

# Targeting microglia-mediated neurotoxicity: the potential of NOX2 inhibitors

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**Abstract** Microglia are key sentinels of central nervous system health, and their dysfunction has been widely implicated in the progressive nature of neurodegenerative diseases. While microglia can produce a host of factors that are toxic to neighboring neurons, NOX2 has been implicated as a common and essential mechanism of microglia-mediated neurotoxicity. Accumulating evidence indicates that activation of the NOX2 enzyme complex in microglia is neurotoxic, both through the production of extracellular reactive oxygen species that damage neighboring neurons as well as the initiation of redox signaling in microglia that amplifies the pro-inflammatory response. More specifically, evidence supports that NOX2 redox signaling enhances microglial sensitivity to pro-inflammatory stimuli, and amplifies the production of neurotoxic cytokines, to promote chronic and neurotoxic microglial activation. Here, we describe the evidence denoting the role of NOX2 in microglia-mediated neurotoxicity with an emphasis on Alzheimer's and Parkinson's disease, describe available inhibitors that have been tested, and detail evidence of the neuroprotective and therapeutic potential of targeting this enzyme complex to regulate microglia.

**Keywords** Parkinson's disease · Alzheimer's disease · Neuroprotection · Microglia · NOX2 inhibition · Redox · ROS · NADPH oxidase

## Introduction

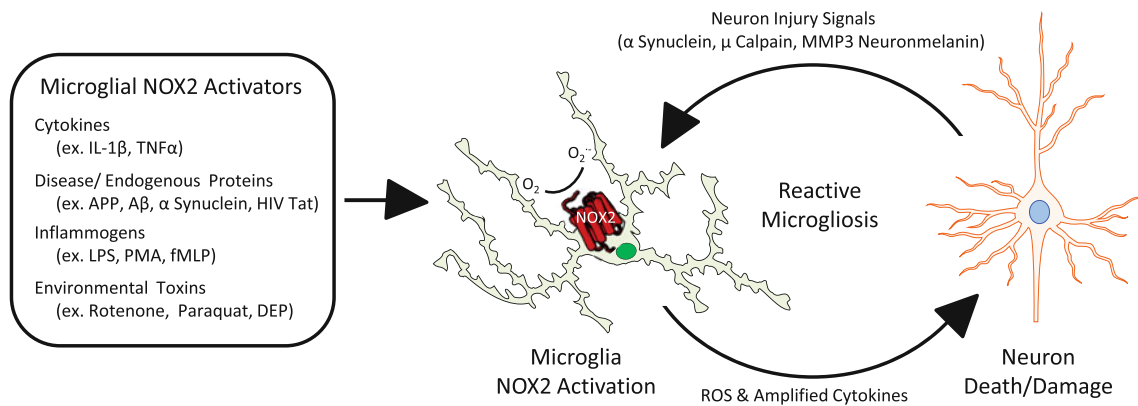
Inflammation, oxidative stress, microglial activation, and progressive neuron damage are common denominators present in most neurodegenerative diseases and some cases of central nervous system (CNS) damage. Increasing evidence supports that microglia, the resident innate immune cells in the brain, are a chronic source of inflammation and reactive oxygen species (ROS) culpable in progressive neuron damage. The NOX2 enzyme complex is present in microglia, has been implicated as a prominent source of microglial ROS in pathology, and is a key mechanism regulating microglia-mediated neurotoxicity. Here, we will detail the role of NOX2 in microglia-mediated neurotoxicity and explore the evidence implicating NOX2 inhibitors as a plausible neuroprotective approach regulating the neurotoxic microglial response.

## Microglia

Microglia are myeloid-derived cells that are essential for normal, healthy central nervous system (CNS) physiology [1]. Comprising approximately 0.5–16.6 % of the adult human brain [2], microglia perform dynamic cellular functions that include synaptic plasticity [3], cleaning of cellular debris [4, 5], neuronal support through the production of growth factors [6–8], wound healing through alternative activation [4, 5], and innate immune defense [9]. In fact, the loss of normal microglial function is deleterious [10]. For example, the inability of microglia to clear toxic disease proteins, such as beta amyloid ( $A\beta$ ), has also been linked to neurodegenerative diseases [10]. In addition, the absence of microglial cells can also be neurotoxic, as deletion of infiltrating macrophages that supply

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**Fig. 1** NOX2 is key for neurotoxic microglial activation. Microglial NOX2 is activated by cytokines, endogenous disease proteins, inflammogens, and soluble neuron injury signals from neuron death/damage (reactive microgliosis), which is implicated in self-

propagating neurotoxicity. Thus, specific inhibition of NOX2 is a promising target with the potential to break the cycle of chronic and neurotoxic microglial activation that is implicated in the progressive nature of neurodegenerative diseases

the expansion of microglial numbers in early stages of spinal injury actually enhances neuron damage [11]. Microglia are even essential for normal behavior, as genetic deletion of a particular microglial subtype has been linked to self-mutilating behavior in mice through compulsive grooming [12]. Thus, therapeutic approaches targeting microglia must focus on regulation of the toxic phenotype with preservation of their beneficial and maintenance functions.

While normal microglial function is mandatory for CNS health, there is increasing evidence that microglia also have an active role in neuropathology in disease, as microglia are activated in several neurodegenerative diseases and are shown to initiate neuron damage, amplify ongoing neurotoxicity, and drive chronic neuron loss over time [13]. As the resident innate immune cell in the brain, microglia actively survey the brain environment and are capable of responding to diverse stimuli, such as neuron damage, disease proteins, and environmental toxins [13]. When activated in response to these assorted triggers, microglia can produce several factors that are toxic to neurons, such as pro-inflammatory factors ( $\text{TNF}\alpha$ ,  $\text{PGE}_2$ , and  $\text{INF}\gamma$ ) and reactive oxygen species ( $\text{NO}$ ,  $\text{H}_2\text{O}_2$ ,  $\text{O}_2^{\cdot-}$ ,  $\text{NOO}^-$ ) contributing to neurodegeneration [14–16]. While limited production of these factors are expected in normal physiology, robust and chronic activation microglia lead to toxic levels that are believed to drive disease [17]. In particular, microglial NOX2 has been implicated as a key mechanism of microglia-mediated neurotoxicity (Fig. 1).

