegenerative disease remains to be determined.

Experiments on manipulation of the x_c^- exchanger in PD models have revealed conflicting results. Repeated injections of the anti-epileptic agent, zonisamide (benzo[d]isoxazol-3-ylmethanesulfonamide), into 6-hydroxydopamine (6(OH)DA)-treated mice improve the cardinal symptoms of PD (Asanuma *et al.*, 2010) by increasing GSH through enhanced activity of the x_c^- exchanger. Similar results using levetiracetam have recently been reported (Miyazaki *et al.*, 2016). On the other hand, Massie and colleagues observed that mice lacking the xCT subunit of the x_c^- exchanger were less susceptible to toxicity by 6(OH)DA, possibly because of reduced glutamate release (Massie *et al.*, 2011). Contrary to expectation, GSH levels in the SN were not reduced in xCT -/- mice (Gout *et al.*, 2001; De Groot and Sontheimer, 2011; Bridges *et al.*, 2012; Sontheimer and Bridges, 2012; Sunitha *et al.*, 2013; Savion *et al.*, 2014; Chen *et al.*, 2015).

New information on the potential of blockade of the x_c^- exchanger for ALS therapy has recently been published (Mesci *et al.*, 2015). Deletion of the xCT subunit in mice confirms that the source of glutamate release from activated microglia is via the x_c^- exchanger. Furthermore, xCT deletion reduces production of microglial pro-inflammatory and neurotoxic factors, including NO, TNFα, IL6, whereas production of anti-inflammatory markers was increased (Mesci *et al.*, 2015). In ALS mice, xCT deletion unexpectedly led to earlier symptom onset, but, ultimately, a significantly slowed disease phase and more surviving motor neurons were observed. It is proposed that this could be used as a clinical approach to slow disease progression after symptoms have appeared.

In summary, the x_c^- exchanger remains an attractive target in thiol redox-based therapy, but much remains to be understood regarding cysteine metabolism in the brain before the full implications of intervention in cysteine homeostasis can be understood. For example, the fact that the transsulfuration pathway is upregulated in response to GSH depletion (Kandil *et al.*, 2010) implies that provision of cysteine from methionine may compensate for a reduction in cystine import via the x_c^- exchanger. A multitargeted approach, such as that used by Chen *et al.* (2015) may ultimately prove more effective. The problem also remains of how to selectively target affected cells whilst avoiding damage to normal sulphur metabolism.

Protein thiol redox state as a potential target in neurodegenerative disease

Alterations in the oxidation state of redox-sensitive proteins are frequently associated with disease-related neurodegeneration. Free thiol groups on proteins are either protected from oxidation by *S*-glutathionylation or, if oxidized to sulphenic acid (Pr-SOH), are converted back to the reduced state (Pr-SH) by protein thiol reductases. Exposure of proteins to high levels of ROS, as may occur in neurodegenerative disease, will generate irreversibly-oxidized sulphinic ($Pr\text{-}SO_2H$) or sulphonic ($Pr\text{-}SO_3H$) acids that cannot be reduced (see Johnson *et al.*, 2015a; McBean *et al.*, 2015; Rojo *et al.*, 2014).

The GSH/GSSG redox couple operates in concert with glutathione peroxidase, glutathione reductase, the thioredoxin system, glutaredoxin and peroxiredoxin to maintain protein thiol redox homeostasis (McBean *et al.*, 2015; Rojo *et al.*, 2014). The thioredoxin system incorporates Trx and its reducing enzyme, thioredoxin reductase (TrxR). Trx has two principal isoforms, Trx1 (cytosolic) and Trx2 (mitochondrial) that associate with their respective reductases, TrxR1 and TrxR2. The Trx system is expressed throughout the brain, particularly in regions of high energy demand and a high rate of production of oxidized metabolites, such as in the SN and subthalamic nucleus. *Post mortem* tissue from AD patients displays a reduction in Trx, but upregulation of TrxR, most likely in compensation for increased ROS production (Akterin *et al.*, 2006). *In vitro*, overexpression of Trx1 protects a neuronal cell line and primary hippocampal neurons from Aβ-induced toxicity. In the MPP^+ model of PD, the expression of Trx1 and 2 is decreased (Silva-Adaya *et al.*, 2014). The content of Grx1 is similarly decreased in *post mortem* PD brain and, in the *Caenorhabditis elegans* model of PD, loss of Grx1 enhances the symptoms of PD (Johnson *et al.*, 2015b). Contrastingly, glutathione peroxidase 3 and glutathione peroxidase 4 levels from PD patients are elevated compared with control subjects and cortical samples from PD patients. To date, notwithstanding the increasing body of work on the antioxidant or neuroprotective effects of the Trx and Grx systems, no therapeutic agents that have the potential to arrest protein oxidation in AD or PD have been developed.

Nrf2-mediated antioxidant action as a therapeutic target in neurodegenerative disease

Nrf2 is considered the master regulator of cell homeostasis that regulates the expression of antioxidant and cytoprotective genes that contain a specific enhancer sequence in their regulatory regions known as antioxidant response elements (ARE) or the electrophile responsive element (EpRE). Nrf2 controls both the basal expression of genes under non-stressed homeostatic conditions and the inducible expression of genes upon redox perturbation. The number of genes regulated by Nrf2 account for more than 1% of the human genome, of which the best known are those related to GSH synthesis, redox regulation and drug metabolism (Ishii *et al.*, 2000; Thimmulappa *et al.*, 2002). However, it is now recognized that Nrf2 may play a role in regulation of genes that contribute to NADPH generation, lipid metabolism, glucose/glycogen metabolism and hydrogen sulphide (H_2S) production (Yates *et al.*, 2009; Wu *et al.*, 2011; Hourihan *et al.*, 2013; Uruno *et al.*, 2013; Hayes and Dinkova-Kostova, 2014). In addition to its capacity to afford cytoprotection against endogenous and environmental stressors, Nrf2 seems to control metabolic reprogramming during stress, thereby contributing to adaptation by upregulating the repair and degradation of macromolecules. Furthermore, Nrf2 modulates intermediary metabolism by virtue of the fact that its targets include other transcription factors (Hayes and Dinkova-Kostova, 2014).

