



Review Article

NADPH oxidases as drug targets and biomarkers in neurodegenerative diseases: What is the evidence?



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ABSTRACT

Neurodegenerative disease are frequently characterized by microglia activation and/or leukocyte infiltration in the parenchyma of the central nervous system and at the molecular level by increased oxidative modifications of proteins, lipids and nucleic acids. NADPH oxidases (NOX) emerged as a novel promising class of pharmacological targets for the treatment of neurodegeneration due to their role in oxidant generation and presumably in regulating microglia activation. The unique function of NOX is the generation of superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2). However in the context of neuroinflammation, they present paradoxical features since $O_2^{\cdot-}/H_2O_2$ generated by NOX and/or secondary reactive oxygen species (ROS) derived from $O_2^{\cdot-}/H_2O_2$ can either lead to neuronal oxidative damage or resolution of inflammation. The role of NOX enzymes has been investigated in many models of neurodegenerative diseases by using either genetic or pharmacological approaches. In the present review we provide a critical assessment of recent findings related to the role of NOX in the CNS as well as how the field has advanced over the last 5 years. In particular, we focus on the data derived from the work of a consortium (Neurinox) funded by the European Commission's Programme 7 (FP7). We discuss the evidence gathered from animal models and human samples linking NOX expression/activity with neuroinflammation in neurodegenerative diseases, such as amyotrophic lateral sclerosis (ALS) and Creutzfeldt–Jakob disease as well as autoimmune demyelinating diseases like multiple sclerosis (MS) and chronic inflammatory demyelinating polyneuropathy (CIDP). We address the possibility to use measurement of the activity of the NOX2 isoform in blood samples as biomarker of disease severity and treatment efficacy in neurodegenerative disease. Finally we clarify key controversial aspects in the field of NOX, such as NOX cellular expression in the brain, measurement of NOX activity, impact of genetic deletion of NOX in animal models of neurodegeneration and specificity of NOX inhibitors.

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1. Introduction

1.1. The family of NADPH oxidases

The family of NADPH oxidases (NOX) contains seven members NOX1, NOX2, NOX3, NOX4, NOX5, DUOX1 and DUOX2 (for review, see [1]). They are membrane proteins comprising 6 transmembrane domains (7 for DUOX1 and 2), which have as sole known function to catalyze the formation of superoxide anion ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2). In terms of mechanism, all NOX isoforms function as electron transporters. Electrons from cytoplasmic NADPH are transferred across membranes by the NOX protein complex, via intermediate flavin adenine dinucleotide and heme prosthetic groups, to reduce molecular oxygen in the extracellular space or in the lumen of intracellular organelles. During this reaction, oxygen and NADPH are consumed while $NADP^+$, intracellular H^+ and extra-cytosolic $O_2^{\cdot -}$ are produced. $O_2^{\cdot -}$ is rapidly dismutated into H_2O_2 either spontaneously or upon catalysis by superoxide dismutases (SOD). While $O_2^{\cdot -}$ is the primary product of the reaction catalyzed by NOX, the kinetics of conversion to H_2O_2 appears to vary among NOX isoforms. For example, only minute amounts of $O_2^{\cdot -}$ and high amounts of H_2O_2 are detected as a result of NOX4 activity and only H_2O_2 is detected from DUOX1 and DUOX2 activities. Each NOX isoform is characterized by specific tissue distribution and mechanism of activation [1].

Because of its high expression and activity in phagocytes, the NOX2 isoform was originally named gp91^{phox} (phox: phagocyte oxidase). NOX2 (encoded by the *CYBB* gene) was formally identified in 1976 [2] and its biochemistry has been extensively studied. At resting state, NOX2 forms a stable but inactive complex with the transmembrane protein, p22^{phox} (*CYBA*). Activation requires protein kinase C-dependent phosphorylation, leading to the translocation of cytosolic subunits p47^{phox} (*NCF1*), p67^{phox} (*NCF2*) and p40^{phox} (*NCF4*) to the cell membrane, where they physically interact with the p22phox-NOX2 complex and one of the small Rho GTP-binding proteins, Rac1 or 2 (Fig. 1). Similarly to NOX2, NOX1 and NOX3 require the interaction with p22^{phox} and specific cytosolic components (NOXO1 (NOX organizer type 1), NOXA1 (NOX activator type 1)) for activity. NOX4 also requires p22^{phox}, but is constitutively active. DUOX1 and 2 require the maturation factors DUOXA1 and 2. NOX5 and DUOXes are activated by direct phosphorylation and/or increase of intracellular Ca^{2+} . Under physiological conditions, NOX are essential mediators of different processes, and mutations and polymorphisms of NOX genes can have notable effects [3]. Different mutations affecting NOX2 holoenzyme cause increased susceptibility to infections by certain pathogens due to deficient neutrophil oxidative burst and impaired bacterial killing in humans and mice, a disease known as chronic granulomatous disease [4]. In addition, a deficiency in functional NOX2 complex has been

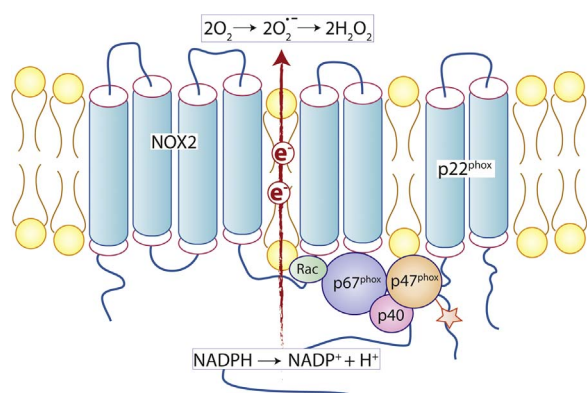


Fig. 1. Schematic structure of the phagocyte NADPH oxidase NOX2. The catalytic complex is formed by the membrane association of NOX2 and p22^{phox}. Upon activation a cytosolic complex is formed and translocates to the membrane in order to form an active $O_2^{\cdot -}$ -generating enzymatic system.

associated with the development of various autoimmune chronic inflammatory conditions [5]. Mutations in one of the component of the DUOX2 complex lead to congenital hypothyroidism while mutations affecting the NOX3 complex lead to balance impairment in rodents. Mutations in NOX1 predispose patients to inflammatory bowel disease [6]. In contrast, loss of function mutations in NOX4, NOX5 or DUOX1 genes have not been reported, and the NOX4 and DUOX1 knockout mice do not display an obvious spontaneous phenotype. While the absence of at least certain NOX-derived oxidants have pathological consequences, excessive oxidant production by NOX enzymes can contribute to oxidative tissue damage in a wide range of diseases, such as cardiovascular diseases [7], pancreatitis [8], and several diabetic complications [9]. Importantly numerous studies have identified the involvement of NOX in various CNS disorders [10].

