

Targeting NOX enzymes in the central nervous system: therapeutic opportunities

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Abstract Among the pathogenic mechanisms underlying central nervous system (CNS) diseases, oxidative stress is almost invariably described. For this reason, numerous attempts have been made to decrease reactive oxygen species (ROS) with the administration of antioxidants as potential therapies for CNS disorders. However, such treatments have always failed in clinical trials. Targeting specific sources of reactive oxygen species in the CNS (e.g. NOX enzymes) represents an alternative promising option. Indeed, NOX enzymes are major generators of ROS, which regulate progression of CNS disorders as diverse as amyotrophic lateral sclerosis, schizophrenia, Alzheimer disease, Parkinson disease, and stroke. On the other hand, in autoimmune demyelinating diseases, ROS generated by NOX enzymes are protective, presumably by dampening the specific immune response. In this review, we discuss the possibility of developing therapeutics targeting NADPH oxidase (NOX) enzymes for the treatment of different CNS pathologies. Specific compounds able to modulate the activation of NOX enzymes, and the consequent production of ROS, could fill the need for disease-modifying drugs for many incurable CNS pathologies.

Keywords NOX NADPH oxidase · Oxidative stress · Antioxidants · Central nervous system · NOX inhibitors · Brain · Alzheimer · Parkinson · Stroke · Schizophrenia · Autoimmune diseases

Introduction: NOX NADPH oxidases and their emerging role in CNS diseases

Central nervous system (CNS) diseases are heterogeneous and have numerous etiologies. However, CNS pathologies as diverse as progressive neurodegenerative diseases, neuropsychopathological disorders, and stroke share many pathogenic mechanisms, such as inflammation, microglia activation, impaired neurotransmission, glutamate-mediated excitotoxicity, mitochondrial dysfunction, apoptosis, and increase of oxidative stress [78, 120]. Among these features of CNS diseases, NOX enzymes are emerging as an important source of oxidants in the CNS and key regulators of neurological pathologies [112, 136, 142].

The NOX family consists of seven members (NOX1, NOX2, NOX3, NOX4, NOX5, DUOX1, and DUOX2), each with a specific tissue distribution. The isoforms present in the CNS are mostly NOX1, NOX2, and NOX4. Despite their similar core structures, NOX isoforms have different mechanisms of activation. NOX1, NOX2, and NOX3 require association with cytosolic components [p47^{phox}, p67^{phox}, NOXO1 (NOX organizer type 1), NOXA1 (NOX activator type 1)], NOX4 is constitutively active, and NOX5 and DUOXes are activated by intracellular Ca²⁺ elevation and direct phosphorylation [8, 42, 84].

NOX enzymes function to transfer electrons across membranes, which then react with oxygen generating the superoxide anion O₂⁻. Superoxide dismutates very rapidly

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to generate hydrogen peroxide and other reactive oxygen species (ROS).

In physiological conditions, ROS generated by NOX enzymes work as defense mechanisms against pathogens and signaling molecules. NOX enzymes are increasingly recognized as key regulators of pathological situations where oxidative stress is involved and leads to tissue oxidative damage, such as pulmonary fibrosis, diabetic nephropathy, and vascular and CNS pathologies [81]. The NOX2 isoform (previously known as gp91^{phox} or the *phagocyte oxidase*) is responsible for the respiratory oxidative burst in neutrophils. However, NOX2 is also expressed in the CNS, where it controls key neuronal functions and neuroinflammatory processes. NOX2 is strongly upregulated in different CNS disorders where it generates large amounts of ROS. NOX2 activation is thought to regulate microglia activation, a hallmark of inflammatory gliosis observed in neuroinflammatory degenerative disorders [17, 85]. As a major source of ROS, NOX2 can induce direct neuronal damage [128] and maintain microglial cells in an activated stage where they produce neurotoxic molecules like peroxynitrite and other inflammatory molecules [94]. A protective role of NOX2 deficiency has been demonstrated in animal models of Parkinson disease (PD), Alzheimer disease (AD), amyotrophic lateral sclerosis (ALS), and stroke (reviewed in [142]). The fact that decreased NOX2-generated ROS is protective in diverse CNS diseases implies that it must regulate a key aspect of the pathological CNS. NOX2 in the microglia is not the only target to be pursued. In fact, NOX2 inhibition also prevents development of psychotic disorders without the microglia-mediated neuroinflammatory components by controlling key neuronal aspects such as neurotransmitter release and GABAergic interneurons function [13]. Although NOX2 deletion leads to decreased signs of oxidative stress, the exact role of NOX enzymes in CNS physiology and pathology is still very incomplete. Recent studies have involved NOX2 in the pathways related to NMDA receptor activation, opening new perspectives of research [20, 143].