Under basal normal conditions, the level of Nrf2 in the cytosol is low, due to constitutive synthesis and degradation of the protein. There are essentially three regulatory mechanisms that control Nrf2: (a) Keap1, (b) regulation by kinases and (c) epigenetic control. The main regulatory mechanism is achieved by the repressor protein, Keap1, which is located mainly in the cytoplasm. Under normal conditions, Keap1 binds to an E3 ubiquitin ligase complex (Rbx-1) via cullin-3, a protein adaptor (Kobayashi *et al.*, 2004). This complex promotes Nrf2 ubiquitination and proteasome degradation (Rushmore *et al.*, 1990; Dhakshinamoorthy and Jaiswal, 2001). However, under cellular stress conditions, oxidative/electrophilic molecules modify the cysteine residues of Keap1 (at least, Cys^{151}, Cys^{273} and Cys^{288}), and the cullin-3/Rbx-1-dependent poly-ubiquitination of Nrf2 assisted by Keap1 is blocked, leading to release of Nrf2 and translocation to the nucleus, where it heterodimerizes with small MAF or JUN proteins. This complex then binds to the EpRE of target genes (Rushmore *et al.*, 1990). The second mechanism of Nrf2 regulation is based on signalling cascades that include various kinases influenced by oxidative stress molecules. For instance, protein kinase RNA – like endoplasmic reticulum kinase and PKA, activated by misfolded proteins or phorbol esters, respectively, as well as PKC, phosphorylate Nrf2, thereby disrupting its association with Keap1 (Huang *et al.*, 2002; Cullinan *et al.*, 2003). Other kinases, such as CK2, Fyn or MAPKs phosphorylate Nrf2 without altering its interaction with Keap1. Interestingly, physiological activation of glycogen synthase kinase-3β (GSK-3β) phosphorylates Fyn, which then phosphorylates Nrf2, triggering its nuclear export and proteasomal degradation (Jain and Jaiswal, 2007). Furthermore, GSK-3β (which is abnormally active in Alzheimer's disease), inhibits Nrf2 by nuclear exclusion. In addition, GSK-3β phosphorylates Nrf2 and activates an alternative degradation pathway that is dependent on a different E3-ligase, named β-TrCP (see Rojo *et al.*, 2014). The physiological relevance of the inhibition of Nrf2 by this kinase is illustrated by two studies in which GSK-3β decreased Nrf2 activity, thereby sensitising neurons against oxidative damage. Interestingly, stimulation of P13K activates Akt, which in turn inhibits GSK-3β and represses Nrf2-driven and ARE-driven genes (Innamorato *et al.*, 2008; Rojo *et al.*, 2008). Furthermore, the kinase MAPK p38 stabilizes the interaction between Keap1 and Nrf2 and promotes breakdown of Nrf2 (Keum *et al.*, 2006). The third regulatory mechanism for Nrf2 includes epigenetic modifications. For example, binding of Nrf2 to EpRE is increased by direct acetylation of Nrf2 by CBP/p300 (Sun *et al.*, 2009). In contrast, methylation of cytosine-guanine islands within the Nrf2 promotor suppresses its expression (Su *et al.*, 2013). Lastly, a number of miRNAs, such as miR153, miR27a, miR142-5p and miR144, have been shown to reduce Nrf2 activation (Narasimhan *et al.*, 2012).

Nrf2 and neurodegenerative disease

The most compelling evidence in favour of a role of Nrf2 in prevention or protection from disease comes from studies with Nrf2-knockout mice. Loss of Nrf2 signalling increases susceptibility to acute toxicity, inflammation and carcinogenesis, due to the inability to mount adaptive responses. With increasing age, these mice show chronic pathologies

related to oxidant and inflammatory stress including cognitive deficits (Jo *et al.*, 2014), depressive disorder (Martin-de-Saavedra *et al.*, 2013b) and increased incidence of lupus-like autoimmune nephritis (Ma *et al.*, 2006). There is evidence in humans where single nucleotide polymorphisms have been identified in the coding and non-coding regions of the gene encoding Nrf2, NFE2L2, and epidemiological studies have revealed significant associations of NFE2L2 haplotypes with risks in pulmonary, gastrointestinal, autoimmune and neurodegenerative diseases (Cho, 2013).

Regarding AD, reduced NAD(P)H quinone oxidoreducase-1 (NQO1), glutathione synthetic enzymes and Nrf2 levels in hippocampal neurons were found in the mouse model that expresses mutated human amyloid precursor protein (APP) and pre-senelin-1 (PS1) genes (APP/PS1 mice) (Kanninen *et al.*, 2008); furthermore, the Nrf2-EpRE pathway was attenuated at the time of Aβ deposition. Importantly, *in vitro* Nrf2 over-expression protected against Aβ-mediated neurotoxicity, as shown by increased expression of Nrf2 target genes and the consequent reduction of oxidative stress. *Post mortem* brains from AD patients show reduced nuclear levels of Nrf2 within hippocampal neurons (Ramsey *et al.*, 2007).

A relationship between Nrf2 and PD has been documented by the following observations: (i) Nrf2 activity decreases with age, and age is one of the main risk factors for PD, (ii) in *post mortem* brains from PD patients, Nrf2 has been localized in nuclei although the induction of phase II enzymes is insufficient to protect neurons, indicating a dysregulation of this pathway (Ramsey *et al.*, 2007), (iii) there is a protective Nrf2 haplotype which delays PD onset, (iv) Nrf2 KO mice are more susceptible to 6(OH)DA and MPTP PD models, and (v) Nrf2 activation protects against different PD models (Lou *et al.*, 2014).

In ALS, studies with *post mortem* tissues have demonstrated that the expression of Nrf2 related genes was diminished in motor neurons of mice expressing mutant SOD1 (Pehar *et al.*, 2005) and in spinal cord of ALS patients (Sarlette *et al.*, 2008). Also, Keap1 was co-localized with intracellular inclusions in motor neurons in the spinal cord of ALS patients (Tanji *et al.*, 2013). In samples of motor cortex of ALS patients, Nrf2 mRNA and protein levels were reduced, whereas Keap1 mRNA expression was increased (Sarlette *et al.*, 2008), suggesting a dysfunction of the Nrf2-EpRE pathway in this disease.

In MS, the expression of DJ-1 (an important protein for Nrf2 stabilization) and Nrf2 are increased in cerebral spinal fluid of patients with relapsing – remitting MS (Hirotani *et al.*, 2008; van Horssen *et al.*, 2010). In fact, increased levels of Nrf2-regulated antioxidant proteins, such as NAD(P)H dehydrogenase (quinone) 1 (NQO1) and haemoxygenase-1 (HO-1) are present in active demyelinating MS lesions, mostly associated with infiltrating macrophages and reactive astrocytes (van Horssen *et al.*, 2010; van Horssen *et al.*, 2008). Furthermore, induction of EAE in Nrf2-knockout mice showed increased severity of the disease (Johnson *et al.*, 2010). It is believed that low levels of Nrf2 or impaired Nrf2 activation in oligodendrocytes may be a cause of selective susceptibility under neuroinflammatory conditions, due to their high degree of vulnerability to oxidative stress (Juurlink *et al.*, 1998).

Overall, Nrf2 inducers are emerging as a valuable tool for treatment of different neurodegenerative conditions where oxidative stress plays a major role. Details of a selection of drugs, including curcumin, carnosic acid, resveratrol, bardoxolone methyl and dimethylfumarate (Figure 3) are discussed in the following text in relation to different neurodegenerative conditions. Most information to date comes from pre-clinical data. The exception is dimethylfumarate (DMF), which has been approved for clinical use in MS patients.