### The NOX2 complex

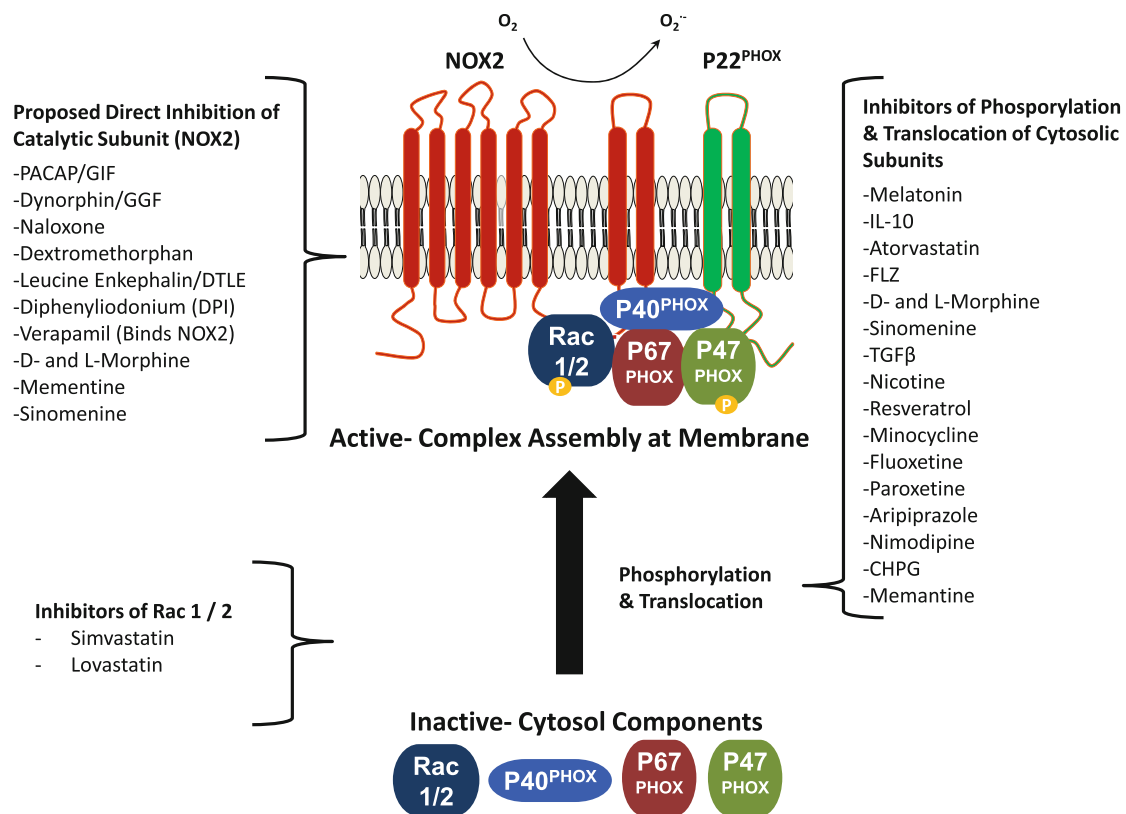
The NOX family of NADPH oxidases is comprised of seven transmembrane proteins (NOX1, NOX2, NOX3,

NOX4, NOX5, DUOX1, and DUOX2) that oxidize intracellular NADPH/NADH, causing electron transport across the membrane and the reduction of molecular oxygen to superoxide [18]. The seven NOX proteins are unique in that they are expressed in different cell populations and require unique combinations of accessory proteins to form the complex necessary for activation [19].

NOX2, also known as  $\text{gp91}^{\text{PHOX}}$ , is the catalytic subunit of the phagocyte NADPH oxidase (PHOX) enzyme complex [20]. As the name PHOX suggests, while NOX2 is known to be expressed in multiple cell types, it is highly expressed in phagocytes including neutrophils and microglia [21, 22], where it is involved in host defense [23], proliferation [24, 25], activated morphology [26], and cell signaling [27–29]. NOX2 is heavily glycosylated with six transmembrane domains and a long cytosolic C-terminus that contains both a NADPH-binding domain as well as a FAD-binding domain, which are required for enzymatic activity [30–32]. While NOX2 is necessary for activation of the enzyme complex, it is not sufficient for generation of superoxide from NADPH and  $\text{O}_2$  in cells [33]. In fact, activation of the NOX2 complex requires a host of organizing and regulating subunits [18, 34].

$\text{P22}^{\text{PHOX}}$  is a membrane protein that colocalizes with NOX2 in the phagosome [35] to form the 1:1 heterodimer flavocytochrome b558 [36, 37]. With no catalytic activity of its own,  $\text{P22}^{\text{PHOX}}$  serves to stabilize NOX2 and organize other PHOX subunits for binding and is essential for catalytic activity of NOX2 [38, 39].

Several cytosolic proteins are also necessary for activation of the NOX2 complex and the successful generation of superoxide.  $\text{P47}^{\text{PHOX}}$  is located in the cytoplasm in quiescent microglia [40], but upon activating stimulus,  $\text{P47}^{\text{PHOX}}$  is phosphorylated [41], then undergoes a conformational change that allows it to translocate to the



**Fig. 2** Inhibitors target microglial NOX2 activation through two mechanisms. Activation of the NOX2 complex occurs in microglia in response to several stimuli and multiple signaling pathways, where activation is due to the assembly of the enzyme complex, as depicted here. Several putative NOX2 inhibitors have been identified that attenuate microglial activation to confer neuroprotection. Here, we classify compounds that have been shown to inhibit microglial NOX2

activity into one of two categories based on whether the molecules were: (1) proposed to directly inhibit the catalytic subunit or (2) indirectly modulate NOX2 through inhibition of phosphorylation and translocation of cytosolic subunits or other pathways. Notably, most of these molecules are not specific for NOX2 and the mechanism of NOX2 inhibition are either poorly understood or unknown at this time

membrane, binding membrane phospholipids (particularly PI3 K [42, 43]) and the proline-rich C-terminus of P22<sup>PHOX</sup> [44]. P47<sup>PHOX</sup> is considered to be an organizer subunit that recruits the activator subunit P67<sup>PHOX</sup> [45]. P67<sup>PHOX</sup> is located in the cytoplasm prior to activation [46]. Upon activation, P67<sup>PHOX</sup> interacts with proline-rich repeats of P47<sup>PHOX</sup> via a C-terminal SH3 domain [47]. Once at the membrane, P67<sup>PHOX</sup> binds to Rac1/2 via an N-terminal tetratricopeptide repeat [48], and presumably interacts with gp91<sup>phox</sup> directly in order to activate the catalytic subunit [49]. P47<sup>PHOX</sup> and P67<sup>PHOX</sup> are mandatory for microglial activation of NOX2 [50]. P40<sup>PHOX</sup>, another cytosolic subunit, also coprecipitates with P47<sup>PHOX</sup> and P67<sup>PHOX</sup> [51], but is dispensable for enzyme activity [52]. However, it is not completely innocuous, and has been implicated in both positive and negative modulation of ROS generation, but neither the required modifications nor a mechanism have been elucidated [53]. Meanwhile, Rac1, a small GTPase both tethers P67<sup>PHOX</sup> to the membrane and is involved in its activation of NOX2 [54]. Thus, the NOX2 complex is comprised of multiple proteins

located both in the membrane and cytosol in microglia that contribute to enzymatic activity (Fig. 2).

Activation of the NOX2 complex occurs in microglia in response to several stimuli (Table 1) and multiple signaling pathways, where activation is due to the assembly of the enzyme complex (Fig. 2). Assembly and activation of the NOX2 complex occurs through sequential events beginning with the phosphorylation of P47<sup>PHOX</sup> at multiple critical serine residues (serines 208 and 283 in the Src homology 3 domain; serine 384 in the C-terminus) [55]. Several kinases have been implicated in P47<sup>PHOX</sup> phosphorylation: protein kinase C (PKC) isoform  $-\delta$ ,  $-\theta$ , or  $-\eta$  [34, 56, 57], protein kinase A (PKA) [58], casein kinase 2 (CK2) [55], and mitogen-activated protein kinases (MAPK) P38 and ERK [58–61]. Following phosphorylation, P47<sup>PHOX</sup> translocates to cytochrome b558 in the cell membrane and acts as recruitment scaffolding for P67<sup>PHOX</sup>, the activating subunit. P67<sup>PHOX</sup> also requires phosphorylation by PKC [62] or the MAPK P38 and ERK2 [63] to initiate activation. Following assembly of the complex, NADPH binds to the c-terminal NADPH binding