1.2. Neuroinflammation and neurodegenerative diseases

The most frequent neurodegenerative diseases (ND) are Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), Huntington's disease (HD) and multiple sclerosis (MS). Symptomatic treatments exist for some ND like PD and potent immunomodulatory drugs show efficacy in MS. However no disease modifying therapies are yet available for ND. The symptoms of ND are largely due to the progressive impairment of neuronal function and vary depending on the type of neuronal cells and brain regions affected (e.g., cortex, hippocampus, substantia nigra, spinal cord, striatum or peripheral nerves) [11–13]. ND can be triggered by cell death, protein aggregation or molecules released by injured neurons. The initiating factors leading to neurodegeneration can vary greatly, but several features are shared in most ND: abnormal aggregation of specific proteins, spatio-temporal spreading of aggregated proteins in the CNS parenchyma, a sustained inflammatory response, and oxidation of lipids, proteins and nucleic acids [14,15]. MS differs from other ND, as it is an autoimmune disease leading to the destruction of the myelin sheath and neuronal loss is secondary to the myelin loss [13]. MS presents typical features of inflammation as it is characterized by infiltration of leukocytes in the brain and spinal cord. In other ND like AD, PD and ALS, the cell types governing neuroinflammation are mostly astrocytes and microglia [14]. Early neuroinflammatory features are mostly inconspicuous, as the classical signs of inflammation (*calor*, *dolor*, *rubor*, and *tumor*) are not observed. Moreover, typical anti-inflammatory drugs, such as non-steroidal anti-inflammatory drugs (most of which inhibit the activity of cyclooxygenase-1 and/or -2) have been unsuccessful in treating ND, or have even been harmful [16]. Similar to peripheral inflammation, neuroinflammation is most likely a primary response of the host aimed at protecting neurons by removing harmful stimuli and initiating healing processes. However, when neuroinflammation is sustained/prolonged, it may become detrimental to neuronal cells and contributes to the progression of ND (for review, see [14]). Thus, identifying novel drug targets that control neuroinflammation represents an important therapeutic approach for ND, although this approach has so far been relatively unexplored [14,17].

1.3. NOX as potential regulators of neuroinflammation

Among the potential regulators of neuroinflammation, NOX represent a novel promising class of targets for the treatment of ND. As indicated, oxidative modifications to proteins, lipids and DNA are almost always observed in ND, but the source of oxidants remains elusive. Increased oxidative stress resulting from increased oxidant formation has been known for many years to be a central feature in ND [18]. It was once thought that oxidants were directly involved in neuronal death due to their toxic properties, but evidence points towards subtler regulatory roles of at least some oxidants [19,20]. Indeed, increased oxidant generation in specific cellular and subcellular compartments leads to oxidative modifications of macromolecules, which modulate

the transcription and other signaling pathways controlling neuroinflammation. Oxidants are key components of the neuroinflammatory response, but therapeutic approaches targeting reactive oxygen species have so far been unsuccessful [18]. In particular, the absence of benefit of antioxidant strategies may be due to the fact that the molecules used lacked *in vivo* efficacy or that the overall effect of antioxidants is complicated by a concomitant attenuation of regulatory role of oxidants. A novel approach consists of targeting a *primary source* of $O_2^{\cdot-}/H_2O_2$ (e.g., NOX) rather than ‘scavenging’ oxidants after they have been formed and/or undergone metabolism.

There are several potential sources of cellular oxidants, as $O_2^{\cdot-}/H_2O_2$ are the byproducts of the activity of mitochondria, peroxisomes and xanthine oxidase among others. Compared with these sources, however, NOX are different in the sense that their sole function is the generation of $O_2^{\cdot-}/H_2O_2$. A role of excessive NOX-formed $O_2^{\cdot-}/H_2O_2$ and secondary oxidants derived from them in CNS pathology is known since the first report that NOX2 gene knockout mice are partially protected from brain ischemia [21]. Similar to neuroinflammation, which can be protective or deleterious when over-activated, NOX present paradoxical features: NOX-derived $O_2^{\cdot-}/H_2O_2$ can lead to oxidative damage and pathology, or be beneficial by regulating key physiological functions, including the resolution of inflammation in autoimmune diseases, including MS. Thus, depending on the pathology, the therapeutic approach should either enhance or mitigate NOX activity (see graphical abstract). We have previously thoroughly reviewed the literature concerning the expression of NOX isoforms in the CNS as well as their presumed contributions to CNS disorders in models of AD, PD, ALS and MS [10,22,23]. With the present review we would like to provide an update on the role of NOX in the CNS as well as how the field has advanced over the last 5 years. In particular, we focus on key findings obtained by the Neurinox consortium (www.neurinox.eu) which had the goal to study some of the most controversial aspects of NOX biology in the CNS.

2. Advances and findings on NOX in the CNS and ND

2.1. NOX expression and localization

The cellular localization of NOX enzymes is still a matter of debate. The scientific literature reports the expression of several NOX isoforms in virtually every cell type of the CNS [23]. However, many studies were performed by using non-validated commercial antibodies without appropriate controls. Validated isoform-specific NOX antibodies are scarce because the lipophilic structure of these proteins makes the generation of specific antibodies difficult. Recent available data however have allowed to deduce the precise cellular localization of NOX isoforms in the CNS by using a combination of qPCR experiments [24], RNAseq analyses of isolated cell types from mouse and human brain [25], and immunohistological signals using the few available specific NOX antibodies, which have been validated using NOX gene knockout mice [24,26].