Nrf2 inducers for the treatment of neurodegenerative disease

Curcumin. Curcumin is the main curcuminoid present in the rhizomes of *Curcuma longa* and is the active ingredient of herbs and spices used in traditional Asian cuisine. Curcumin contains electrophilic α,β unsaturated carbonyl groups that selectively react with nucleophiles, such as cysteine-thiols present at Keap1, thus releasing Nrf2 (Balogun *et al.*, 2003). Curcumin is a highly lipophilic natural compound that crosses the blood–brain barrier and concentrates mainly in the hippocampus (Tsai *et al.*, 2011). In AD related models, curcumin inhibits Aβ oligomers and

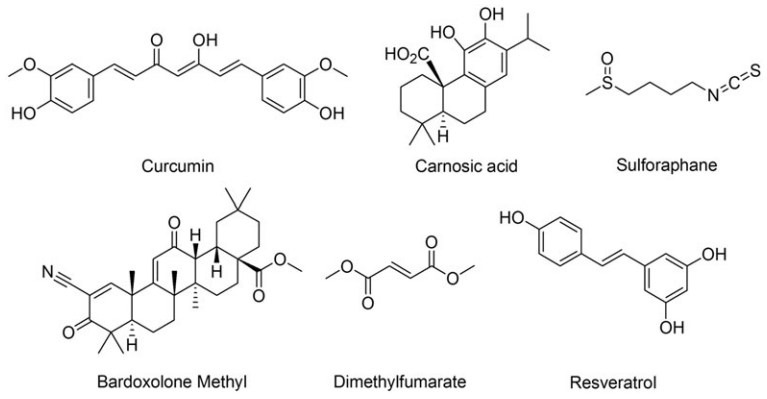

Figure 3
Chemical structures of various Nrf2 inducers.

restored neurite damage (Yang *et al.*, 2005; Garcia-Alloza *et al.*, 2007). Most interesting is that epidemiological studies have demonstrated that populations with a high level of consumption of curcumin show lower incidence of AD and better performance in Mini-Mental State Examination (Chandra *et al.*, 2001; Ng *et al.*, 2006). Based on these promising findings, curcumin has been tested in AD patients (Ono *et al.*, 2004). Several clinical trials have been performed, or are currently recruiting patients. The results have been limited, most probably because of its poor bioavailability (Anand *et al.*, 2007). Therefore, new curcumin derivatives are being synthesized in order to improve its pharmacological properties (Hu *et al.*, 2011; Orlando *et al.*, 2012; Pulido-Moran *et al.*, 2016).

In regard to ALS, preclinical data indicate that curcumin increases Nrf2 nuclear translocation, thereby inducing the expression of its target genes HO-1, NQO1 and GCLc in primary spinal cord astrocytes (Jiang *et al.*, 2011). Also, curcumin has been shown to reduce oxidative stress and mitochondrial dysfunction in motoneurons expressing mutant TDP-43 (Dong *et al.*, 2014).

Curcumin has also been tested as a potential treatment for MS. Its immunomodulatory and anti-inflammatory properties have been assessed in several models and are mostly mediated by its ability to inhibit COX-2, iNOS and the NF-κB transcription pathway (Menon and Sudheer, 2007). In the EAE model, curcumin showed behavioural improvement and reduction in the number of inflammatory cells infiltrating the spinal cord (Xie *et al.*, 2009). Furthermore, curcumin reduced IL-17 and TNFα production and up-regulated IL-4, IL-10 and CD4 + CD25 + −Foxp3+ Treg cells in the EAE model. These results suggest that curcumin augments Th2/Treg responses in EAE in the CNS (Kanakasabai *et al.*, 2012). In order to improve curcumin's bioavailability, a new polymerized form of nano-curcumin was evaluated in the EAE model; the results showed corrected balance of pro-inflammatory and anti-inflammatory gene expression, decreased oxidative stress markers, improved myelination and increased progenitor cell markers (Mohajeri *et al.*, 2015).

Carnosic acid. Carnosic acid (CA) is an active ingredient in the extract of *Rosmarinus officinalis* that improves long-term memory performance in rats (Ozarowski *et al.*, 2013). CA crosses the blood–brain barrier to up-regulate endogenous antioxidant enzymes via Nrf2 activation and promotes overexpression of phase II enzymes, GCL and HO-1 (Satoh *et al.*, 2008). In relation to AD, CA reduced $A\beta_{1-42}$ secretion by 71% in human U373MG astrocytoma cells and by 61% in SH-SY5Y neuroblastoma cells (Meng *et al.*, 2013; Yoshida *et al.*, 2014). Application of CA (10 $mg \cdot kg^{-1}$) to an AD animal model *in vivo* reduced cell death in the CA1 region of the hippocampus. With regard to PD, CA provided neuroprotection of human neuroblastoma SH-SY5Y cells against 6(OH)DA-mediated toxicity (Wu *et al.*, 2015) and against dieldrin-induced toxicity in the neuronal dopaminergic SN4741 cell line (Park *et al.*, 2008b). This activity was further confirmed *in vivo* by the improvement of behavioural performance in rats intrastriatally injected with 6-OHDA (Wu *et al.*, 2015). In the ALS transgenic mice model G93 A-hSOD1, administration of rosemary extract, in which rosmarinic and carnosic acid are present, led to improved motor performance and prolonged mean survival, compared with control conditions (Shimojo *et al.*, 2010).

Resveratrol. Resveratrol is a natural polyphenolic compound especially abundant in the skin of red grapes, which activates the Nrf2-EpRE pathway (Hsieh *et al.*, 2006). Resveratrol has shown beneficial effects in *in vitro* and *in vivo* models related to AD (Ma *et al.*, 2014; Savaskan *et al.*, 2003). In humans, a phase III Clinical Trial of resveratrol has been performed to study its effect in mild–moderate AD. Additional clinical trials are presently recruiting participants. Although resveratrol has the ability to cross the blood brain barrier to reach the brain, it metabolizes very rapidly, which reduces its half-life and bioavailability (Davinelli *et al.*, 2012). A variety of approaches have been taken to bypass this problem: one is synthesis of new resveratrol derivatives with improved pharmacokinetics, such as pterostilbene, which shows a greater efficiency in modulating cellular stress and cognition than its parent compound (Joseph *et al.*, 2008; Chang *et al.*, 2012). Another is use of resveratrol-loaded polysorbate 80-coated poly(lactide) nanoparticles. The latter was evaluated in the MPTP *in vivo* model of PD, where it showed significant neuroprotection and improved behavioural and neurochemical indices (da Rocha Lindner *et al.*, 2015b).

Bardoxolone methyl. Bardoxolone methyl is a triterpenoid derivative, 2 cyano-3,12-dioxooleana-1,9-dien-28-oic acid methyl ester (CDDO-Me), with potent Nrf2-inducing activity. It was first studied as a treatment for diabetic nephropathy (Wang *et al.*, 2014). The initial excitement about this compound was hampered by the observation that in the phase III clinical trial, BEACON, there was a small yet significant increase in heart failure in the treated group compared to the control group. There is no clear explanation for these adverse events; it was speculated that fluid retention, increased afterload and higher heart rate could have contributed to the heart failure in the bardoxolone-treated group, although direct toxic effects cannot be discounted (Chin *et al.*, 2014). Despite these adverse effects, bardoxolone methyl is now being tested in pulmonary arterial hypertension, melanoma and Friedreich's ataxia.