**Table 1** Triggers of NOX2 activation in microglia

NOX2 trigger/toxin	Implicated CNS disease/condition	References
Cytokines (elevated in neurodegenerative diseases)		
Tumor necrosis factor $\alpha$	Multiple CNS diseases	[25]
Interleukin-1 $\beta$	Multiple CNS diseases	[25]
Interleukin-4	Multiple CNS diseases	[87]
Interleukin-13	Multiple CNS diseases	[88]
Disease protein/endogenous compounds		
Amyloid precursor protein/ $\beta$ amyloid	Alzheimer's disease	[24, 89, 190, 191]
$\alpha$ Synuclein	Parkinson's disease	[90, 233]
Myelin	Multiple sclerosis	[92]
Human immunodeficiency virus (HIV)-Tat	HAND	[93, 94]
Fibrillogenic prion peptide PrP106-126	Prion disease	[95]
Neuromelanin	Parkinson's disease	[99]
Prothrombin kringle-2/thrombin	Stroke	[234, 235]
Angiotensin II	Parkinson's disease	
Substance P	Parkinson's disease	[101]
Gangliosides	Multiple CNS diseases	[236]
Environmental toxins (linked to CNS disease)		
Paraquat	Parkinson's disease	[102]
Rotenone	Parkinson's disease	[103]
Deildrin	Parkinson's disease	[104]
Diesel exhaust particles (air pollution)	Parkinson's disease	[105]
Lindane	Parkinson's disease	[106]
Mancozeb	Parkinson's disease	[107]
Maneb	Parkinson's disease	[107]
Neuron damage/death		
Reactive microgliosis in response to MPTP/MPP <sup>+</sup>	Parkinson's disease	[98, 143, 155, 237]
Extracellular $\mu$ calpain	Parkinson's disease	[98]
Reactive microgliosis in response to 6-hydroxydopamine	Parkinson's disease	[164]

**Table 1** continued

NOX2 trigger/toxin	Implicated CNS disease/condition	References
Extracellular metalloproteinase 3	Parkinson's disease	[97]
Controlled cortical impact	Traumatic brain injury	[67]
Transient middle cerebral artery occlusion model	Ischemia injury	[134]
Spinal nerve injury	Neuropathic pain	[136]
Pro-inflammatory trigger/other		
Zinc	Multiple CNS diseases	[238]
Lipopolysaccharide	Multiple CNS diseases	[26, 78–81]
Phorbol 12-myristate 13-acetate (PMA)	N/A	[50, 82, 83]
Zymosan	N/A	[50, 84]
Encephalomyocard Virus	Encephalitis	[85]
<i>N</i> -formyl-methionyl-leucyl-phenylalanine	Parkinson's disease	[83, 86]
Mycobacterium tuberculosis	N/A	[239]

Several compounds and conditions reported to activate microglial NOX2 are listed along with the central nervous system diseases/conditions believed to be affected

*Tat* Trans-activator of transcription protein, *HAND* HIV-associated neurocognitive disorder, *N/A* not applicable

domain spanning four folds of the c-terminal region of NOX2, and a single FAD molecule binds across two folds [31, 64]. FAD is thought to be transiently reduced by a single electron from NADPH, after which the two heme groups sequentially pass an electron, moving it to the outside of the membrane, where molecular O<sub>2</sub> is the final electron acceptor and is converted to superoxide (O<sub>2</sub><sup>-</sup>) [18].

### NOX2 in central nervous system cells

The NOX family of proteins is expressed in diverse cell types throughout the brain in a cell-specific and localized manner [65]. Microglia have been shown to express all the necessary subunits of NOX2 both in vivo and in vitro [66, 67]. Microglial NOX2 is involved in host defense [23], proliferation [24], and regulation of cell signaling via redox signaling mechanisms [27–29]. Neurons, the primary communicators in the CNS which are damaged in both AD and PD, express NOX2 [68, 69] in a variety of brain regions [65], where the enzyme complex has been implicated in neuronal apoptosis [69], learning and memory [70], long-term potentiation [71], and in neuronal

myelination signals [72, 73]. Astrocytes, which are involved in maintaining CNS structure, trophic and metabolic support of neurons, neurotransmission [74], and inflammation, also express NOX2 [75], where it is involved in cell signaling [27] and cell survival [76], and may also contribute toward inducible neuroinflammation [75]. At present, whether NOX2 is expressed in oligodendrocytes is unknown. Recent work shows not only that NOX2 is expressed in adult hippocampal stem/progenitor cells but also that NOX2-generated ROS regulate proliferation signals through redox signaling in response to NOX2-derived ROS [77]. For a more detailed review of NOX homologues in the CNS, see [65]. Given that multiple NOX homologues are present in the brain and employ many common subunits for function, and the fact that NOX2 is involved in cellular functions independent of microglia, the specificity of NOX2 inhibitors and the timing of drug administration will be important to confer accuracy when targeting the deleterious microglial response.

Microglial NOX2 is activated by a surprisingly long list of compounds and events (Table 1). As expected, classical triggers of the innate immune response, such as LPS [26, 78–81], Phorbol 12-myristate 13-acetate (PMA) [50, 82, 83], zymosan [50, 84], encephalomyocardus virus [85], and *N*-formylmethionine leucyl-phenylalanine (fMLP) [83, 86] activate the enzyme complex. Consistent with the expected phenotype of phagocytic cells, cytokines are also reported to initiate microglial NOX2 activation, including TNF $\alpha$  [25], Interleukin-1 $\beta$  [25], Interleukin-4 [87], and Interleukin-13 [88]. However, disease proteins found in the CNS, such as A $\beta$  [43, 89],  $\alpha$  synuclein [90, 91], myelin [92], HIV tat [93, 94], and fibrillogenic prion peptide PrP106-126 [95], are also known to initiate microglial superoxide production through NOX2 activation. In fact, NOX2 is implicated in reactive microgliosis (the microglial response to neuronal death/damage), a mechanism contributing to the progressive nature of many neurodegenerative diseases [96]. More specifically, several neuron injury factors have been identified that activate microglial NOX2 to further propagate additional neurotoxicity, such as matrix metalloproteinase-3 (MMP3) [97],  $\mu$  calpain [98], neuromelanin [99], and  $\alpha$  synuclein [90, 91]. Even endogenous neuropeptides are capable of activating microglial NOX2, including angiotensin II [100] and substance P [101], or inhibiting it, such as dynorphin [101], suggesting this enzyme may be tightly regulated in the CNS under normal physiological conditions. Environmental toxins have also been reported to reach the brain and activate microglial NOX2 to produce ROS, including paraquat [102], rotenone [103], dieldrin [104], diesel exhaust particles [105], lindane [106], mancozeb [107], and maneb [107]. Thus, triggers of microglial NOX2 activation extend well past traditional immunological stimuli and include environmental toxins,

neuromodulators, neuronal death, and CNS disease pathways (Table 1).

### Microglial NOX2: dual modes of neurotoxicity

There is increasing evidence that activation of microglial NOX2 is culpable in neuronal damage. Microglial NOX2-induced neurotoxicity is believed to occur through two mechanisms: the production of extracellular ROS that directly damages neurons and intracellular signaling that primes microglia to enhance the pro-inflammatory response and propagate neurotoxicity (Fig. 1).