2.1.1. NOX2 and NOX4 are the main NOX isoforms expressed in the CNS

Remarkably, NOX expression data indicate that under physiological conditions most NOX isoforms are expressed only to a negligible extent in the CNS. Specifically, qPCR shows that NOX1, NOX3, NOX5, DUOX1 and DUOX2 are undetectable or at the limit of detection in the human brain as well as in the mouse spinal cord and cerebral cortex [24,25]. NOX4 is detected in CNS tissue, and RNAseq data indicate that it is expressed mostly in the mouse endothelium, while very low amounts are also present in fetal astrocytes and oligodendrocytes. Similarly, NOX1 and NOX5 are expressed in the vasculature [27], but no clear evidence indicates that they are present in CNS parenchyma. Interestingly, DUOX1 was detected at high levels in the CNS by qPCR and RNAseq, most likely in microglia, however only in the mouse brain [24,25]. DUOX1 is known to play a role as maturation factor of

DUOX1, but since DUOX1 is absent in microglia, it may have a presently unknown role in mouse microglia. Nonetheless, the most relevant NOX isoform in the CNS in terms of expression is NOX2. Moreover, NOX2 expression is strikingly enhanced in the CNS of patients and mouse models of ND [24,26].

2.1.2. NOX2 and its subunits are enriched in microglia

Several lines of evidence indicate that, in the CNS, NOX2 is expressed mostly in microglia. First of all, NOX2 and subunits are key markers of phagocytes and are under the control of the transcriptional factor PU.1, the main transcriptional regulator of phagocytes [28]. In humans, the MoAb 48 monoclonal NOX2 antibody [29] shows distinct cellular co-localization of NOX2 with the microglial marker Iba-1, but not with markers for astrocytes (GFAP) or neurons (MAP2 or NF) [26]. Although no suitable antibody is available for NOX2 immunohistochemistry in mice, the following indirect evidence indicate microglial expression of NOX2: (i) concomitant increase of NOX2 expression and its subunits p47^{phox}, p67^{phox}, p40^{phox}, rac1 and p22^{phox} with the microglial markers CD68, Iba-1 and CD11b in a mouse model of ALS [24]; (ii) absence of NOX2 detection in organotypic cerebellar slices where microglia was specifically ablated by ganciclovir in *tga20/TK* mice [22]; (iii) enrichment of NOX2 in microglial populations as confirmed by transcriptional profiling of isolated brain cells [25].

2.1.3. NOX2 is present in neural adult stem cells

In addition to microglia, NOX2 is also expressed in small populations of neural stem cells of the dentate gyrus and subventricular zone of the adult brain, where NOX2 plays a role in the proliferation and multipotency of mouse neural stem cells [30,31,98].

Fig. 2 summarizes NOX expression in the cells of the CNS. Our analysis cannot exclude the possibility that, in specific circumstances (e.g., during neuronal development) or in particular subpopulations of cells or brain areas, the expression of NOX isoforms can be elicited, e.g., NOX1 in substantia nigra [32] or NOX4 in pericytes [33]. Nevertheless, in ND, the expression of NOX2 increases greatly, largely due to microglia activation or CNS invasion by peripheral myeloid cells. The latter cannot easily be distinguished from resident microglia, especially in pathological situations associated with blood-brain-barrier damage. In any case, it is unlikely that NOX2 is a key source of oxidants in mature neurons. Instead, alternative sources of neuronal oxidants might

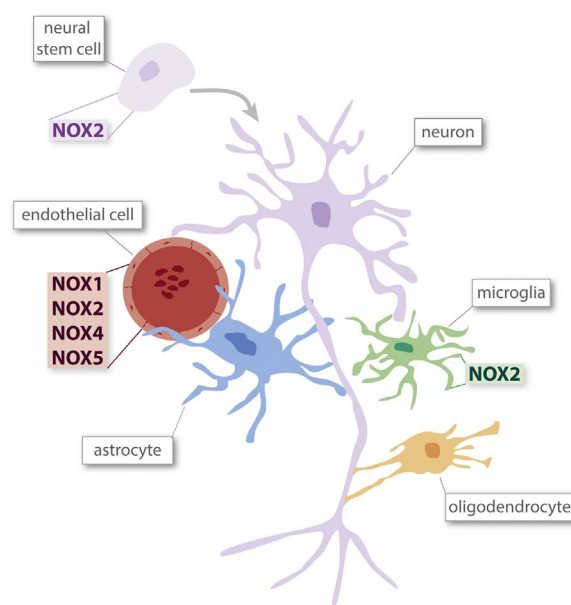


Fig. 2. Cellular localization of NOX2 in the CNS. NOX2 is expressed by microglia and invading monocytes/macrophages as well as adult neural stem cells. All other NOX isoforms are present in brain vascular cells.

derive from mitochondria [34], MICAL proteins [35], monoamine oxidase [36] and cytochrome P450 [37] among others.

Having clarified that NOX2 expression is closely related to microglia activity offers the possibility to better understand and interpret the impact of NOX2 genetic or pharmacological targeting. In light of the multifaceted and often discordant consequences of microglia contribution in ND, it could then be reasoned that NOX2-derived oxidants in these cells can similarly exert beneficial and detrimental effects, depending on the particular context.

3. Small molecules targeting NOX enzymes

3.1. NOX inhibitors

Most information on the role of NOX in the CNS derives from studies using the natural product apocynin. Numerous published studies report a striking beneficial effect of apocynin on ND in animal models, via decreasing microglia activation and improving symptoms and survival (reviewed in [22,38]). However, there are some notable exception to such an apparent beneficial effect, including an ALS mouse model where apocynin is inactive [39] and the Tg19959 mice model of AD, where apocynin is deleterious [40]. Importantly, we [41] and others [42] have shown that apocynin is not a NOX inhibitor in the sense that it does not show affinity for the NOX enzyme. Apocynin may appear as a NOX inhibitor at high concentrations because either it interferes with peroxidase-dependent assays for oxidants [43] or may inhibit the translocation of p47^{phox} in neutrophils, thereby indirectly inhibiting NOX2 activation [44]. A recent study using a mitochondria-targeted apocynin argues for mitochondrial oxidant scavenging as the basis for its anti-inflammatory and neuroprotective effects in a mouse model of PD [45]. Thus, studies documenting a therapeutic effect of apocynin in models of neurodegenerative diseases do not necessarily imply a role of NOX.