Another striking aspect of the complexity of the role of NOX2 is the fact that NOX2 plays an anti-inflammatory role in autoimmune-mediated diseases including autoimmune neuroinflammation. Indeed, an insufficient ROS production by systemic antigen presenting cells, such as mononuclear phagocytes, will favor autoimmune neuroinflammation. Genetic studies identified a polymorphism in the *Ncf1* gene coding for p47^{phox}, a regulatory cytosolic factor of NOX-dependent oxidant production, which is associated with susceptibility in rheumatoid arthritis models and other autoimmune disorders [116], including experimental allergic encephalomyelitis, an animal model of multiple sclerosis (MS) [62], and experimental allergic neuritis, a model of Guillain Barré syndrome [58]. The link

between *Ncf1* and disease susceptibility was further confirmed in humans because circulating leukocytes of patients affected by MS [110] and other autoimmune neuropathies [109] have a lower capacity for ROS generation. These unexpected findings appear to be due to down-regulation of T cells by NOX2-dependent ROS generation in response to autoantigen presentation [63].

To summarize, there are two possible therapeutic options using NOX enzymes as pharmacological targets (Fig. 1): (1) in CNS diseases like stroke, PD, AD, ALS, with a strong glutamate excitotoxicity component, the inhibition of NOX enzymes is expected to be beneficial, while (2) when the neuroinflammatory process is mediated by peripheral immune cells, activation of NOX2 in antigen presenting cells of the immune system is predicted to bring a therapeutic effect.

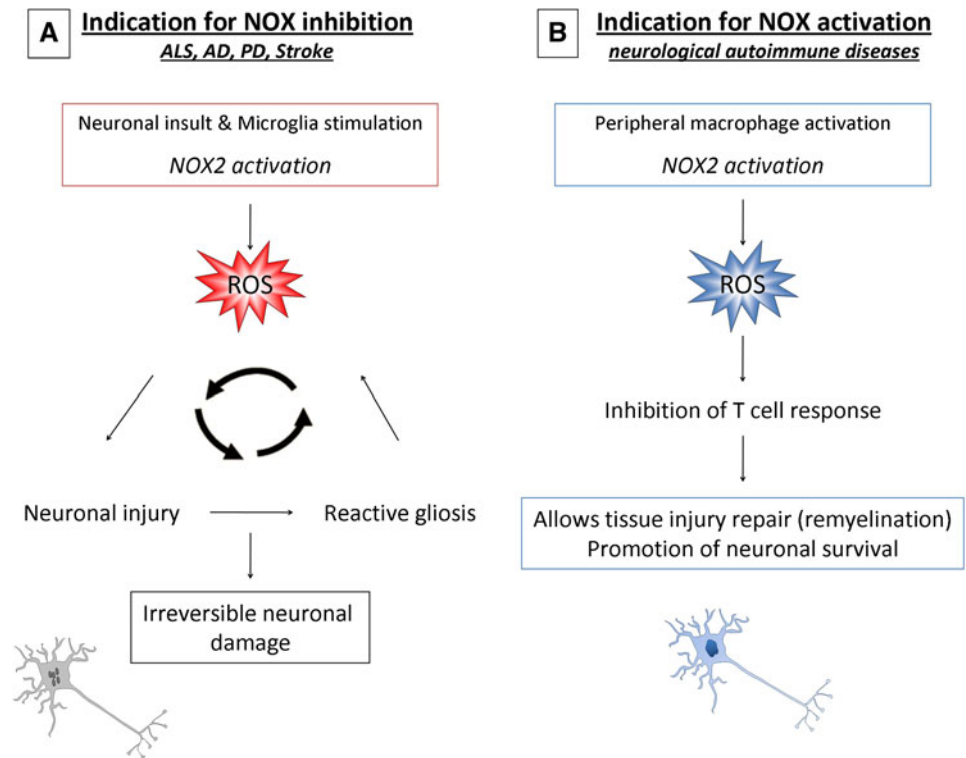
Although it has been known for a long time that mitochondria are an important source of ROS in CNS disease [113, 2], the role of NOX enzymes in CNS pathologies is rapidly emerging. However, because of the presence of at least three NOX isoforms in the CNS and the absence of studies using specific NOX inhibitors, we focus here on disease models where proof-of-principle studies using knock-out or mutant mice are available. We discuss current therapies and the potential for NOX-based therapeutics either decreasing NOX activity or, on the contrary, enhancing low NOX activity in CNS autoimmune disorders. Finally, we discuss past and emerging small molecules targeting NOX enzymes and their possible advantages and risks in CNS-based pathologies.