Microglial NOX2 has been implicated as chronic source of ROS in pathological CNS conditions. NOX2 generates extracellular superoxide ( $O_2^-$ ), which is highly reactive and is rapidly dismuted, either spontaneously or by the enzyme superoxide dismutase (SOD) [108], to yield the cell-soluble molecule hydrogen peroxide ( $H_2O_2$ ). However,  $H_2O_2$  is reduced to water and the hydroxyl radical ( $-OH$ ) through the Fenton reaction [109, 110]. The hydroxyl radical is one of the strongest known oxidizing species and a powerful host-defense mechanism because of its ability to damage pathogens [109, 110]. NOX2 ROS is generated both in the phagosome and at the membrane in phagocytes, where concentrations of superoxide in the phagosome can surpass 1 M in addition to other reactive species [111]. Because NOX2 is located at the cellular membrane, NOX2 activation has been implicated in damage in surrounding tissues, particularly neurons [112, 113]. Peroxynitrite ( $ONOO^-$ ), a product of NO and superoxide, is also noted to be toxic to neurons [113, 114]. Excessive levels of ROS that exceed endogenous, compensatory anti-oxidant levels can lead to oxidative modification in neurons responsible for dysfunction of proteins, nucleic acids, and lipids [113, 115]. While many neurons can adjust to and regulate spikes in ROS, there are select populations of neurons in the brain that are vulnerable, which is implicated in neurodegenerative disease [115].

Redox signaling occurs when a biological signaling pathway is modified by free radical species such as a ROS or nitric oxide in a manner characteristic of a messenger, modulating or impacting the signaling in a way that is not explicable simply by oxidative tissue damage [116]. As ROS and other free radical species are highly reactive in comparison to uncharged molecules, redox signaling occurs frequently within a cell, less frequently between cells that are in contact, and rarely between cells separated by a space of even a few microns. One mechanism by which ROS can influence signaling is through redox-sensitive target proteins such as protein tyrosine phosphatases [117], which can be reversibly oxidized at vulnerable cysteine residues, temporarily inactivating proteins. In



addition, many transcription factors have been shown to be redox-sensitive, including members of the AP-1 [118] and NF- $\kappa$ B [119] families, Nrf2 [120], p53 [121], and glucocorticoid receptor [122]. There is evidence for ROS involvement in cell–cell contact [123, 124], in regulation of NOX2 subunits Rac1 and P47<sup>PHOX</sup> [125], and in many other cellular processes.

Importantly, NOX2 redox signaling has been shown to play an essential role in the microglial pro-inflammatory response and associated neurotoxicity. More specifically, NOX2 has been shown regulate intracellular ROS levels in microglia to result in both amplification of the production of pro-inflammatory cytokines, such as TNF  $\alpha$  [26] or PGE<sub>2</sub> [126] and priming of microglia to be sensitive to additional stimuli [13]. For example, NOX2 inhibitors suppress LPS-induced expression of cytokines (IL-1 $\beta$ , IL-6, and TNF $\alpha$ ), iNOS expression, MAP kinases, and NF $\kappa$ B phosphorylation [27]. Further supporting this premise, NOX2<sup>-/-</sup> mice have shown drastically reduced microglial activation, cytokine levels, and neurotoxicity with intranigral LPS injection [26]. In addition, evidence supports activation of NOX2 shifts the microglia to a primed phenotype that can result in a neurotoxic response to otherwise benign stimuli. Ongoing neuron damage and rotenone [127, 128], both of which activate NOX2 (Table 1), have been shown to synergistically amplify the microglial pro-inflammatory response to LPS and neurotoxicity in vitro. Interestingly, paraquat, another initiator of microglial NOX2 (Table 1), appears to exert toxicity in vivo only when combined with more than one stimulus of microglial activation. Rather, priming appears to be necessary for neuronal damage, where paraquat must be administered in at least two doses or in combination with another pro-inflammatory stimulus, such as LPS [129]. Notably, paraquat priming only occurs in mice with functional NOX2 [129]. Thus, current evidence supports that NOX2 regulates microglial priming, enhancing the microglial response to diverse stimuli, which may contribute to both the magnitude of the microglial response and the chronic nature of microglial activation in the brain.

### Microglial NOX2 in neurodegenerative diseases

Microglial NOX2 is implicated in the ongoing pathology of several neurodegenerative diseases and CNS disorders/conditions, including amyotrophic lateral sclerosis [130, 131], multiple sclerosis [132], pathologically altered behavior [133], ischemic stroke [134, 135], neuropathic pain [136, 137], HIV-associated dementia [93, 138], neurotrauma [67, 139], schizophrenic behavior [140, 141], Alzheimer's disease (AD) [142], and Parkinson's disease (PD) [143]. In the current review, we focus on the

relevance of NOX2 inhibition for the two most prevalent neurodegenerative diseases, AD and PD. Notably, as a common cause of dementia in the elderly and the most prevalent neurodegenerative disease [144], AD affects more than 4 million people in the United States and an estimated 27 million are affected worldwide [145]. PD is a devastating movement disorder and is the second most prevalent neurodegenerative disease [144], affecting 1–2 % of the population over the age of 50 [146]. These numbers for both of these diseases are expected to swell in the future with the aging population. Analysis of post-mortem brains from both AD and PD patients reveal activated microglia [14], where this microglial activation has been implicated in the progressive nature of each disease [16, 96, 147, 148]. Interestingly, observations in AD brains found marked increases in P47<sup>PHOX</sup> and P67<sup>PHOX</sup> translocation to the membrane in microglia when compared to control brains [142]. Further, mild cognitive impairment has been associated with increased expression of P40<sup>PHOX</sup>, P47<sup>PHOX</sup>, and P67<sup>PHOX</sup> in AD brains [149]. In PD brains, the NOX2 protein itself is upregulated in the brain region that is selectively damaged, the *substantia nigra* [143]. Given the number of individuals affected by AD and PD, the strong link between microglial NOX2 and ongoing pathology driving these diseases, and the fact that current treatment is only palliative, targeting of NOX2 may be especially beneficial for AD and PD. However, the majority of mechanistic information regarding the role of NOX2 in microglia-mediated neurotoxicity has been derived from animal and cell culture studies.

### NOX2 in Parkinson's disease models

Increasing evidence points to NOX2 as a critical mechanism of neuron damage in several models of PD (Table 2). 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is a classic in vivo and in vitro model used to explore selective dopaminergic (DA) neurotoxicity, a predominating characteristic in PD [150]. MPTP-induced parkinsonian symptoms were first observed in human patients by Langston et al. in 1984, and experiments in C57 mice followed [151, 152]. MPTP is converted by monoamine oxidase B, to 1-methyl-4-phenylpyridine (MPP<sup>+</sup>) by astrocytes, which is a metabolite that is then selectively toxic to DA neurons. Selectivity is conferred when the dopamine active transporter moves MPP<sup>+</sup> into the DA neuron, where it interferes with complex I and III of the mitochondrial electron transport chain to cause DA neuron death [153, 154]. Microglia are clearly activated in vivo in response to MPTP-induced lesions in the SN [97, 155], and NOX2 is activated in microglia that are localized with the neuron damage [143]. Reconstituted cell cultures have

**Table 2** Genetic deletion of NOX2 in murine models of Alzheimer's and Parkinson's disease is anti-inflammatory and neuroprotective

Mouse disease model	Effect	References
Alzheimer's disease		
A $\beta$ <sub>1-40</sub> superfusion	Gp91 <sup>-/-</sup> mice showed lower ROS and reduced cerebrovascular dysfunction	[181]
Tg2576/Cybb <sup>-/-</sup> mice	Lack of gp91 protected against neurovascular and behavioral dysfunction	[184]
Parkinson's disease		
Paraquat	Gp91 <sup>-/-</sup> mice showed reduced microglial activation and DA neurotoxicity	[102, 129]
Neuromelanin	Gp91 <sup>-/-</sup> mice showed reduced neuroinflammation and DA neurotoxicity	[99]
LPS	Gp91 <sup>-/-</sup> mice showed reduced microglial activation and DA neurotoxicity	[26]
MPTP	Gp91 <sup>-/-</sup> mice showed microglial activation and DA neurotoxicity	[143, 156]