The case of apocynin highlights the challenges faced when searching for NOX inhibitors. This is the reason why we [41,46,47] and others [48] have developed a strict flowchart for identification and characterization of NOX inhibitory molecules [49]. This approach is designed to exclude false positives including those resulting from cell toxicity and interference with oxidant-measuring systems (Fig. 3). This flowchart emphasizes the requisite of (i) using NOX cell free assays for screening small molecules and determines the mode of action of identified inhibitors, (ii) use several detection systems to measure the activity of NADPH oxidases, ideally by separately measuring O₂⁻ and H₂O₂, (iii) use of methods allowing measuring NOX-dependent substrate consumption (i.e. O₂ and NADPH) to exclude molecules interfering with ROS detection systems and (iv) importantly for the development of NOX based drugs, clear documentation of in vivo pharmacokinetics and pharmacodynamics parameters. Bona fide NOX inhibitors should have the two following characteristics: i) they should inhibit NOX-derived oxidants in both cellular and NOX purified membranes and ii) they should block oxygen consumption in cells and/or NADPH consumption in NOX purified membranes. Below is an exhaustive list of small molecules NOX inhibitors which have passed this flowchart.

(i) Diphenyleneiodonium (DPI) potently inhibits all NOX isoforms with high affinity [46], most likely by irreversible binding to the cytosolic FAD domain of NOX [50]. However DPI lacks drug-like properties due to mitochondria toxicity and low solubility. DPI is not specific for NOX as it inhibits other electron transport chains, including NO synthase, xanthine oxidase among others. Nonetheless DPI is a useful pharmacological tool in vitro and should be systematically used as a reference NOX inhibitor. As a rule of thumb, a signal not inhibited by DPI is most likely not due to NOX [49]. However caution is still warranted in interpretation since a lucigenin bioluminescent signal inhibited by DPI was recently

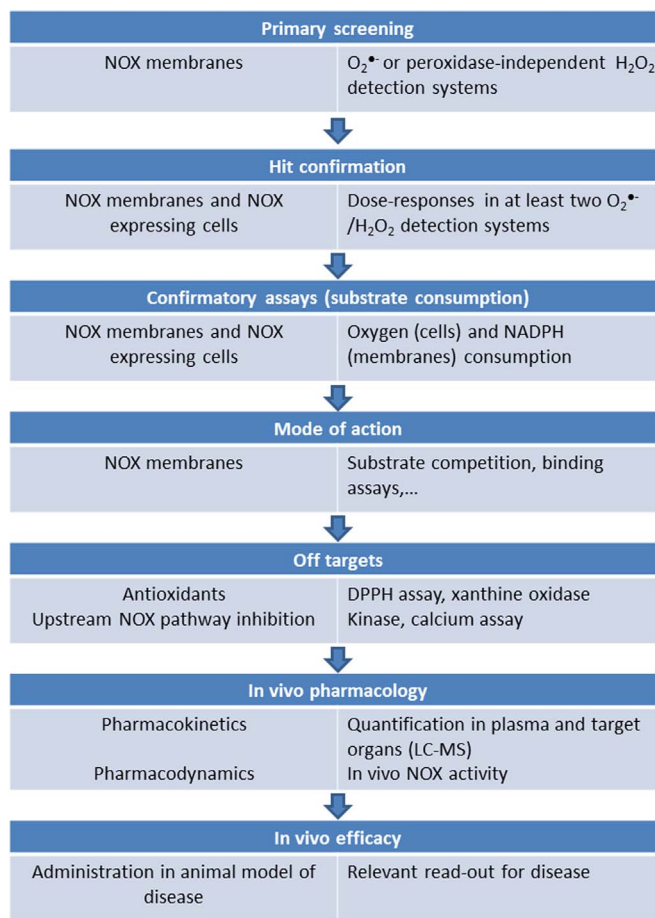


Fig. 3. Flow-chart for identification of bona fide NOX inhibitory drugs.

shown to be due to cytochrome P450 [51].

- (ii) GSK2795039, a novel drug-like small molecule, is the first identified NOX inhibitor to show isoform-specificity. Its mode of action is through competition for the NADPH binding site of NOX2. GSK2795039 is orally available, non-toxic, CNS-permeable and inhibits NOX2 activity in vivo [41]. GSK2795039 has not yet been tested in an animal model of ND.
- (iii) A subset of *N*-substituted phenothiazines - but not non-substituted phenothiazines - inhibits NOX activity [47]. *N*-substituted phenothiazines have been used for decades as anti-psychotic agents, and upon administration can give rise to micromolar concentrations in the CNS. It remains unclear how *N*-substituted phenothiazines inhibit NOX, although they do not show isoform specificity. *N*-substituted phenothiazines, including thioridazine and perphenazine, are known to be promiscuous molecules with numerous potential modes of action in the CNS. However, since they are already used as human therapeutics, they offer an attractive perspective for potential repurposing in ND (see below for their use in a mouse model of ALS).
- (iv) Ebselen and ebselen-sulfur analogues (e.g. thr101) were identified as inhibitors of the interaction between p22phox and p47phox in a high throughput screening [52]. These molecules inhibit NOX2 in a membrane assay and in whole cells expressing NOX1, NOX4 and NOX5. Although low concentration of ebselen is toxic to cells, it was confirmed as bona fide inhibitor of NOX2 in precedent [53] and subsequent [48] studies. Ebselen is known for a long time for its neuroprotective properties, in particular against stroke [54]. Unfortunately ebselen has multiple potential pharmacological targets with a strong redox component [55] and is thus not very useful to demonstrate a NOX-dependent activity.

(v) Celastrol is a natural extract derived from *Tripterygium wilfordii*, a plant used in traditional Chinese medicine. Celastrol inhibits NOX2-derived ROS and substrate consumption in NOX2 membrane assays and cells expressing NOX1, NOX2, NOX4 and NOX5, including NOX2-dependent oxygen consumption in neutrophils and NADPH in NOX2 membrane assay [41,46]. However, its practicality as a NOX inhibitor is limited due to cell toxicity, the fact that it is colored (orange) and thus interferes with several assays. Similarly to ebselen, it is a promiscuous molecule with multiple described mode of action and is therefore not very useful to demonstrate a NOX-dependent activity.