In the context of AD, bardoxolone methyl showed improved spatial memory retention and decreased microgliosis, oxidative stress and Aβ burden in the TG19959 AD transgenic mouse, signifying its potential as a therapeutic agent in this disease (Dumont *et al.*, 2009).

The mechanism of action of bardoxolone consists of causing a reversible Michael addition of SH groups in Keap-1 to attenuate Nrf2 degradation; as mentioned earlier, this is one of the main defence systems against redox stress. Some compounds, such as RTA 408, a novel synthetic triterpenoid related to bardoxolone, are cytotoxic in cancer cells by this mechanism (Probst *et al.*, 2015). It is therefore postulated that some of these compounds could act as 'chemical irritants' to activate body defences.

Sulforaphane. Another interesting compound is sulforaphane, which is found in cruciferous vegetables, such as broccoli. Due to its electrophilic structure, sulforaphane interacts

with specific redox sensitive cysteine residues in Keap1, which disrupts the Keap1/Nrf2 complex and leads to the increase in Nrf2 protein levels and transcriptional activity (Dinkova-Kostova and Talalay, 2008). Sulforaphane (5 μM) exerts protection against Aβ-toxicity via activation of the phase II antioxidant response *in vitro* (Lee *et al.*, 2013) and ameliorates cognitive deficit in AD animal models (Kim *et al.*, 2013; Zhang *et al.*, 2014). In the AD-model induced by the combination of D-galactose and aluminium, sulforaphane reduced the number of lesions, decreased the levels of aluminium in the brain and reduced spatial memory deficits and amyloid plaques (Kim *et al.*, 2013). In PD, sulforaphane protected against MPTP-induced toxicity by increasing Nrf2 protein levels in basal ganglia, upregulating HO-1 and NQO1 enzymes and decreasing astrogliosis, microgliosis and the release of pro-inflammatory cytokines (Jazwa *et al.*, 2011). In MS, sulforaphane treatment inhibits progression of disease in EAE mice, again via activation of the Nrf2 pathway and reduction in oxidative stress and inflammation. Furthermore, it reduced demyelination and CNS infiltration and inhibited production of MMP-9 (metalloproteinase that degrades extracellular matrix proteins), which contributes to BBB preservation (Li *et al.*, 2013).

Most clinical studies performed with sulforaphane have used a highly standardized formulation of broccoli sprout extracts. In contrast to dimethylfumarate (DMF) and bardoxolone methyl, two major drawbacks for sulforaphane for clinical use are the absence of a pure formulation and the lack of commercial value that could attract significant investment by pharmaceutical companies. Nevertheless, several attempts are being made to provide proof of concept that Nrf2 targeting with sulforaphane has therapeutic value. Currently, at least 32 studies are assessing its clinical efficacy in chronic diseases such as cancer, asthma, autism, schizophrenia, chronic kidney disease, type 2 diabetes and cystic fibrosis.

Dimethylfumarate (DMF). The most successful case reported so far of an indirect antioxidant targeting Nrf2 is the dimethyl ester derivative of fumaric acid, DMF. As early as 1994, topical and oral administration of DMF (commercial name, 'Fumaderm') was used to treat psoriasis (Altmeyer *et al.*, 1994). In the EAE model, DMF inhibits progression of MS symptoms, including macrophage inflammation in the spinal cord and decreased expression of the pro-inflammatory cytokine IL-1β (Schilling *et al.*, 2006). In Nrf2-deficient murine motoneuron cultures, the protective effect of DMF is completely abolished (Linker *et al.*, 2011a), supporting the role of Nrf2 in its mechanism of action. DMF crosses the gastrointestinal barrier, after which it is converted into its active metabolite, monomethyl fumarate (MMF), which binds to Keap1. This binding disrupts the Keap1/Nrf2 interaction and leads to up-regulation of the transcriptional Nrf2 signature of antioxidant genes. MMF is more potent than DMF in activating Nrf2, but is probably metabolized more rapidly. In an attempt to allow DMF to bypass gastric metabolism, it has been packaged in an oral delayed release formulation (BG-12, known commercially as Tecfidera ®) (Bomprezzi, 2015). It has shown positive results in two clinical trials and has been recently approved by the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) as first-line therapy for relapsing–remitting MS (Xu *et al.*, 2015). A new formulation of MMF ('ALKS 8700') has completed a phase 1 trial as a pro-drug for MS (NIH Trial number NCT02201849), but results have not been posted. The immune modulatory effect of DMF has been exploited more recently for other autoimmune diseases such as lupus erythematosus (Tsianakas *et al.*, 2014) and asthma (Seidel and Roth, 2013).

Despite these promising results, its use seems to be limited by significant adverse effects and regular monitoring requirement (Dubey *et al.*, 2016) due to the fact that some patients with MS, chronically treated with DMF, have shown reduction of total leukocyte and lymphocyte counts (Spencer *et al.*, 2015), and progressive multifocal leukoencephalopathy (Faulkner, 2015).

In conclusion, Nrf2 inducers can be of potential interest for neurodegenerative diseases because of their antioxidant and antinflammatory effects. However, their use in clinic has been hampered by different circumstances. For example, curcumin has poor bioavailability and bardoxolone has major side effects. Until today, the only drug with this mechanism of action used clinically is DMF. However, long term treatment with this drug exhibits, in a subset of patients, severe side effects such as lymphopenia and progressive multifocal leukoencephalopathy. Therefore new derivatives of these compounds are being synthesized and developed with the hope of improving their pharmacokinetic and safety profile.

NOX-based therapies in neurodegenerative disease

The NOX family of enzymes are a major source of ROS in the brain, and there is now little doubt of their association with pathological ROS production in a range of neurodegenerative disorders. The NOX enzymes use NADPH as electron donor in the reduction of molecular oxygen to the superoxide anion, O_2^-. Of the seven mammalian subtypes of the enzyme, four isoforms (NOX1 through NOX4) have received greatest attention in the context of physiological and pathological function of the CNS. All four isoforms are expressed in neurons, mostly as inducible enzymes associated with disease states. NOX4, and to a lesser extent NOX1 and NOX2, is expressed in astrocytes and is particularly evident in glioma cells. The expression of NOX2 is high in activated microglial cells, but low in the inactivated state. NOX1 and NOX4 are also expressed in microglia. However, full evaluation of the cellular, regional and inter-species distribution of NOX's is hampered by the absence of reliable antibodies (Nayernia *et al.*, 2014). Full details of the subunit structure, subtypes and cellular localisation of the enzymes can be found in a number of excellent and informative review articles (Infanger *et al.*, 2006; Sorce and Krause, 2009; Sorce *et al.*, 2012; Nayernia *et al.*, 2014).

The association of NOX with a wide spectrum of neurodegenerative disorders constitutes a compelling case for development of therapeutic strategies that modulate their activity. The therapeutic opportunities presented by targeting NOX in diseases of the CNS have been discussed

comprehensively elsewhere (Sorce *et al.*, 2012; Nayernia *et al.*, 2014). Here, we provide an update on recent research and the development of NOX-based therapeutic agents. There is a growing body of evidence illustrating the beneficial effects of targeting NOX in several animal models of neurodegenerative disorders. However, advances into the arena of clinical intervention are still at an early stage.