Genetic ablation of NOX2 in mice is associated with anti-inflammatory and neuroprotective effects in mouse models of Alzheimer's and Parkinson's disease

DA dopaminergic, A $\beta$  beta amyloid, LPS Lipopolysaccharide, MPTP 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, ROS reactive oxygen species

shown that while microglia are not activated by MPP<sup>+</sup> alone, microglia are activated in the presence of MPP<sup>+</sup> damaged neurons [98, 156]. Thus, it is not surprising that MPP<sup>+</sup> in cultures is more toxic to DA neurons in the presence of microglia [98, 157]. However, NOX2 inhibitors or genetic deletion of the catalytic subunit NOX2 show that this additional microglia-mediated neurotoxicity is dependent on NOX2 [98, 143, 157–159]. Together, these studies indicate that the microglial response to neuronal damage (reactive microgliosis) is toxic and point to an active role for microglial NOX2 in chronic neurotoxicity (Fig. 2).

2,4,5-trihydroxyphenylethylamine (**6-OHDA**) is another established model used in the study of PD. 6-OHDA does not cross the blood–brain barrier, but upon direct injection into the brain, it efficiently produces lesions of DA neurons in the SNPC and has been used for decades to model PD [160]. Interestingly, 6-OHDA is selectively toxic to DA neurons by more than one route. More specifically, 6-OHDA is internalized by the dopamine active transporter [161], where once inside the neuron it can both directly induce oxidative stress [162] and dysregulate complexes I and IV in the mitochondrial transport chain [163]. Recent studies show that neuronal damage due to 6-OHDA activates microglia, induces expression of NOX2 subunits, induces apocynin-inhibitable NOX2 activity, and causes superoxide production, where these phenomenon cause additional DA neurotoxicity [164, 165]. Thus, evidence supports that neuronal damage in response to 6-OHDA also initiates neurotoxic reactive microgliosis, which is regulated by NOX2.

In addition, several environmental toxins have been linked to Parkinson's disease and as such, are used as experimental models for this disease [166]. *N,N'*-dimethyl-4,4'-bipyridinium dichloride (**paraquat**) is a widely used herbicide structurally similar to MPTP, but in vitro studies reveal that unlike MPTP, lower concentrations of paraquat

are not toxic to DA neurons in the absence of microglia [167]. In fact, paraquat is shown to be a potent inducer of microglial NOX2 activity and consequent superoxide production, where both in vivo [129] and in vitro [167] studies confirm this is the primary mechanism of DA neurotoxicity. **Rotenone** is another environmental compound associated with PD and is a plant-derived insecticide that is known to inhibit the transfer of electrons from iron–sulfur centers in complex I of the MTC to ubiquinone (Coenzyme Q<sub>10</sub>). However, in vitro evidence suggests that, at low concentrations, rotenone is selectively toxic to DA neurons, but only in the presence of microglia [168]. Further analysis showed that microglia are activated by rotenone to produce superoxide, which is selectively toxic to DA neurons, and completely dependent on NOX2, as NOX2<sup>-/-</sup> cultures are protected from rotenone [128, 169]. With different molecular structures and predicted sites of action, it may at first seem surprising that rotenone and paraquat converge on the common NOX2 pathway of microglia-mediated neurotoxicity. However, several other structurally unrelated environmental toxins have also been identified that are selectively toxic to DA neurons through NOX2, as noted in Table 1, supporting that NOX2 activation may be a common mechanism of neurotoxic microglial activation.

Other PD models that use pro-inflammatory triggers of microglial NOX2 activation are also available. Bacterial lipopolysaccharide (**LPS**) is a structural component of the cell wall of Gram-negative bacteria and a potent inflammogen. Importantly, LPS is not directly toxic to DA neurons and LPS activates microglia to produce ROS through iNOS [170] and NADPH oxidase [128], where the role of NOX2 in LPS-induced neurotoxicity is confirmed in vivo [26].  $\alpha$  **Synuclein** is a disease protein linked to PD, where A30P and A53T  $\alpha$  synuclein mutant mice are a common genetic model for PD. The mutations result in elevated and advanced aggregation of  $\alpha$ -synuclein with

age. Aggregated wild-type, mutant A30P, and mutant A53T  $\alpha$  synuclein have been shown in vitro to activate microglia via the Mac1 receptor [91]. These  $\alpha$  synuclein aggregates were not toxic to DA neurons in the absence of microglia, nor in microglia deficient in NOX2 catalytic subunit of NADPH oxidase [90]. Together, these findings further support the common mechanism of microglial NOX-mediated neurotoxicity.

### NOX2 in Alzheimer's disease models

Microglia are localized in and around senile plaques and neurofibrillary tangles [171, 172] in AD, where they become activated [173], and are noted to produce neurotoxic factors, including nitric oxide (NO) [174], superoxide [89, 175], and TNF $\alpha$  [176]. The amyloid hypothesis holds that A $\beta$ , a key component of senile plaques, has a causative role in AD pathology, where microglia-mediated neurotoxicity has been implicated as a key mechanism [89, 177]. Indeed, A $\beta$  recruits and activates microglia [172, 173], further supporting a role for both A $\beta$  and microglia in AD progression [178]. Unfortunately, the same receptor complex necessary for microglia to recognize and phagocytize A $\beta$  fibrils is also culpable in activation of microglial NOX2 and the consequent production of superoxide [179, 180], supporting that microglia NOX2 is a source of oxidative stress in AD [180]. Although not all available animal AD models are based on the amyloid hypothesis, the ones we will discuss here employ some derivation of this principle. Of note, choosing the correct AD model to test anti-inflammatory compounds, such as NOX2 inhibitors, is complicated and fraught with problems, as many mouse AD models fail to exhibit neuroinflammation at levels comparable to human patients [148], and the timeline and degree of inflammatory parameters may vary by model. Further, while some studies have explored the effects of NOX2 inhibitors on plaque load, neuroprotection, and memory, few AD animal models can directly confirm NOX2 activation in the brain, let alone specific NOX2 activation in microglia that is similar to AD patients, despite clear elevation of A $\beta$  and plaque deposition. However, regardless of these limitations, with judicious use and cautious interpretation, appropriate and interesting models are available to test anti-inflammatory compounds.