Recently, the team of Zielonka and Kalyanaraman have developed their own flow-chart to identify NOX2 inhibitors using HL60 cells, a human neutrophil-like cell with high levels of NOX2 expression and activity [48,56]. They use several distinct read-outs for $O_2^{\cdot-}$ and H_2O_2 in high throughput format and confirm the hits by measuring toxicity and NOX2-dependent oxygen consumption by HL60 cells. This approach confirmed the activity of DPI as well as ebselen and analogues but infirmed the NOX inhibitory of apocynin. It also allowed the identification of several novel small molecules, including a novel small molecule of great interest Thioxo-dihydroquinazolin-one (aka compound 43) [48]. Identified molecules now awaits confirmation and potential mode of action in NOX2 semi-recombinant assays.

The favorable impact of antioxidants and anti-inflammatory molecules like apocynin adds further evidence that targeting neuroinflammation and oxidative stress may be beneficial in ND. The potential therapeutic values of apocynin and other reported non-validated NOX inhibitors is not questioned, but their efficacy in a model of ND is not sufficient evidence of a role of NOX and should be interpreted with caution. Only the use of CNS-permeable and fully validated NOX inhibitors, such as GSK2795039 will provide valid information on the role of NOX2 in ND.

3.2. NOX activators

Only few attempts have so far been made to identify substances inducing NOX2 activity for treating inflammatory disease although small molecules that activate ROS production have previously been identified [57–61]. Quinolinone derivatives are a new class of molecules that enhance NOX2 function has been recently developed aiming for treatment of CNS autoimmune diseases [62,99]. Identified NOX agonists are currently in a phase of optimization for bioavailability and pharmacokinetics. They have anti-inflammatory properties and are able to decrease the pro-inflammatory role of TNF- α in the low nanomolar range. Future studies will show whether they are suitable for clinical use as first in class NOX2 activators for treatment of autoimmune diseases.

3.3. Mechanisms regulated by NOX2 in peripheral circulating cells and autoimmune disease

In reference to the scheme in the graphical abstract, a large part of NeuroInox effort was dedicated to the study of the oxidants generated by NOX enzymes in the context of autoimmune diseases, including MS, and other peripheral autoimmune demyelinating diseases such as chronic inflammatory demyelinating polyneuropathy (CIDP) and Guillain-Barré syndrome (GBS). More specifically, NOX2 activity in macrophages and other antigen presenting cells has an anti-inflammatory effect and regulate activation of autoreactive T cells [63]. This unexpected fact was originally discovered in a genetic study performed to identify the most important polymorphic loci controlling autoimmune chronic inflammatory diseases, using models for rheumatoid arthritis and multiple sclerosis [64]. This led to the identification of a polymorphism in the coding sequence of Ncf1 (neutrophil cytosolic factor 1), the gene coding for p47^{phox}, a subunit essential to

control NOX2-dependent formation of $O_2^{\cdot-}$ [64,65]. It has been estimated that approximately half of inbred rat strains and half of the wild rat population carries an allele in Ncf1 that leads to a significantly lower NOX2-mediated oxidative burst [66]. The regulatory role of oxidants is likely to be conserved between species. Consistent with this notion, there is also an extensive polymorphism in the human Ncf1 region which is associated with autoimmune diseases [67]. Importantly, this allele confers strong protection against autoimmune diseases. This finding opened the provocative possibility that NOX agonists could serve as therapeutics in autoimmune disorders [68]. Since then, a protective role of NOX2-derived oxidants has been reproduced in several different models of autoimmune disorders, including models for MS and Guillain Barré [69–71]. The NOX2-dependent anti-inflammatory effect is mediated by several different pathways: (i) downregulation of autoreactive T cells during antigen presentation [63]; (ii) autocrine downregulation of inflammatory macrophages [5,72]; (iii) downregulation of STAT1 mediated activation of the interferon pathway [73,74]; and (iv) promotion of a protective effect by neutrophil extracellular traps (NETS) formed by neutrophils [75]. Further studies aimed to identify both the chemical nature of the oxidants involved and the respective roles played by these potentially protective pathways in MS and peripheral neuropathies are needed. Indeed, it is still unclear at present if excess oxidants in the CNS are counteracting the protective effect of the peripheral inflammatory attack, thereby promoting neurodegeneration [76].

3.4. Genetic and pharmacological inhibition of NOX in models of ND

The contribution of NOX-derived oxidants to the development of ND was studied in detail in two models of ND known for their intense neuroinflammatory reaction: ALS and Creutzfeldt–Jakob disease (CJD).

ALS is a severe ND affecting motoneurons and leading to complete paralysis and death. ALS is a typical neuroinflammatory disease characterized by an increase in pro-inflammatory markers, microgliosis and astrocytosis. In most cases, the underlying causes of ALS are unknown. However, the identification of novel ALS-causing mutations indicates that mechanisms leading to the aggregation of specific proteins and RNA pathology (e.g., TDP-43, SOD1, FUS and c9orf72) are a key pathological event in ALS. There are no treatments for ALS, and Riluzole, a small molecule known to decrease glutamate levels in the CNS, is the only drug prescribed for ALS patients, although it has very modest efficacy. In both patients and animal models of ALS, the expression of microglial NOX2 is strongly increased, specifically in affected regions of the spinal cord [24]. Previous studies by the Engelhardt group have shown that NOX1 and NOX2 deficiency confers prolonged survival of transgenic mice expressing an ALS-causing mutation (SOD^{G93A} mice), the most commonly used model for this disease [77]. NOX inhibitors appeared as most promising therapeutics to slow down ALS progression. As mentioned above, thioridazine and perphenazine have NOX inhibitory activity and as they are used in the treatment of humans, they represent attractive drugs for rapid initiation of clinical trials in ALS patients [47]. However, administration of thioridazine and perphenazine to SOD^{G93A} mice did not improve survival, although they showed some benefits in secondary read-outs, such as motor function for thioridazine and weigh loss for perphenazine [47]. Thioridazine decreased $O_2^{\cdot-}$ levels and microglia activation markers in spinal cords of SOD^{G93A} mice, yet crossing of SOD^{G93A} mice with NOX2 or NOX1 gene knockout mice did not affect disease onset and progression, nor microgliosis, astrogliosis or motoneuron survival [24]. These results question initial reports [77], showing a prolonged lifespan in ALS models related to NOX2 and NOX1 deficiency.