Beneficial effect of NOX inhibition: potential CNS indications

Amyotrophic lateral sclerosis (ALS)

ALS is a neurodegenerative disease of the motoneurons that leads to complete paralysis and death. Its prevalence is around 2 per 100,000. Several therapeutic approaches have been proposed for the treatment of this disease with varying efficacy. Today, there are no treatments that can arrest, or even substantially delay, the progression of ALS. The only drug approved to treat ALS patients is RiluzoleTM, which is thought to inhibit the presynaptic release of glutamate. However, its therapeutic benefit is modest as in randomized controlled trials, it prolonged survival by approximately 4 months [15, 79], a statistical benefit which is subjectively not perceived by patients, family members, or physicians [50]. ALS has a strong inflammatory component with, for instance, increased expression of COX-2, but the benefit of COX-2 inhibitors, such as rofecoxib and celecoxib for the treatment of ALS, remains uncertain.

Fig. 1 Schematic overview of possible therapeutic indications for NOX targeting compounds. **a** In diseases characterized by neurodegeneration and neuroinflammation such as Alzheimer, Parkinson, Amyotrophic lateral sclerosis, and stroke, excessive NOX2 activation in injured neurons and activated microglia cells leads to production of ROS and oxidative damage. **b** In neurological autoimmune diseases, such as multiple sclerosis and Guillain Barré syndrome, NOX2 activation inhibits the excessive T cell response and contributes to remyelination and neuronal survival



A protective effect of numerous compounds has been described in animal models of ALS (anti-inflammatory drugs, anti-glutamate agents, neurotrophic factors, antioxidant, anti-apoptotic, gene inducers, autophagy inducers), but none of them significantly prolonged survival or improved quality of life when translated to ALS patients. Signs of oxidative stress are observed in ALS rodent models and in ALS patients, such as 8-oxodeoxyguanosine (8-oxodG), urinary 15-F(2t)-isoprostane, protein carbonylation, and markers of lipid peroxidation [5, 7, 104, 106]. The most studied form of ALS is a familial ALS (FALS) in which the antioxidant enzyme SOD1 is mutated (SOD1^{G93A}). However, surprisingly, the increase of oxidative stress is not due to the lack of function of SOD1, but rather to aggregation of mutant protein [124], leading to a toxic gain of function of the mutated SOD1 protein, which in turn has been proposed to activate NOX enzymes [54]. NOX2 and subunits are strongly upregulated in both ALS mice and patients colocalizing with microglial markers [164]. Arguments in favor of a beneficial effect of NOX inhibition comes from experiments where transgenic mice expressing human SOD1^{G93A} were bred with mice deficient for NOX1 or NOX2, which showed lifespan increases of 33 and 97 days, respectively [97]. Another study using a similar approach has shown that NOX2-deficiency in SOD1^{G93A} overexpressing mice decreased the production of microglial-derived ROS, delayed neurodegeneration and prolonged survival, although more modestly than the other study (13 days) [164]. Because of absence of treatment and

the inexorable development of ALS, there is a real need for testing new targets for the pharmacological approach of ALS. In spite of important differences in the protective potential of NOX inhibition in ALS mice, these preliminary proof-of-principle experiments converge at identifying NOX enzymes as primary target for a potential treatment of ALS.