Several AD animal models show that inhibition of NOX2 is neuroprotective (Tables 2, 3). For example, neocortical superfusion of A $\beta$ <sub>1-40</sub> causes free radical production and cerebrovascular dysregulation in the wild-type mouse cortex, where studies have shown ROS were not generated in response to A $\beta$  in NOX2<sup>-/-</sup> mice [181]. The Tg(APP-SWE)2576Kha (TG2576) mouse model is a commonly modified transgenic mouse that over-expresses the human

APP695.SWE mutation (K670N and M671L) amyloid precursor protein (APP) gene under the control of the hamster prion protein promoter [182]. These mice are normal at 3 months, but acquire learning and memory in spatial reference deficit after 9 months, concomitant with augmented A $\beta$  accumulation and plaque formation [183]. In a recent in vivo study using the Tg2576 mice crossed with Cybb<sup>-/-</sup> mice (mice missing functional NOX2), analyses showed that cerebrovascular alterations, amyloid deposition, and behavioral deficits observed in 12-15 month old TG2576 mice were absent in TG2576/Cybb<sup>-/-</sup> mice. Interestingly, the plaque load was not affected in TG2576/Cybb<sup>-/-</sup> mice, indicating that behavior deficits in this mouse model were somehow independent of plaque deposition [184]. Another genetic AD mouse model, the Tg(PRNP-APP<sup>SweInd</sup>)19959Dwst (TG19959) mouse, expresses mutant human amyloid protein precursor with both the Swedish (K670N/M671L) and the Indiana (V717F) mutations, under the control of the Syrian hamster prion protein promoter. This particular model demonstrates cognitive deficits and A $\beta$  pathology quite early, at 3 months of age [185]. Microglial activation of NOX2 has not been established in these mice, but apocynin did show protection from AD pathology (both plaque load and cognitive deficit) [185]. Further, the N141I-PS2 presenilin mutant animal model of AD demonstrates that the NOX2 inhibitors, DPI and apocynin, abrogated neurotoxicity was in these mice as well [186]. Thus, while NOX2 inhibition has an effect in most animal AD models tested, each model seems to have a unique phenotype of AD pathology, and NOX2 activation has not been confirmed in most models tested.

The importance of assessing NOX2 activation prior to initiation of testing NOX2 inhibition was evidenced by a recent study using transgenic mice overexpressing human amyloid precursor protein with the Swedish and London mutations (hAPP(751)<sub>SL</sub>). Amyloid depositions begin to occur as plaques 3-4 months in hAPP(751)<sub>SL</sub> mice, with accumulation in the hippocampus commencing at 7 months [187-189]. During this study, mice were given apocynin from 4 to 8 months of age, before significant damage had occurred.

Apocynin was shown to significantly reduce microglial number and A $\beta$  plaque size, with no effects found on measures of synaptic damage or learning and memory, supporting a disconnect between plaque deposition and neuronal damage/behavior in this model. Upon closer analysis, despite the high level of A $\beta$ , accumulation of A $\beta$  protein deposits, and behavioral deficits associated with this model [187-189], hAPP(751)<sub>SL</sub> mice showed little evidence of neuroinflammation and oxidative stress in saline control animals at 8 months, making reduction by any inhibitors improbable. Thus, the effects of apocynin were likely independent of any anti-inflammatory effects, emphasizing the importance of choice in the AD animal model when testing NOX2 inhibitors.



**Table 3** NOX2 inhibition promotes healthy microglial function and neuroprotection

NOX2 inhibitor	Disease model	Effect	References
<b>Alzheimer's disease</b>			
Ibuprofen	B6-R1.40 mice	Enhanced A $\beta$ clearance and reduced ROS	[222]
Apocynin	hAPP(751)(SL) mice	Reduced plaque size and microglial number	[240]
Nicotine	fA $\beta$ (1-42), in vitro	Reduced microglial NOX2 activation	[241]
Melatonin	fA $\beta$ (1-42), in vitro	Inhibits p47 <sup>PHOX</sup> phosphorylation	[242]
Simvastatin and lovastatin	fA $\beta$ (1-42), in vitro	Inhibits NOX2 activation via Rac1 inhibition	[215]
<b>Parkinson's disease</b>			
Apocynin	MPTP, MPP <sup>+</sup> , in vitro	Anti-inflammatory and neuroprotective	[156]
Diphenyliodonium (DPI)	LPS, in vitro	Inhibited microglial activation; neuroprotective	[194]
Interleukin-10	LPS, in vitro	Anti-inflammatory and neuroprotective	[243]
Transforming growth factor $\beta$ 1	LPS and MPP <sup>+</sup> , in vitro	Anti-inflammatory and neuroprotective	[228]
Morphine	LPS and MPP <sup>+</sup> , in vitro	Anti-inflammatory and neuroprotective	[195]
PACAP	LPS and MPP <sup>+</sup> , in vitro	Anti-inflammatory and neuroprotective	[200]
Leucine enkephalin	LPS, in vitro	Anti-inflammatory and neuroprotective	[199]
Dynorphin	LPS, in vitro	Anti-inflammatory and neuroprotective	[101, 244]
Dextromethorphan	LPS and MPP <sup>+</sup> , in vitro, and MPTP, in vitro	Anti-inflammatory and neuroprotective	[198, 245]
Naloxone	LPS, in vitro	Binds NOX2; anti-inflammatory and neuroprotective	[244]
Sinomenine	LPS and MPP <sup>+</sup> , in vitro	Anti-inflammatory and neuroprotective	[196]
Glyceryl nonivamide	6-hydroxydopamine, in vitro	Anti-inflammatory, neuroprotective	[246]
Resveratrol	LPS, in vitro	Reduced microglial NOX2 activation; attenuated microglia-mediated neurotoxicity	[224]
FLZ	LPS and MPP <sup>+</sup> , in vitro	Anti-inflammatory and neuroprotective	[28]
Minocycline	Nigral thrombin injection, mice	NOX2 inhibition, anti-inflammatory and neuroprotective	[247]
Fluoxetine (antidepressant)	MPTP, in vitro	Only protective in presence of microglia; anti-inflammatory and neuroprotective	[159]
Verapamil	LPS, in vitro	Binds to NOX2; anti-inflammatory and neuroprotective	[201]
Paroxetine	MPTP, mice	Reduced NOX2 activation, loss of nigrostriatal DA neurons, and glial activation	[248]
Nimodipine	LPS and MPTP, in vitro	Decreased pro-inflammatory cytokines and ROS production; neuroprotective	[249]
<b>Ischemia</b>			
LY367385 (mGluR1 antagonist)	Transient focal ischemia, rat	Decreased NOX2 activation, decreased expression of the NOX2 complex subunits	[250]
Andrographolide	Hypoxia, mice	Reduced NOX2 activation, Anti-inflammatory and neuroprotective	[251]
Apocynin	Cerebral Ischemia, mice	Blocked IL-1 $\beta$ potentiation of pial venule permeability	[252]
Apocynin	Cerebral Ischemia, mice	50 % less brain infarction and 70 % less cleaved spectrin	[253]
Atorvastatin (statin)	Transient focal ischemia, rat	Anti-inflammatory and neuroprotective	[254]
<b>Other</b>			
Aripiprazole (atypical antipsychotic)	PMA, in vitro	Anti-inflammatory and neuroprotective	[255]
(RS)-2-chloro-5-hydroxyphenylglycine	LPS, in vitro	Reduced microglial NOX2 activation and expression	[256]
Cannabidiol (non-psychoactive cannabinoid)	LPS, retinal microglia cultures	Reduced NOX2-derived ROS and lowered production of pro-inflammatory factors	[257]

At present, highly specific NOX2 inhibitors that regulate microglial activation of confer neuroprotection are unavailable. The compounds listed here are potential inhibitors that have not confirmed direct interaction with NOX2 enzyme components. These molecules have demonstrated the ability to impact NOX2 function, which was determined by loss of function in the presence of other NOX2 inhibitors, NOX2 genetic ablation, or demonstration of the ability to impair assembly of the NOX2 complex. Notably, microglia have the ability to produce ROS through other mechanisms, and all ROS-inhibiting compounds were not included. Further, the compounds listed may have many neuroprotective effects outside of what is noted in this table, which only focuses on NOX2-dependent effects