In PD, loss of dopamine in the SN correlates with an increase in expression of NOX in SN neurons and microglial cells. The rat dopaminergic N27 cell line displays constitutive expression of most NOX isoforms, but a selective increase in NOX1 is observed following treatment of cells with 6(OH) DA (Choi *et al.*, 2012). NOX1 expression was also identified in the nucleus of dopaminergic SN neurons of PD patients, *post mortem*. Hernandes *et al.* assessed the association between NOX activation with PD progression using the 6(OH)DA mouse model of the disease (Hernandes *et al.*, 2013). Mice lacking the catalytic subunit of NOX2 (gp91 (phox −/−)) were protected from dopaminergic cell loss, and microglial cell activation was abolished. The results support a role for NOX2 in the 6(OH)DA-induced degeneration of DA neurons. Taken together, these studies suggest that inhibition of NOX1 and/or NOX2 could be viable targets for arresting ROS production in PD.

Other recent developments in the PD/NOX field come from exploring the influence of angiotensin receptor activation on NOX-derived ROS production. Following from rodent studies, in which dopamine loss and changes in NOX activity in the SN correlate with expression of the angiotensin AT_1 receptor, Zawada and colleagues measured the distribution of AT_1 receptors and NOX4 in different nigral compartments in post mortem samples from PD patients (Zawada *et al.*, 2015). The ratio of nuclear/total neuron AT_1 expression increased according to disease progression and was associated with increased levels of NOX4. It is proposed that AT_1 receptor antagonists offer a viable approach to modify NOX-mediated ROS production and slow the progression of the disease. On the other hand, and in line with the opposing effects of AT_2 receptors, the AT_2 agonist, CGP42112, inhibits NOX activation and protects against oxidative stress in the rotenone model of PD *in vitro* (Lu *et al.*, 2015). These results are promising but further work is required to clarify the therapeutic potential of targeting the renin-angiotensin system in PD.

A new focus in the search for antioxidant approaches for treatment of PD is the enzyme MPO. This is a ROS-generating enzyme that is expressed in microglia. In recent work, Jucaite and co-workers used a selective and irreversible MPO inhibitor, AZD3241, to assess the effect of MPO blockade on microglial activity in PD. Patients received 600 mg orally twice daily for 8 weeks, during which time microglial activity was determined using positron emission tomography to map binding of a probe to a microglial marker translocator protein. The drug was well tolerated and, by the end of the study, a reduction in the binding of the probe to the translocator protein was recorded. Following this confirmation that MPO blockade affects microglial activity, further studies on the efficacy of this treatment in neurodegenerative disorders are warranted (Jucaite *et al.*, 2015).

In regard to ALS, the focus is more clearly on the therapeutic benefits of inactivation of NOX. For example, NOX2 activity in 83 ALS patients was assessed by measuring neutrophil oxidative burst in fresh blood samples by flow cytometry (Marrali *et al.*, 2014). Although the activity of the enzyme was independent of gender and disease duration, a lower NOX2 level correlated with a longer survival time from disease onset. It was concluded that modulation of NOX2 may have value in arresting the rate of progression of the disease. Similar conclusions have been drawn from work on the SOD1 G93 A mouse model of ALS. Mutant SOD1 protein directly activates NOX2 in microglia, due to protein misfolding (Apolloni *et al.*, 2013). Furthermore, stimulation of the P2X7 purinergic receptor with 2′3′-O-(benzoyl-benzoyl) ATP enhanced NOX2 activity by stimulating the translocation of the p67phox subunit to the membrane, with a consequent increase in ROS production. This process was inhibited following P2X7 receptor knock-out, or by using pharmacological antagonists of the receptor, signifying that P2X7 receptors may be a promising target to limit oxidative stress in the disease (Apolloni *et al.*, 2013).

It should be noted that NOX derived ROS production is a very conserved response mechanism towards microbial infections and other stress signals (Aguirre and Lambeth, 2010). It is therefore important that possible negative effects on resistance to infection are taken into account when using NOX inhibitors, especially so in AD, where there is a connection between infections and cognition (Honjo *et al.*, 2013), although this connection may very well be ROS-mediated (Trumbull *et al.*, 2012).

NOX inhibitors

In the past decade, NOX inhibitors have attracted increasing interest and researchers are reaching a point where they may be on the verge of clinical applications (Altenhöfer *et al.*, 2014b). The range of NOX inhibitors has been extensively reviewed previously (Kim *et al.*, 2011, 2012, 2014b). Peptide-based inhibitors such as gp91ds-tat (Rey *et al.*, 2001) have been much used in experimental studies. However, due to the non-optimal absorption, distribution, metabolism and excretion/toxicity (ADME(T)) properties of peptides, they are less attractive as treatment options. However, continuous progress in novel methods for peptide delivery and targeting may increase their viability in future but, for now, low MW NOX inhibitors are closer to market. A selection of inhibitors is described in Figure 4 and Table 1.

Traditionally, NOX activity has been inhibited with compounds such as diphenyleneiodonium, a non-specific inhibitor of flavin-containing enzymes, and apocynin (Stolk *et al.*, 1994), a compound believed to interfere with p47-phox binding to other components of the NOX complex. However, neither of these are specific (Aldieri *et al.*, 2008a) and are not generally considered as treatment options for NOX inhibition. In addition, apocynin appears to require oxidative activation before inhibiting NOX enzymes, and both apocynin and the analogue diapocynin are more likely to act as ROS scavengers, at least *in vitro* (Heumuller *et al.*, 2008). It should, however, be mentioned that both apocynin and diapocynin have been used successfully *in vivo* (for example, Dranka *et al.*, 2013 and Hernandes *et al.*, 2013), and some researchers have called for clinical studies (Drummond & Sobey, 2014). However, it is not known whether their effects were based on NOX inhibition