Alzheimer disease (AD)

Alzheimer disease (AD) represents the most common single cause of age-associated dementia worldwide. Although AD etiology is unclear, neurodegeneration in AD patient brain is characterized by protein misfolding and accumulation of A β (derived from amyloid precursor protein APP) and *tau* (a microtubule-associated protein) in plaques and intraneuronal neurofibrillary tangles, respectively. Formation of these abnormal protein aggregates leads to severe neuronal death and synaptic loss, associated with microglial and astrocytic activation. Excessive production of inflammatory mediators and ROS by activated glial cells and damaged neurons contribute to reduced neuronal survival and disease progression.

Due to the decreased levels of neurotransmitters, and especially of acetylcholine, in AD patient brain, the main therapy currently used for AD consists in the administration of acetylcholinesterase inhibitors, such as tacrine, donepezil, rivastigmine, and galantamine [103]. Another drug also commonly used for AD treatment is memantine

[103], which acts as a non-competitive antagonist of the NMDA receptor, thereby reducing excitotoxicity due to the excessive glutamate release associated with neurodegeneration [132]. Although these treatments can reduce AD symptoms and improve cognitive functions, they do not contribute to the resolution of disease or arrest its progression [103]. Current approaches to develop better therapies include inhibition of $A\beta$ aggregation and/or production, as well as inhibition of $A\beta$ /tau-dependent neurotoxic effects [24]. Indeed, preventing the protein misfolding or its direct consequences would represent a more efficacious way to reduce or even reverse the progression of the disease.

The involvement of NOX activation in the pathological mechanisms of AD has been described in several studies based on in vitro experiments with cultures of microglia, astrocytes, and neurons (reviewed in [142]). Moreover, disease progression was prevented in a mouse model of AD (overexpressing the Swedish mutation of APP, leading to $A\beta$ fragment accumulations) after breeding with NOX2-deficient mice. Deletion of NOX2 gene reduced the pathologic effects of $A\beta$ amyloid fragments, rather than the spontaneous accumulation of plaques in the mouse brain [119]. There is also evidence that NOX enzymes are expressed and activated in AD patients as compared to healthy controls: translocation of NOX2-associated subunits (p47phox, p67phox) to the membrane [140], as well as increased NOX2 activation in the frontal and temporal cortex of AD patients with mild cognitive impairment [3]. Interestingly, in this recent study, only the expression of NOX2 regulatory cytosolic subunits (p47phox, p67phox, and p40phox) were increased in AD patients, while the expression of membrane-associated proteins (p22phox and NOX2) remained stable [3]. In addition, increased levels of NOX1 and NOX3 mRNA were found in early stage AD patients [33], suggesting that other isoforms might contribute to this pathology. Nevertheless, in spite of increasingly recognised importance of NOX in AD, free radicals of specific mitochondrial origin and uncoupling of endothelial nitric oxide synthase are likely to be important mediators of the general oxidant status observed in AD [102].

Parkinson disease (PD)

Among neurodegenerative diseases, Parkinson disease is the disorder for which the best options for symptomatic treatment exist, at least for the initial phases. Neurodegeneration occurs in the *substantia nigra*, leading to loss of dopaminergic neurons, which is associated with motor dysfunctions. Typical Parkinson-related symptoms include shaking, rigidity, or slowness, and they can be diagnosed early in the course of the disease [135]. Treatment