PACAP pituitary adenylate cyclase-activating polypeptide, LPS lipopolysaccharide, ROS reactive oxygen species, MPTP 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, MPP<sup>+</sup> 1-methyl-4-phenylpyridinium, A $\beta$  beta amyloid, FLZ N-[2-(4-hydroxy-phenyl)-ethyl]-2-(2,5-dimethoxy-phenyl)-3-(3-methoxy-4-hydroxy-phenyl)-acrylamide

In vitro studies modeling components of AD and microglial NOX2 activation tend to be more consistent and clear cut when compared to animal models. There is

extensive direct and indirect support that aggregated/fibrillar A $\beta$  activates NOX2 in microglia in vitro and that NOX2 inhibition reduce superoxide production [24, 89,

[190, 191]. In addition to basic primary cell culture studies, more creative in vitro models are also available. For example, NOX2 in a monocyte-lineage cell was firmly implicated in neurotoxicity in response to A $\beta$  in a co-culture study with the neuroblastoma cell line SH-SY5Y [190]. More specifically, SH-SY5Y cells expressing APP with three aggregation-inducing mutations (Swedish double mutation at positions 670/671 combined with the London V717I mutation) were co-cultured with microglia from wildtype or NOX2<sup>-/-</sup> microglia cell lines (PLB-985-wt or PLB-985 X-CGD). Wild-type microglia were activated by unidentified factors, presumably due to chronic A $\beta$  production, and the neuroblastoma cells were killed. However, microglia deficient in functional NOX2 were incapable of killing the neuroblastoma cells [192], supporting the role of phagocytic NOX2 in neurotoxicity. Thus, multiple tools are available to test the ability of NOX2 inhibitors to modulate microglia-mediated neurotoxicity in AD models.

### Classification of microglial NOX2 inhibition

At present, current treatment for both AD and PD is largely unable to halt disease progression, emphasizing the need for new approaches to both disease prevention and intervention. Inhibition of NOX2 has been implicated as an ideal therapeutic target in diverse diseases, as it is involved in a variety of pathological processes throughout the body in addition to CNS effects [193]. However, given the strong support for the role of NOX2 in pathological microglial activation driving CNS disease, here we report on **potential NOX2 inhibitors** tested in AD and PD models. Most of the compounds discussed are considered **potential inhibitors** because the mechanism of actions have yet to be confirmed as directly interacting with members of the NOX2 complex [193]. The potential NOX2 inhibitors discussed here have demonstrated the ability to modify general measures of NOX2 activity (i.e., ROS production and assembly of the NOX2 enzyme complex), attenuate neurotoxic microglial activation, promote beneficial microglial activities (i.e., A $\beta$  clearance and phagocytosis), and/or are neuroprotective through inhibition of microglial activation (Table 3). We have classified these potential NOX2 inhibitors by two general molecular approaches to the inhibition (Fig. 2): (1) direct NOX2 inhibition involving documented/hypothesized interaction with the NOX2 catalytic subunit to impede enzymatic activation; and (2) indirect NOX2 inhibition, which refers to everything else, including prevention of P47<sup>PHOX</sup>/P67<sup>PHOX</sup> phosphorylation and translocation, where the lack of assembly of the enzyme complex fails

to activate NOX2, but the precise mechanisms are unknown. While these categories likely oversimplify NOX2 inhibition, the majority of studies attempting to identify the mechanism of inhibition are limited by current understanding of the enzyme, in addition to the lack of specificity of available inhibitors, and this classification serves to help organize this rapidly expanding class of compounds.

### Direct inhibition of microglial NOX2

#### Gp91ds-tat & DPI

At present, no compounds have been tested to conclusively show that they affect microglial NOX2 enzyme function through direct interactions with the catalytic subunit. However, several potential molecules have been proposed to work through this mechanism. For example, DPI is an established inhibitor of flavoproteins that was once implicated as a specific, direct inhibitor of NOX2. While DPI clearly modulates microglial function and attenuates NOX2-derived ROS [194], it is now well known that the inhibitor is not specific for NOX2 and likely impacts NOX2 through multiple other mechanisms. Interestingly, gp91ds-tat is a peptide inhibitor of NOX2 (gp91ds-tat) that is believed to directly bind to NOX2 to impair enzymatic activity [184]. Unfortunately, at present, no studies have assessed the efficacy of gp91ds-tat on microglial function. We mention it here, because gp91ds-tat has been tested in an in vivo AD disease model with short-term administration, where gp91ds-tat treatment was sufficient to rescue to the diseased phenotype of aged TG2576 mice, suggesting that further inquiry may be warranted [184].

#### Opioids and related molecules

The majority of compounds tested in microglia that are proposed to work through direct interaction with NOX2 can best be classified as opioids and opioid-related molecules. However, as increasing numbers of related molecules are identified, the structural similarities remain, but the related compounds have extended past strict morphinan and opioid classifications. All the referenced compounds are structurally similar molecules with similar anti-inflammatory properties, neuroprotective effects, and a unique bi-modal dose response curve. Yet some of the molecules are opioid receptor agonists, while others are antagonists. Further, the concentrations at which they are effective are very tightly maintained across the range of molecules, suggesting a common mode of action. The compounds in this category include: both L- and

D-morphine [195]; the morphinan analog sinomenine [196]; the mu-opioid receptor competitive antagonist naloxone [197]; the L-isomer of levorphanol dextromorphan [198]; the dynorphin opioid peptide and its minimal 3-residue peptide gly-gly-phe (GGF) [101, 197]; enkephalin (a natural opioid peptide similar to dynorphin) [199]; pituitary adenylate cyclase-activating polypeptide (PACAP) 38, PACAP 27, and its minimal internal peptide, gly-ile-phe (GIF) [200]; the natural opioid peptide leu-enkephalin [199]; DT-leu-enkephalin, which is unable to bind the kappa opioid receptor [199]; and verapamil [201]. Many of the molecules were analyzed for chemical similarities in various studies using pharmacophore representations of essential features, yielding the common model features of a hydrogen bond acceptor, hydrogen bond donor, ionizable, and sometimes hydrophobic moieties [101]. This suggests a common binding site(s), perhaps on NOX2 itself, rather than an opioid receptor and subsequent signaling, especially considering that both opioid receptor agonists and antagonists have the same effect, and that both naloxone [198, 199] and verapamil [201] have been found bound to NOX2 [198, 199]. Memantine, an NMDA receptor antagonist used in AD treatment, is protective of DA neurons in LPS-mediated toxicity and has been suggested to belong to this class of compounds [202]. However, because memantine alone significantly increased dopamine uptake in neuron/glia cultures (a measure of DA neuron health and function) and an increase in glia-derived neurotrophic factor (GDNF) expression, it is also proposed that much of memantine's protective effect is due to induction of neurotrophic factors from astroglia [202]. Regardless, it is clear these compounds are not specific for NOX2, as they are known ligands for several other receptors with documented effects, and some of the inhibitors reported in this category also impair the NOX2 complex assembly.

### Indirect inhibition of microglial NOX2

This is the largest and most diverse category of potential microglial NOX2 inhibitors (Fig. 2), as targeting this aspect of NOX2 activation could occur through a multitude of pathways that include inhibiting the phosphorylation and translocation of the cytosolic subunits directly, ablating receptor signaling, or impairing kinase signaling that leads to initiation of NOX2 complex assembly. Of course, the disadvantage of this NOX2 inhibition strategy is the risk of losing specificity and gaining the potential for unwanted side-effects. Indeed, most inhibitors in this category are not specific for NOX2 effects. None the less, several promising molecules in this category have been identified.