Creutzfeldt–Jakob disease (CJD) is an incurable ND characterized at the neuropathological level by a massive neuronal loss conferring a spongiform aspect of the brain [78]. The causative agent of CJD is the misfolding of prion proteins which propagates among neurons and leads to a neuroinflammatory environment and increased microglial

NOX2 expression [26]. Interestingly, NOX2 gene knockout mice infected with prions did not show decreased microgliosis, but showed a transient improvement of motor function (rotarod), decreased oxidants, significant survival improvement and improvement of vacuoles formation in the brain at terminal stage. Similarly, NOX2 greatly contributes to neurotoxicity in mice treated with a neurotoxic anti-prion antibody [79].

Together, these data show that NOX2 expression is strongly increased in ND and most likely contributes to oxidant formation associated with neurodegenerative mechanisms, although it does not seem to limit microglia activation and microgliosis. Whereas increased NOX2 activity and oxidant generation are shared between CJD and ALS, NOX2 deletion slightly improves survival only in CJD. This suggests that the neurotoxic cascade of CJD and ALS are different, and that targeting NOX2-derived oxidants might be more efficient in CJD than in ALS. Remarkably, while antioxidant-based therapies have been strikingly unsuccessful in ALS, prion pathogenesis was shown to converge in oxidant formation and to be efficiently mitigated both *ex vivo* and *in vivo* by antioxidants [80]. Therefore, novel specific NOX2 inhibitors with blood brain barrier permeability and a defined mode of action such as GSK2795039 await further testing in prion pathogenesis and other ND [41,81].

3.5. Measurement of NOX activity *in vivo*

Measuring NOX activity in tissues remains a challenge. In the literature of ND the most commonly used approach is to use a CNS tissue homogenate to which lucigenin and NADPH are added and lucigenin-enhanced chemiluminescence is then determined as a measure of $O_2^{\cdot-}$. However, the method of lucigenin-enhanced chemiluminescence has well known pitfalls and limitations (see, e.g., [82]). In addition, Rezende et al. [51,83] recently reported that the signal measured in this assay is due to cytochrome P450, rendering it inappropriate for determination of NOX activity.

The major product of NOX is $O_2^{\cdot-}$, and hydroethidine or dihydroethidium [84] is considered the ‘gold standard’ for $O_2^{\cdot-}$ determination in biological systems [85]. Importantly, previous work by Kalyanaram and co-workers has established that the fluorescence of ethidium, commonly the most abundant oxidation product of hydroethidine in biological systems, is not specific for $O_2^{\cdot-}$ [84]. Instead 2-hydroxyethidium is the $O_2^{\cdot-}$ specific product. Unfortunately, ethidium and 2-hydroxyethidium have very similar fluorescent properties, so that fluorescence is not a specific readout for $O_2^{\cdot-}$ in biological systems. Rather, a combination of chromatographic separation of ethidium and 2-hydroxyethidium coupled with fluorescent or mass spectrometry based detection is required for the selective determination of $O_2^{\cdot-}$. Using LC-MS/MS and administering hydroethidine into the spinal cord revealed that thioridazine decreased $O_2^{\cdot-}$ in the spinal cord of SOD1^{G93A} mice *in vivo* [24]. Despite this successful demonstration of a localized decrease in $O_2^{\cdot-}$ *in vivo*, there remain a number of challenges. First, such approach cannot provide direct information on NOX activity, as the method at best measures steady state concentration of $O_2^{\cdot-}$, that is determined by the rates of both formation and ‘removal’ of this reactive species. ‘Removal’ in this context includes both dismutation of $O_2^{\cdot-}$ as well as reaction of $O_2^{\cdot-}$ with other reactive species (e.g., nitric oxide) giving rise to secondary oxidants. Second, the concentration of 2-hydroxyethidium detected following *in vivo* administration of hydroethidine represents a snapshot only of what is likely a complex and changing process as ND develops. Similarly, the route of administration of hydroethidine likely affects the products detected subsequently. Therefore, for such a probe-based approach to measure $O_2^{\cdot-}$, each experimental model requires careful ‘optimization’ with regards to timing of probe administration relative to disease process, route of probe administration, and exposure time prior to tissue harvest and analytical analyses of unreacted and reacted probe. Third, the biochemical approach cannot provide true information on the spatial

compartmentalization of NOX activity.

As of today, no method allowing for the live measurement of reactive oxygen species or NOX activity in the CNS is available. Such a probe would help understanding the respective sources of oxidants, their dynamics, prognosis value of NOX2 activity and contribution to ND.

3.6. NOX and oxidative biomarkers in NDs

Despite the challenges mentioned above, a large part of Neurinox was dedicated to evaluate NOX activity and oxidized biomarkers in ND patients. In a clinical study, NOX2 activity from peripheral neutrophils and monocytes was directly measured in fresh whole blood of a cohort of 83 ALS patients, and age- and gender-matched healthy controls. The assay is based on flow cytometry and uses the cell permeable fluorescent dye DHR₁₂₃, an unspecific probe used for diagnosis of chronic granulomatous disease. Upon addition of a specific activator, no difference was observed between patients and healthy controls, however, inside the ALS group, low DHR₁₂₃ oxidation in leukocytes was significantly associated with a longer survival [86]. This represents an important finding in the field of ALS because this may be a prognosis biomarker of the severity of ALS. More importantly, such a measure could be implemented as surrogate biomarker to address the benefit of a drug in clinical studies (Fig. 4A-C).

Chronic inflammatory demyelinating polyneuropathy (CIDP) is a neurological disorder characterized by damaged myelin sheath of the peripheral nerves leading to progressive weakness and impaired sensory function in the legs and arms. Intravenous immunoglobulin (IVIg) therapy is used as a first-line therapy and usually provides substantial benefit to patients. A prospective clinical study enrolled 30 CIDP patients treated with IVIg and 30 control subjects for whom NOX2 activity/DHR₁₂₃ oxidation was measured in neutrophils and monocytes from freshly collected blood. At diagnosis NOX2 activity was significantly increased in CIDP patients compared to controls. However, following IVIg therapy, NOX2 activity was even more increased compared to basal levels [87]. The exact cause of this observation is unclear, but the results are consistent with therapeutic improvement in autoimmune demyelination being associated with enhanced NOX2. Because of the simplicity and robustness of this assay, it could be included systematically in clinical settings for ND. This would potentially provide key information on inclusion criteria and the response to a drug.