consists of the administration of levodopa, a precursor of dopamine, to compensate for the dopamine reduction. Although levodopa alleviates early movement dysfunctions, it does not provide a real cure to prevent the progressive degeneration of dopaminergic neurons. Similarly, other available treatments include dopamine receptor agonists, or monoamine oxidase (MAO-B) and catechol-O-methyl transferase (COMT) inhibitors, which reduce the dopamine metabolism and increase its availability. These drugs are often used alone at the first appearance of symptoms, while, at later stage, they are used in combination with levodopa, which remains more efficacious, but is associated with more undesirable side effects, such as dyskinesia (Table 1). Both genetic and environmental factors have been implicated in the etiology of Parkinson [135]. However, misfolding and deposition of toxic α -synuclein aggregates seem to be an initiating pathogenic event of the disease, whereas the progressive degeneration is due to glial activation [53]. Oxidative damage is thought to contribute to these mechanisms, and mitochondrial dysfunction has been proposed to be a major source of ROS [55]. Yet, there is experimental evidence supporting a role for NOX enzymes. Most of the data derive from in vitro studies, indicating a major involvement of NOX2 in microglia-dependent dopaminergic neurotoxicity (reviewed in [142]). In addition, a direct expression of NOX enzymes in dopaminergic neurons might play a role, while microglia serve to amplify the neurotoxic stimuli [17]. In line with these findings, decreased death of dopaminergic neurons in NOX2-deficient mice as compared to wild-type (WT) was detected following LPS-injections in the *substantia nigra* [129] and systemic administration of MPTP [170] or the herbicide paraquat [127]. A recent study identified a possible mechanism by which NOX2 could be involved in paraquat toxicity. In its native form, paraquat is a divalent cation and is not a substrate of dopaminergic transporters. It therefore requires reduction into a monovalent cation by microglial NOX2 in order to be a substrate for dopamine transporters leading to its accumulation in dopaminergic neurons [130]. Whether oxidative modification of α -synuclein or other Parkinson causative agents increase their toxicity to dopamine neurons remains unknown. Two recent studies show evidence of a role of neuronal NOX1 in Parkinson disease [28, 31]. At this stage, the role of different NOX isoforms is not clear, but one could speculate that a cross-talk exists between neuronal NOX (probably NOX1) and microglial NOX2, which would act by amplifying the neuronal damage and regulating a neuroinflammatory response. In order to evaluate the exact effect of different isoforms, backcross of NOX-deficient animals with genetic models of PD should be performed [35].

Table 1 Present main therapies for CNS diseases with documented information involving NOX enzymes

Disease	Drugs	Pharmacodynamic	Effect	Therapeutic benefit	Main issues	Comments	Ref.
Alzheimer	Tacrine, donepezil, rivastigmine, galantamine	Acetylcholinesterase inhibitors	Increase the level of acetylcholine	Improvements of cognitive functions	Benefic effects are often modest and only temporary	These drugs do not decrease disease progression	[24, 103]
	Memantine	Non-competitive NMDA receptor antagonist	Protects from glutamate excitotoxicity				
ALS	Riluzole	Not defined	Protects from glutamate excitotoxicity	Increase survival by 3 months	Absence of subjective benefits while drug is expensive	Unique therapeutic option, well tolerated	[105]
Parkinson	Levodopa (L-DOPA)	Precursor of dopamine	Increase dopamine levels	Improvement of motor symptoms	Dyskinesia, dystonia, motor fluctuation	Drugs often used in combination	[26, 121]
	Sinemet or Madopar	Dopamine agonists	Binds to postsynaptic dopamine receptor: mimicks the effect of dopamine		Lower efficacy than L-dopa, but less side effects		
	Apomorphine, bromocriptine, cabergoline, dihydroergocryptine, lisuride, pergolide, priribedil, pramipexole, ropinirole and rotigotine.						
Schizophrenia	Selegiline	Monoamine oxidase inhibitors	Increase level of dopamine				
	rasaligine						
Schizophrenia	Antipsychotics	Mainly D2 receptor antagonists	Decrease dopamine action	Reduce positive symptoms	Severe side effects (sedation, extrapyramidal motor disabilities) low compliance, drug resistance	No improvement of social functionality no effect on negative symptoms	[71]
Ischemic stroke	IV or IA tissue plasminogen activator (t-PA) within 3 – 4.5 h of symptom onset	Thrombolysis	Decrease vessel occlusion	Recanalization	Risk of intracerebral hemorrhage	Treatment is limited to early phases after stroke, and does not reduce the brain damage	[99]
	MERC1 device	Mechanical thrombectomy			Still under clinical investigation		
Obstructive sleep apnea	Application of devices to induce a continuous positive airway pressure	Delivering compressed air to avoid obstruction of the upper airway		Reduce or prevent apnea	Non-compliance	Patients consider the device uncomfortable	[51]