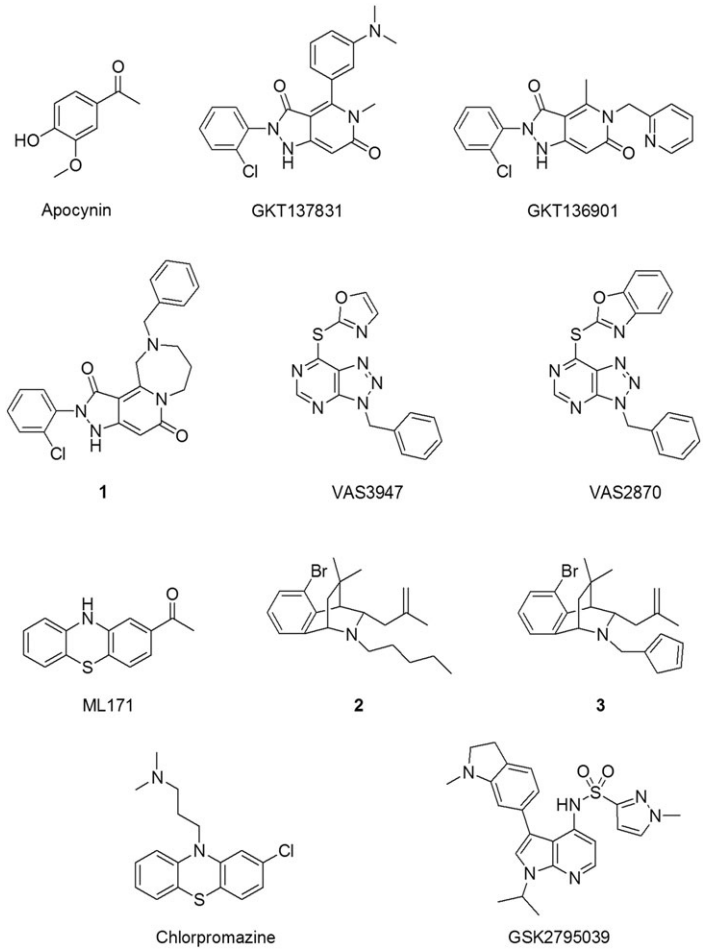

Figure 4
Selected NOX inhibitors.

(Drummond and Sobey, 2014). More recently, several laboratories have identified more specific NOX inhibitors that are more promising as therapeutic agents. For instance, GenKyoTex has developed several series of pyrazolopyridine diones (Laleu *et al.*, 2010; Page *et al.*, 2010; Gaggini *et al.*, 2011; Brandes *et al.*, 2013) and pyrazolo piperidine (Page *et al.*, 2011) derivatives with varying NOX specificity. Recently, a phase II study of the most advanced compound, GKT137831, was marked as completed, although the results have not yet been released (2016). Although the primary endpoint was not met, it showed a good safety profile and patients with diabetic kidney disease had significantly reduced level of circulating liver enzymes and inflammatory markers (NIH Trials number NCT 02010242). GKT137831 has a NOX1/4 inhibitory effect with a K_i of around 100 nM and a 10-fold or 4-fold selectivity over NOX2 and NOX5, respectively, and is essentially inactive towards xanthine oxidase (Aoyama *et al.*, 2012). Also, GKT136901 (Laleu *et al.*, 2010) and compound **1**, described in Gaggini *et al.*, (2011), absorption, distribution, metabolism and excretion/toxicity (ADME(T)) properties. GKT136901 has been shown to scavenge peroxynitrite (Schildknecht *et al.*, 2014). The mechanism of the scavenging is not known except for the fact that GKT136901 is consumed in the process. The phenomenon could very likely be present also for the other compounds in this series.

Vasopharm Biotech has described a series of triazolopyrimidine derivatives (Tegtmeier *et al.*, 2005), with special focus on VAS2870 (ten Freyhaus *et al.*, 2006; Gatto *et al.*, 2013a) and VAS3947 (Wind *et al.*, 2010), which are both considered to be pan-NOX inhibitors with limited off-target effects, even though some have been reported (Laleu *et al.*, 2010; Sun *et al.*, 2012). However, the broad-spectrum NOX effects of these compounds will render them less likely to be used in a clinical setting. ML171 is a NOX1 specific inhibitor with nanomolar potency and 12-fold to 20-fold specificity over the other NOX isoforms and minimal off target effects (Gianni *et al.*, 2010). However, these results have recently been questioned by Serendenina *et al.* (2015) in a study that combined a multitude of readouts to minimize assay interference effects. In contrast, they describe N-substituted phenothiazines as NOX inhibitors with varying specificities. Some of these were independently found to inhibit NOX2 by Zielonka *et al.* (2016). One of the compounds discussed in both these papers is chlorpromazine that, interestingly enough, was used as a negative control in the work on

Table 1

Summary of potency, specificity and ADME(T) properties of selected NOX inhibitors

Compound	Potency[a]	NOX specificity[b]	ADME(T)/Off target effects[c]	Reference
Apocynin	~30 μM (IC_{50}; neutrophils)	–	H_2O_2 scavenging	(Stolk *et al.*, 1994)
GKT137831	110 nM (K_i; NOX4)	NOX1,NOX4 > NOX5 > NOX2	Good ADME(T), no known off target effects	(Aoyama *et al.*, 2012)
GKT136901	160 nM (K_i; NOX1)	NOX1,NOX4> > NOX2	Good ADME(T), no known off target effects	(Laleu *et al.*, 2010)
1	72 nM (K_i; NOX4)	NOX1,NOX4 > NOX5 > NOX2	Good ADME(T), no known off target effects	(Gaggini *et al.*, 2011)
VAS2870	77 nM (IC_{50}; neutrophils)	–	No effect on XO and eNOS	(Gatto *et al.*, 2013a)
VAS3947	2 μM (IC_{50}; HL60 cells)	NOX2 > NOX1, NOX4	No effect on XO and eNOS	(Wind *et al.*, 2010)
ML171	0.25 μM (IC_{50}; NOX1)	NOX1> > NOX3, NOX2,NOX4	No or low effects on XO and several CNS targets	(Gianni *et al.*, 2010)
2	20 μM (IC_{50}; NOX2)	NOX2 > NOX1, NOX4,NOX5	No effect on XO or ROS scavenging	(Cifuentes-Pagano *et al.*, 2012)
3	38 μM (IC_{50}; NOX2)	NOX2 > NOX1, NOX4,NOX5	No effect on XO or ROS scavenging	(Cifuentes-Pagano *et al.*, 2012)
GSK2795039	0.3 μM (IC_{50}; NOX2)	NOX2> > NOX1, NOX3,NOX4,NOX5	No or very low effect on a set of secondary assays, such as XO and reduction potential	(Hirano *et al.*, 2015)
Chlorpromazine	4.3 μM (IC_{50}; NOX2)	NOX2 > NOX1, NOX3 > NOX4, NOX5	Inhibition of dopamine D_2 receptors	(Seredenina *et al.*, 2015)

[a]Potency is reported as K_i or IC_{50} values for the isoform or cell type where the potency is highest.
[b]The NOX isoforms are ordered according to compound potency. Isoforms separated with comma have similar potency, > indicates 3–5 fold selectivity, > > indicates a selectivity over 10 fold. A dash indicates that the selectivities have not been reported.
[c]Summary of the reported effects on non-NOX enzymes. XO, xanthine oxidase

ML171 (Gianni *et al.*, 2010). The phenothiazines, like chlorpromazine, are used as antipsychotics and are known to pass the blood brain barrier, which increases their potential suitability for repurposing, in the event that the observed effects translate to human use. In other work, researchers from the University of Pittsburgh have developed NOX2-specific tetrahydroisoquinoline inhibitors, such as compounds **2** and **3** (Cifuentes-Pagano *et al.*, 2012; Pagano *et al.*, 2014) that do not inhibit xanthine oxidase or scavenge ROS. Very recently, GSK2795039 was described as a selective NOX2 inhibitor with more than 30-fold selectivity over other NOX enzymes and good PK properties (Hirano *et al.*, 2015). In addition to these inhibitors, there is a plethora of reported NOX inhibitors targeting different isozymes. However, less is known about the specificities of these compounds within the NOX family, as well as towards other off-target effects.