Measuring NOX2 activity in whole blood is informative of a stage of disease. However, as previously stated, there are currently no methods/probes available to measure $O_2^{\cdot-}$ or H_2O_2 in human CNS. For these reasons, determining the role of NOX in human ND continues to rely on indirect measures, such as assessment of oxidative stress. The Neurinox consortium determined F_2 -isoprostanes as well as enzymatic oxidation products of arachidonic acid (by LC-MS/MS) in cerebrospinal fluid and plasma of patients with progressive multiple sclerosis. Compared with controls, plasma concentrations of F_2 -isoprostanes and prostaglandin $F_{2\alpha}$ (PGF_{2 α}) were decreased with increasing disability score [88]. This was in contrast to the situation in cerebrospinal fluid, where the concentrations of PGF_{2 α} , but not F_2 -isoprostanes, were significantly higher in patients with progressive disease than controls. Cerebrospinal fluid PGF_{2 α} was reduced with natalizumab and methylprednisolone treatment, suggesting that PGF_{2 α} levels in the CSF represents reliable surrogate biomarkers for evaluation of the efficacy of a drug (Fig. 4D, E). Note that PGF_{2 α} levels remained unaffected by the use of nonsteroidal anti-inflammatory drug in secondary progressive MS. Interestingly, cerebrospinal fluid PGF_{2 α} did not associate with validated cerebrospinal fluid markers of inflammation or markers of brain damage that themselves did not associate with the disability score [88]. These results suggest that MS progression is associated with low rather than high systemic oxidative activity, and that this may play a role in immune dysregulation with central nervous system inflammation

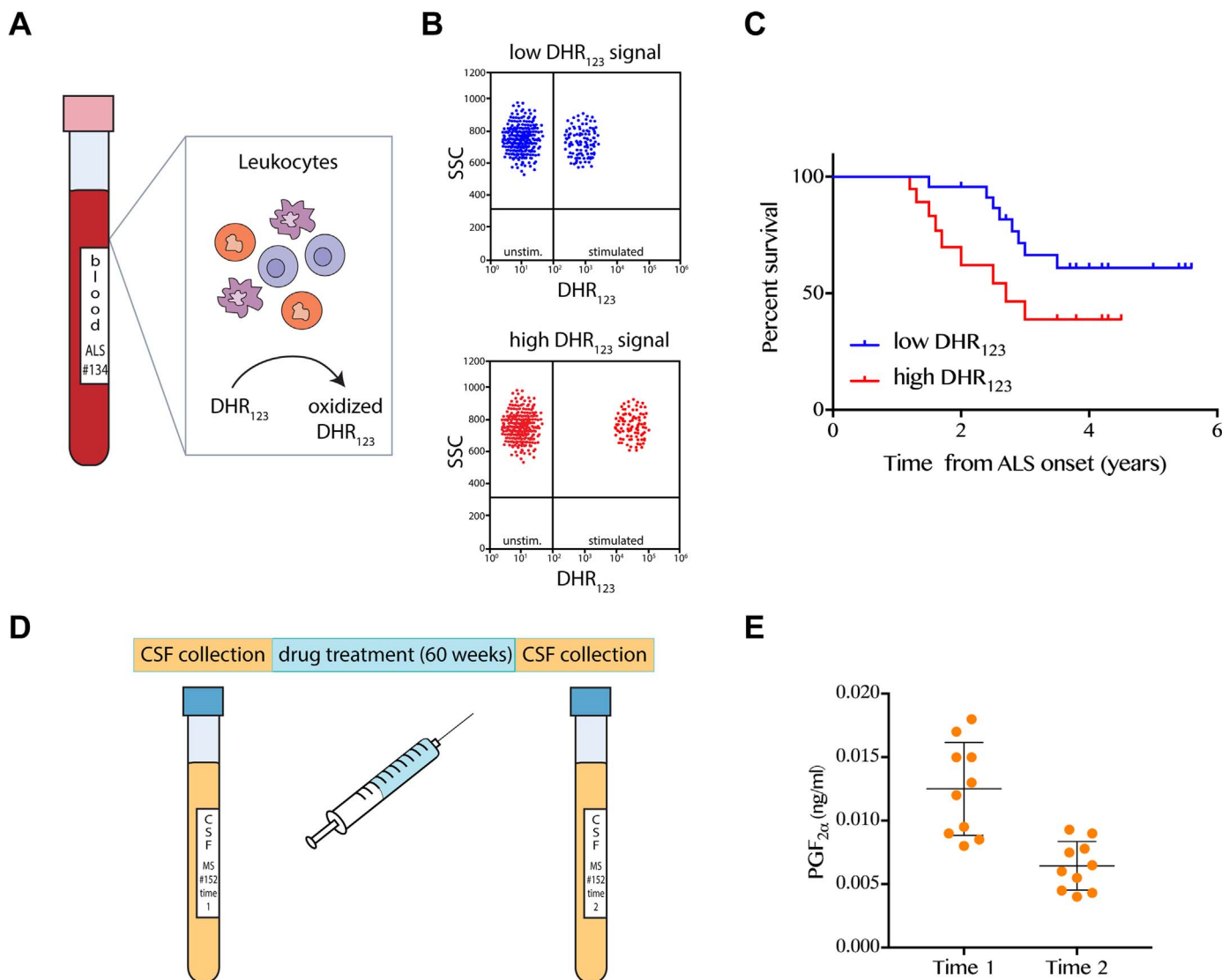


Fig. 4. Measurement of redox activity in physiological fluids of patients affected by neurodegenerative disease as predictors of disease severity and potential efficacy of a drug (surrogate biomarkers). NOX2 activity can be measured directly in whole blood using DHR₁₂₃ (A) followed by flow cytometry (B). High levels of NOX2 activity is associated with decreased survival in ALS patients (C) [86]. Quantification of oxidation products of arachidonic acid in the cerebrospinal fluid of MS patients (D) shows that it can serve as surrogate biomarkers of the activity of a drug treating MS (E) [88].

accompanied by increased local cyclooxygenase-dependent lipid oxidation.