Table 1 continued

Disease	Drugs	Pharmacodynamic	Effect	Therapeutic benefit	Main issues	Comments	Ref.
Multiple sclerosis	Interferon (IFN)- β	Recombinant interferon β	Prevent inflammation	Reduction in the rate of MS relapses, and to slow the progression of disability in MS	Lymphopenia, severe depression and injection site necrosis	Real efficacy is controversial	[14, 40, 162]
	Glatiramer acetate	Random polymer of four amino acids of myelin basic protein	Immunomodulator: decoy diverting an autoimmune response against myelin	Decreases frequency of relapses and slow progression of disease	Reaction at injection site	Long term improvement and similar efficiency as interferon Beta	[25]
	Natalizumab	Humanised monoclonal antibody to $\alpha 4$ -integrin	Decrease penetration of T lymphocytes in the CNS	Reduces progression and decreases relapsing rate	Development of progressive multifocal leukoencephalopathy	Use limited to severe relapsing remitting MS and not responding to interferonBeta	[6, 64]
	Mitoxantrone	Type II topoisomerase inhibitor	Immunosuppressant	Especially used in secondary progressive MS	Cardiotoxicity and risk of leukemia	Non specific; antineoplastic agent	[98, 100]
	Fingolimod	Binding of sphingosine 1-phosphate receptor	Immunosuppressant: Inhibit T lymphocytes excavation from lymphoid tissue	Decreases relapsing rate	Increased infection risk	Novel pharmacological approach; Recently approved by the FDA (2010)	[48, 125]
Guillain Barré	Plasma exchange and Intravenous immuno globulin	N/A	Diversion of autoimmune response	reduction of stays in intensive care unit and long-term disability	Randomised trials have low statistical power due to low number of patients	Emergency action, not a treatment	[59, 93]

Schizophrenia

Schizophrenia is a severe psychiatric disorder characterized by three main symptoms: positive, such as hallucinations and delusions, negative, such as loss of motivation and blunted emotions, and cognitive impairment mainly due to deficits in working memory and attention [22]. Although symptoms of schizophrenia usually appear in the late second or third decade of life, it is considered to be a neurodevelopmental mental disorder. Both genetic and environmental factors contribute to the development of schizophrenia, but a complete picture of pathogenic events is still not clear. Alterations in glutamate and dopamine neurotransmission seem to be the main cause of the symptoms, but it is not known how these abnormalities develop [87]. Treatments for schizophrenia are limited and, in general, consist of drugs that act mainly on positive symptoms. The introduction of chlorpromazine in 1954 as a first compound for the treatment of schizophrenia opened the era of psychopharmacology. Similar drugs were developed in the 1950s and 1960s (typical antipsychotics), finally giving the possibility of controlling symptoms in schizophrenic patients and limiting behavioral abnormalities. Although these molecules are known to have different pharmacological actions [146], their antipsychotic effect is thought to be due to the inhibition of the D2 dopamine receptor. However, severe side effects are associated with these treatments, such as a strong sedative action or extra-pyramidal motor control disabilities, leading to a syndrome per se. The subsequent development of other antipsychotic therapies led to the discovery of different compounds (atypical antipsychotics), but with similar mechanisms of action. These drugs prevent positive symptoms, and, even if attenuated, they cause important side effects, especially related to metabolic syndromes and extrapyramidal symptoms [71]. Due to severe side effects, the treatment with antipsychotics (typical or atypical) is often associated with low compliance of the patient and non-adherence to the therapy. In addition, patients develop drug resistance. For these reasons, there is a real need for new treatments involving novel targets with increased efficacy and better tolerability. New therapies should also aim at targeting negative symptoms and cognitive deficits, and reducing the functional and social impairment, which prevent schizophrenic patients from living a normal life [71].

The role of NOX enzymes in the pathogenesis of schizophrenia has been shown in two different experimental animal models. By inducing schizophrenia-like symptoms in mice with subchronic administration of subanesthetic doses of ketamine, Behrens and colleagues first demonstrated that NOX2-dependent ROS production induces a loss of parvalbumin in interneurons [11, 12].