Taken together, the future of NOX inhibiting compounds looks very interesting, with several groups making progress on novel, more specific inhibitors and clinical results showing good safety profiles, together with some positive results. Of high importance is also the fact that known CNS active drugs have been shown to inhibit NOX enzymes, raising hopes that repurposing will become a reality.

Reactive nitrogen species as a therapeutic target in neurodegenerative disease

NO-mediated *S*-nitrosylation (the chemical reaction is *S*-nitrosation, but the 'ylation' suffix is commonly used in biology) entails the addition or transfer of a nitroso group to a free sulphydryl group in a protein, forming *S*-nitrosocysteine. This happens as part of normal cellular signalling, but dysregulation of *S*-nitrosylation (termed 'nitrosative stress') is being increasingly linked to human neurodegenerative disorders (Chung, 2006; Cobb and Cole, 2015; Lee *et al.*, 2016).

Another significant reaction involving NO is nitration of protein tyrosine residues to 3-nitro-tyrosine. Frequently, this occurs when excess ROS and NO form peroxynitrite ($ONOO^-$) and has been deemed a biomarker of reactive nitrogen species (RNS)-mediated cellular damage (Radi, 2013). In this case, the process is referred to as 'nitrative stress' and commonly occurs in neurodegenerative disorders because both ROS and RNS are in oversupply. In some instances, 'nitroxidative stress' is used as a collective term for nitrosative, nitrative and oxidative stress (Lancaster, 2006).

Information is accumulating on the association between nitrative stress and neurodegenerative disease, but using this knowledge to design therapeutic strategies is at an early stage of development. For example, α-synuclein accumulation in PD increases both ROS and RNS. In fact, exogenous α-synuclein increases NO synthesis via NMDA receptor-induced activation of iNOS and increased NO in rat brain experiments (Wilkaniec *et al.*, 2013). Concurrent stimulation of superoxide by α-synuclein increases production of ONOO−. Lee and colleagues tested several antioxidants, including superoxide dismutase, catalase, 2,2,6,6-tetramethyl-1-piperidinoxyl (TEMPO), NAC, dimethylthiourea and uric acid in preventing protein tyrosine nitration caused by ROS and NO in primary neuronal cultures (Lee *et al.*, 2016). Of these, TEMPO was the most effective at promoting clearance of ROS/RNS and inhibition of stress signalling pathways. It was concluded that TEMPO offers potential for development of combined reduction in ROS and RNS in neurodegenerative disease.

Experimentally-induced nitrative stress using the peroxynitrite donor, 3-morpholinosyndnonimine, was examined in SH-SY5Y neuroblastoma cells and mouse motor neuron – neuroblastoma fusion NSC-34 cell lines (Kupershmidt *et al.*, 2010). Notably, Fe III and Mn III corroles (metal complexes structurally similar to porphyrin compounds) increased cell survival. The water solubility, low effective concentration and non-toxic properties of the metallocorroles rendered them attractive as therapeutic agents able to decompose ROS and RNS and limit neuronal loss in a number of neurodegenerative disorders. Okun and colleagues undertook further investigation of the structure–activity relationships of the antioxidant properties of metallocorroles, concluding that the C(10)-anysil-substituted complex was most effective (Okun and Gross, 2012). To date, the metallocorroles, together with other peroxynitrite decomposition catalysts, have demonstrated preclinical efficacy, oral availability and reduced toxicity risk (Slosky and Vanderah, 2015). Work is ongoing to generate compounds with greater oral availability and peroxynitrite selectivity, with the aim of providing safe, effective and selective modulators of nitrative stress (Okun and Gross, 2012).

An alternative strategy to combat the toxic effects of RNS is to limit synthesis of NO by inhibition of iNOS, for example, using aminoguanidine (Díaz *et al.*, 2014). Systemic administration of aminoguanidine to rats (100 $mg \cdot kg^{-1}$/day for 4 days) improved the spatial memory deficits caused by Aβ injection into the temporal cortex. The effect was accompanied by decreased levels of pro-inflammatory cytokines and nitrite, plus a reduction in reactive gliosis. At the time of writing, the most recent report on aminoguanidine relates to its use in two animal models of schizophrenia (Lafioniatis *et al.*, 2016). In this study, aminoguanidine (25 and 50 $mg \cdot kg^{-1}$) attenuated the extinction of recognition memory that is characteristic of the disorder. However, aminoguanidine had no effect on ketamine-induced social isolation in the animals, even at the higher dose of 100 $mg \cdot kg^{-1}$. These observations led to the conclusion that aminoguanidine may be effective in reducing memory impairments in patients with neurodegenerative disease.

Another approach to reduce nitrative stress in neurodegenerative disease is to use cerium oxide nanoparticles, known as nanoceria. These particles scavenge superoxide anions, hydrogen peroxide and peroxide by switching between their Ce^{3+} and Ce^{4+} states (Dowding *et al.*, 2014). The potential of nanoceria to reduce oxidative and nitrative stress in neurodegenerative disease is considerable: they are taken up by neurons and accumulate at both the plasma membrane and the outer mitochondrial membrane and have been shown to reduce endogenous peroxinitrite formation, Aβ-induced mitochondrial fragmentation and neuronal death in experimental systems (Dowding *et al.*, 2014).

Other work, likely to be the focus of future investigations, has examined cross-talk between inflammatory cytokine production, astrocyte activation and generation of NO. For instance, fingolimod (Gilenya®, Novartis; a sphingosine-1-phosphate receptor antagonist approved as oral therapy in MS) inhibits astrocyte activation and NO production following administration to the EAE mouse model of MS, thereby providing neuroprotection (Colombo *et al.*, 2014). Similar neuroprotective effects of fingolimod have been observed in a mouse model of AD (Aytan *et al.*, 2016). However, use of fingolimod in clinical situations for AD may be some way off, due to recent evidence of lesions in the brain of patients taking fingomilod for MS, as identified by magnetic resonance imaging (Fragoso and Sato, 2016).

Other redox components as therapeutic targets in neurodegenerative disease

Li and colleagues explored the potential of inhibition of MPO as a redox-based therapeutic target in neurodegenerative disease (Li *et al.*, 2015). MPO catalyses the reaction between hydrogen peroxide and halides that generates hypochlorite. Hypochlorite is a powerful oxidant and chlorinator of tyrosine residues in proteins, forming 3-chloro-tyrosine. MPO is expressed in microglia and has been implicated in a number of diseases, including AD and PD. However, the development of inhibitors to target MPO in neurodegenerative disease is at an early stage. For example, Li and colleagues synthesized and tested over 50 low MW MPO inhibitors and a number were identified with IC_{50} values in the high nanomolar range. For example, AZD3241 is a novel selective and irreversible inhibitor of MPO that has undergone a phase 2a randomized placebo controlled study on PD patients (Jucaite *et al.*, 2015). Using positron emission tomography, it was confirmed that AZD3241 targets microglial cells. Jucaite and colleagues concluded that further studies on the efficacy of AZD3241 in neurodegenerative disorders were warranted.