4. Other redox systems in the CNS

The therapeutic approach of targeting proteins involved in redox dysregulation in ND is not limited to NADPH oxidases and several redox systems represent valid pharmacological targets. Dimethyl fumarate, a small molecule activator of Nrf2, a key transcription factor of the antioxidant response, has shown promising results for the treatment of certain forms of MS [89]. Similarly, mitochondrial targeting of specific antioxidants [90] and apocynin [91] have been reported to yield promising results in preclinical models of PD. Small molecules inhibiting myeloperoxidase are in clinical trials for PD [92]. In the CNS, H₂O₂ can be generated by the MICAL (molecule interacting with CasL) family of enzymes, in particular by MICAL-2 which controls dendrite and synapse formation [93]. In addition to NOX, several sources of O₂^{•-}/H₂O₂, including nitric oxide synthase, monoamine oxidase, xanthine oxidase are present in the CNS, mainly in microglia [94]. Reversible cysteine oxidation by thioredoxins and peroxiredoxins are increasingly recognized as a fine-tuning redox regulation of CNS homeostasis, similar to what is

known for phosphorylation. Fig. 5 summarizes the main localization of several of these oxidant generators and redox regulators.

5. Concluding remarks

Our recent work has clarified NOX localization in the CNS, identified novel small molecule NOX inhibitors/activators and state-of-the-art methods to measure O₂^{•-} in vivo, showed a strong association between NOX2 and disease progression in ND, and indicated that NOX2 expression and activity parallels microgliosis and neuroinflammation in ND. Importantly, however, inhibition of NOX provided at best only limited beneficial effects in mouse models of ALS and CJD. Although some neurodegenerative processes (e.g. following traumatic brain injury) may benefit from NOX2 inhibition, it appears that this is not a disease-modifying approach. The question as to whether Nox2-generated oxidants is beneficial, not functional or detrimental in neuropathology should be addressed in priority using NOX2-deficient mice in animal models of ND.

Despite the scientific progress, no current therapeutic is able to halt neurodegenerative processes, in part due to difficulties in our understanding of the mechanisms underlying the development of ND. Indeed,

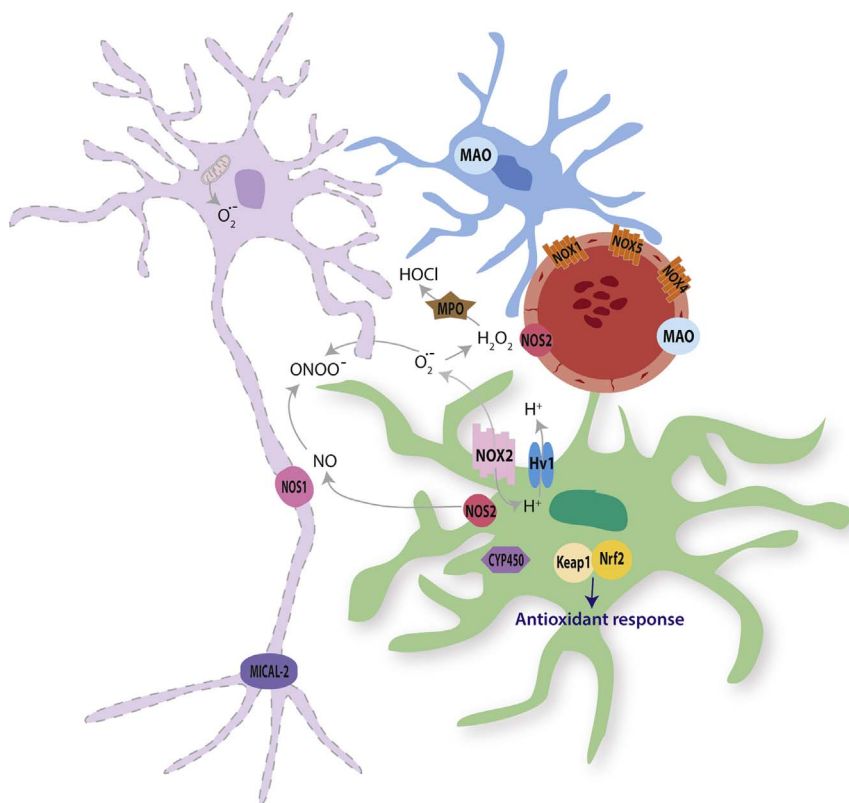


Fig. 5. Oxidant generating and redox regulating systems potentially involved in oxidative stress-mediated neuroinflammation (see text for details).

the identification of potential therapeutic targets derives from neuropathological observations and identification of disease-causing mutations. However, the fact that a particular pathological characteristic, such as oxidative stress, is associated with disease progression does not mean that it is a cause of the disease (see review [19]). For example, the presence of β -amyloid aggregates is the most clear pathological signs related to AD, however, it is not yet clear whether therapies decreasing β -amyloid aggregates in AD patients translate into symptomatic improvement and doubts arise about the validity of the original hypothesis implying amyloid as major cause of the disorder [95–97]. Similarly, the increase in number of glial cells with altered morphological status in brain sections of different patients affected by ND has supported the idea that this reaction contributes to disease progression. Experimental evidence has shown that the pathogenic mechanisms are more complex. Indeed, microglia can exert both detrimental and protective functions, depending on the particular pathological context [14]. It appears that similar conclusions can be taken for NOX in ND. NOX2 up-regulation is indeed a common feature of ND and a sign of a neuroinflammatory response, but NOX2 inhibition is not a disease-modifying treatment. NOX2 upregulation, increased oxidant generation, microgliosis and neuroinflammation are all associated factors of ND, but this evidence does not necessarily mean that they are a cause, as they can be rather a consequence of the neurodegenerative process. However, one of the key findings of our studies shows that a correlation between NOX2 activity and disease progression or response to treatments in patients is measurable in the blood, which makes NOX2 a promising biomarker for future evaluation of therapies for ND. Understanding the kinetics of oxidant formation and metabolism, the relative role of various oxidant-generating systems and their inter-dependence in the fine redox regulation will pave the way for long awaited therapeutics targeting oxidative stress in CNS disorders.

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