### Natural compounds and herbal supplements

Apocynin (4'-Hydroxy-3'-methoxyacetophenone) was originally isolated from the medicinal plant *Picrorhiza kurroa* and is widely used as a non-specific NOX2 inhibitor that prevents translocation of P47<sup>PHOX</sup> and P67<sup>PHOX</sup> to the membrane. However, apocynin has also been implicated as an antioxidant because it inhibits ROS in nonphagocytic cells [203], although there is some support that the molecule may exert predominantly NOX2 effects in vivo [193]. Apocynin has been tested in several AD and PD models, as noted above. In addition, the squamosamide derivative FLZ is another molecule in this category, as it has been shown to impede P47<sup>PHOX</sup> translocation in microglia [28]. FLZ was protective in vitro against LPS-induced DA neurotoxicity and in vivo in the MPTP model of DA neuron loss in the *substantia nigra, pars compacta* (SNpc) [28]. Melatonin (*N*-acetyl-5-methoxytryptamine) is another commercially available supplement found to be effective in preventing phosphorylation of P47<sup>PHOX</sup>, translocation of P47<sup>PHOX</sup> and P67<sup>PHOX</sup> to the membrane, and generation of ROS in primary microglia by aggregated A $\beta$ . In addition, EUK134 [204], the green tea polyphenol (–)-epigallocatechin-3-gallate [205], has also been shown to inhibit microglial ROS production. Further, docosahexaenoic acid (DHA) is an omega III fatty acid component of fish oil and a peroxisome proliferation-activating receptor (PPAR) agonist [206]. DHA inhibits NOX2 activation induced by angiotensin II in models of hypertension, although, interestingly, it may activate NOX2 in polymorphonuclear monocytes [207]. In microglia, DHA suppresses neuroinflammation [208], possibly by integrating into the phospholipids bilayer and modifying presentation of receptors like TLR4 and CD14, which form a receptor complex that can activate NOX2 in response to LPS [209]. The capacity of DHA to act as a ligand to PPAR and to integrate into and modify dynamics of lipid bilayers leaves a wide field of possible routes to modify NOX2 activity.

### Statins

Statins are cholesterol-lowering drugs that have been proposed as potential therapeutics in PD and AD. While there are conflicting reports, some epidemiological studies show that individuals taking statins for cholesterol are at lower risk for PD [210] and AD [211]. Mechanistically, statins compete with hydroxymethylglutaryl-coenzyme A (HMG CoA), which generates mevalonic acid. HMG CoA activity causes prenylation of Rac1, impairing GTPase activity, providing a route for statin modulation of NOX2 activity [212–214]. Simvastatin has been shown to reduce glial activation, oxidative stress, and DA neurotoxicity [215]. Further, the statins lovastatin and simvastatin were found to

be protective of  $A\beta$ -induced microglial activation and superoxide generation through inhibition of Rac1 activation and subsequent NADPH oxidase activity [216]. However, direct interaction with the specific members of the NOX2 enzyme complex has yet to be identified, and the effects on Rac1 support an indirect mechanism of statin NOX2 inhibition.

#### Non-steroidal anti-inflammatory drugs (NSAIDs)

There has been increasing interest in the role of NSAIDs in PD and AD. Epidemiological studies conflict, with some concluding no correlation [217] and others reporting marginal protection from PD by ibuprofen but not other NSAID, aspirin, or acetaminophen [218–220]. Mechanistically, experiments performed by Landreth et al. show that ibuprofen inhibits activation of iNOS in microglia, as well as phosphorylation of P38 MAPK and Rac1 activation, both of which have been shown to be upstream of NOX2 [221, 222]. Importantly, the beneficial effects of NSAIDs in AD mouse models was confirmed with ibuprofen in R1.40 human swiss mutation APP-over-expressing mice. Administration of ibuprofen for 9 months ameliorated measures of oxidative stress and plaque load [222]. While the effect on cognitive decline was not reported, they did demonstrate in microglial cultures that ibuprofen inhibited assembly of the NOX2 complex, independent of COX2 inhibition [222]. While the mechanisms of action are clearly not specific for NOX2, these findings warrant further inquiry into the potential therapeutic efficacy of NSAIDs.

#### Targeting upstream kinases

The major inhibitable activating pathways upstream of NOX2 consists of the MAPK family of kinases [58–61], PKC [34, 56, 57], PAK [223], PKA [58], and CK2 [55]. Of these, the greatest amount of work has been done with the MAPK and PKC families. The MAPK P38, ERK1/2, and JNK are phosphorylated (and presumably activated) in rat primary microglia-enriched neuron glia cultures by LPS. This phosphorylation is coincident with microglia- and NOX2-mediated DA neuron-specific toxicity. The DA-specific toxicity is ameliorated with comparable efficiency by NOX2 inhibitors, inhibitors of the MAPK, and the thus far targetless anti-inflammatory molecules compound A and resveratrol [224, 225]. For example both the above-mentioned inhibitors have been shown to inhibit cyclooxygenase-1 and -2 expression at 20  $\mu$ M [226], and SB203580 has been shown to inhibit phosphoinositide-dependent protein kinase 1 (PDK1) at 3–5  $\mu$ M [227]. In fact, many molecules may impact these signaling pathways

to modify microglial NOX2. For example, transforming growth factor  $\beta$ 1 (TGF $\beta$ 1) is a neuroprotective cytokine tested in vitro in LPS and MPP<sup>+</sup> models of DA neurotoxicity [228]. More specifically, TGF $\beta$ 1 has been shown to attenuate LPS-induced phosphorylation of ERK 1 and 2 and alleviate membrane translocation of P47<sup>PHOX</sup> in microglia [228]. Further analysis revealed that the ERK phosphorylation inhibitor U0126I was also protective of LPS-induced DA neurotoxicity [228]. While targeting signaling upstream of NOX2 may be therapeutically relevant, this approach to NOX2 inhibition will likely lead to many non-specific effects.

#### Iron chelation

Iron accumulation in the *substantia nigra* is implicated in the pathology of PD and iron chelators have been proposed as therapeutics [229]. With reference to NOX2, the catalytic subunits require the occupation of two heme groups with iron atoms which are used to convey the electron through the membrane to extracellular molecular oxygen. Thus, iron sequestration with chelating molecules is a strategy for inhibition of NOX2 activity [230]. The iron chelator desferrioxamine (DFO) not only ameliorates microbe- and LPS-induced NADPH oxidase activity in vivo, but it suppressed expression of P22<sup>PHOX</sup> in subcutaneous tissue, though this may have been a secondary effect of decreased ROS and altered redox signaling [231, 232]. Inhibition of NADPH oxidase by iron chelation in microglia has not been investigated, but may prove to be an important part of the observed benefit of iron chelation in PD.

#### Summary and conclusions

NOX2 may be an ideal therapeutic target for promoting healthy microglial function and attenuating the chronic production of neurotoxic microglial ROS underlying progressive neuron damage, which is particularly relevant to Parkinson's disease and Alzheimer's disease. More specifically, current data support that inhibition of microglial NOX2 is neuroprotective and can halt the progression of chronic neuron damage. However, while in vitro and animal studies using potential NOX2 inhibitors hold promise, most available compounds indirectly target NOX2, increasing the probability of side effects, and potentially decreasing consistency across experimental models and reducing therapeutic utility. Thus, future efforts must focus on the development of more specific and perhaps potent NOX2 inhibitors that are able to reach the brain and are safe for human use.

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