New developments in understanding the pathogenesis of AD include the 'Inverse Warburg Hypothesis', which is a bioenergetics model that places metabolic dysfunction as a central element in development of the disease (Demetrius *et al.*, 2014). If this proves to be the case, as seems likely, then therapeutic agents that are based upon modulating the availability of oxidisable substrates, on the one hand, and ROS homeostasis on the other, may be most beneficial. Finally, it is also worth mentioning that, although exercise is an excellent generator of radicals, it has been proven to

have the greatest effect in preventing progression of mild cognitive impairment to AD (Fiatarone Singh *et al.*, 2014).

Mitochondrial dysfunction as a source of ROS and RNS

Dranka and colleagues sought to determine the relationship between mitochondrial dysfunction and oxidant production in a number of PD disease models – finding that mitochondrial dysfunction was a more significant cause of degeneration than oxidative damage in these models (Dranka *et al.*, 2012). Similarly, Haddad and co-workers recently reviewed the evidence that mitochondrial stressors may cause PD and reached the conclusion that more information is needed to understand how mitochondria contribute to the pathogenesis of PD (Haddad and Nakamura, 2015). Nonetheless, mitochondria-targeting antioxidants are emerging as new therapeutic agents in treatments for neurodegenerative disease. Chief amongst these is MitoQ (mitoquinone mesylate: [10-(4,5-dimethoxy-2-methyl-3,6-dioxo-1,4-cyclohexadienl-yl) decyl triphenylphosphonium methanesulfonate]), which is ubiquinone covalently bound to a triphenylphosphonium cation. MitoQ readily permeates the blood brain barrier and neuronal membranes, where it concentrates within mitochondria. MitoQ was assessed for prevention of AD pathology in mouse cortical neurons in culture and a triple transgenic mouse model of AD (McManus *et al.*, 2011). MitoQ successfully attenuated Aβ-induced toxicity in cortical neurons and prevented the loss in mitochondrial membrane potential. Moreover, treatment of mice for 5 months with MitoQ provided protection against neurodegeneration in the AD mouse model *in vivo* (McManus *et al.*, 2011). MitoQ was also tested in the 6(OH) DA model of PD (SH-SY56Y cell cultures) (Solesio *et al.*, 2013c). Again, pre-treatment with MitoQ prevented redox-related mitochondrial dysfunction.

Using the same rationale of boosting the antioxidant capacity of mitochondria, Dranka and colleagues tested a mitochondrial-permeable form of the antioxidant apocynin (mito-apocynin) in the transgenic mouse model of PD (Dranka *et al.*, 2014). Significant deterioration in physical ability and increased hyposmia in the mice was prevented by treatment with mito-apocynin (3 $mg \cdot kg^{-1}$) three times a week.

Acetyl-L-carnitine and α-lipoic acid are two compounds that target mitochondria and have been tested in their capacity as antioxidants to improve mitochondrial function in a PD animal model (Zaitone *et al.*, 2012). Acetyl-L-carnitine is an essential component in the α-oxidation of fatty acids to generate ATP. α-Lipoic acid is a co-enzyme, but also has antioxidant properties and readily crosses the blood brain barrier. Treatment of rats with acetyl-L-carnitine and α-lipoic acid, either alone or in combination, significantly improved rotenone-induced neurotoxicity (Zaitone *et al.*, 2012). It was concluded that these agents are promising candidates for neuroprotection in the early stages of PD.

Collectively, the results from experiments with mitochondrial antioxidants suggest that mitochondria-targeting therapeutic agents may have a place in neurodegenerative disease treatment. Onyango and colleagues provide an excellent review on mitochondrial dysfunction in AD and discuss the rationale behind the search for bioenergetics-based therapies. A number of investigations are ongoing that include potentiation of energy production, scavenging ROS and curtailing oxidative damage (Onyango *et al.*, 2016). Several of these avenues of experimentation are promising and progression beyond pre-clinical experiments is eagerly awaited.

Conclusion and future perspectives

Much progress has been made in designing strategies to combat the loss of redox homeostasis in neurodegenerative disorders. It is becoming increasingly clear that several diseases share common pathways of ROS-related damage and much can be gained from comparative studies that incorporate a range of disorders. As the origins and targets of ROS are better understood, therapies must evolve that address the deficiencies in ROS signalling more effectively. In this review, we have chosen to discuss, on the one hand, three major strategies that we believe best address gaps in knowledge and, on the other, more effective therapies. Nonetheless, the challenges are considerable, particularly in the arena of polypharmacy and improved drug delivery. Therapeutic approaches that will address both the inflammatory and oxidative-nitrosative pathways in the neuropathogenesis of age-related neurodegeneration are urgently needed (Daulatzai, 2016). Similarly, improvements in drug delivery and targeting will greatly assist in enhancing bioavailablity and aid in the development of more effective therapeutic agents. For example, Hagl and colleagues review improvements in the bioavailability of curcumin compounds as potential treatments for neurodegenerative disease (Hagl *et al.*, 2015). Curcumin micelles had improved effectiveness in protecting PC12 cells from nitrosative stress, compared with curcumin alone. It is expected that similar developments using other antioxidants will emerge in the near future. There are still significant gaps in our understanding of brain thiol redox biochemistry. It is only relatively recently accepted that the brain contains a functional transsulphuration pathway, but its significance in generating sulphur-based compounds with antioxidant properties remains to be fully understood. In their well-focussed and timely review, Hensley and Denton describe how the broad-spectrum enzymes of the transsulphuration pathway generate a range of compounds, including H_2S and the unusual amino acid, lanthionine (Hensley & Denton, 2015). Derivatives of the transamination product of this amino acid, lanthionine ketimine, have potent antioxidant, neuroprotective, neurotrophic and antioxidant actions. The opportunities for development of thiol-based therapeutics for treatment of neurodegenerative disorders based on these compounds must be considerable.

The Nrf2-EpRE pathway is an intrinsic mechanism of defence against oxidative stress which acts by inducing the expression of a wide variety of cytoprotective and detoxificant genes. There is much evidence for the protective role of the Nrf2-EpRE pathway in different neurodegenerative conditions, as it reduces oxidative stress and neuroinflammation. The proof of concept that Nrf2 induction can be a good strategy to develop drugs for neurodegenerative conditions is

exemplified by DMF, which is already in the clinic for the treatment of MS. Therefore, the development of Nrf2 inducers opens a new direction for the treatment of patients with neurodegenerative diseases.

With the emergence of clinical data for NOX inhibitors, interest is increasing. In addition, novel, highly selective inhibitors have been shown to have effect in a variety of animal models. Together, these results describe a future where detrimental ROS can be inhibited at the production stage, without disturbing essential ROS signalling.

Acknowledgements

M. G. L. acknowledges the support of the Spanish Ministry of Economy and Competence grant ref. SAF2015-63935R.

All authors were supported by the European Cooperation in Science and Technology (COST Action BM1203/EU-ROS).

Conflict of interest

F. K. W. is an employee of Redoxis AB, a Swedish company that develops low MW NOX modulators.

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