Parvalbumin is a Ca^{2+} binding protein, which normally regulates activity of GABAergic interneurons, thereby modulating the glutamatergic transmission. Decrease in parvalbumin expression has been detected in the brain of schizophrenic patients, suggesting an alteration in the control of excitatory glutamatergic neurotransmission, possibly related to schizophrenia symptoms [86]. By analyzing the acute effects of ketamine on mouse behavior and neurotransmission, it has been found that an increased production of ROS by NOX2 can be determinant to initially trigger the increase in glutamate and dopamine release [52, 143]. In contrast, the repetitive ketamine exposure leads to adaptive alteration of the post-synaptic NMDA receptor [143], possibly as a consequence of parvalbumin decrease [76]. The beneficial effect of NOX2 inhibition is not limited to the psychosis induced by ketamine, as similar observations were made in a model of social isolation in the rat. In rats grown in social isolation for 7 weeks after weaning, NOX2 is up-regulated concomitantly with behavioral alterations, and signs of oxidative stress and loss of parvalbumin. Treatment with apocynin, a non-specific NOX inhibitor, was able to prevent all these effects [136]. Since the social isolation of young rats after weaning induces a prolonged stress during the development of the CNS [83], these findings show in two unrelated models using two different species that NOX2-dependent generation of ROS can play a role in the pathogenesis of schizophrenia. However, at this stage, although signs of oxidative stress are known in schizophrenic patients, NOX2 expression pattern in post mortem specimens or even increased NOX2 activity in peripheral leukocytes has so far not been documented. NOX inhibition in animal models of psychosis does not only completely prevent behavioral changes but also blunts signs of oxidative stress as well as the histopathological and neurochemical alterations observed in these models. Development of CNS-targeted NOX inhibitors therefore represents an extremely promising alternative approach to existing therapies currently used in the treatment of psychotic disorders.

Stroke

Stroke is a leading cause of death and permanent disability worldwide. The majority of strokes are ischemic, due to the occlusion of a vessel in the brain. Intervention in these cases is mainly based on enzymatic or mechanical removal of the occlusion to restore blood flow. Even if beneficial, this treatment can be performed in patients only 3–4 h after the occurrence of stroke, because of the associated severe risk of inducing a hemorrhage [99]. Since it is difficult to rescue the infarcted area, possible therapies are designed to reduce further development of tissue damage and cognitive

Table 2 Apocynin use in vivo in experimental CNS disorder models

Disease	Model	Species	Dose	Way/duration admin	Therapeutic benefit	References
AD	Transgenic mice overexpressing human APP with two mutations (Tg19959)	Mice	300 mg/kg	In the drinking water from 1 to 5 months of age	No improvement of behavior and neuropathologic signs (amyloid deposition, microgliosis and tau phosphorylation)	[34]
PD	Transgenic mice overexpressing human APP with the Swedish and London mutations hAPP(751)(SL)	Mice	10 mg/kg	Daily gavage from 4 to 8 months of age	Reduced plaque size and microglia proliferation in the cortex	[95]
	Sequential injections i.p. of paraquat (10 mg/kg/3 days)	Mice	200 mg/kg	i.p.	Decrease in dopaminergic neuronal loss and microglia proliferation in the substantia nigra	[31]
ALS	<i>SOD1^{G93A}</i>	<i>SOD1^{G93A}</i> transgenic mice	30, 150, and 300 mg/kg/day	In drinking water starting at day 14 of age	Increased survival by 56, 80 and 113 days respectively	[54]
			150, 300 and 750 mg/kg/day	In drinking water starting at day 21 and 100 of age	No increased survival for any treatment protocol	[152]
			150 mg/kg	Way not specified, starting at day 50 of age	Increased survival by 5 days	[89]
Schizophrenia	i.p. subanesthetic ketamine injection (30 mg/kg) on two consecutive days at around 4 pm	C57BL/6 J mice	5 mg/kg/day	Per os in the drinking water for a total of seven days before ketamine injections	Decreased oxidative stress and loss of parvalbumin	[11]
	Post-weaning social isolation for 7 weeks	Wistar rats	5 mg/kg/day	Per os in drinking water for 7 weeks	Decreased oxidative stress, prevented parvalbumin loss and behavioural alterations	[136]
Ischemic stroke	2 h MCA occlusion with a poly-L-lysine-coated monofilament suture followed by 22 h reperfusion	C57BL/6 J mice	2.5 mg/kg	i.v. 30 min before reperfusion	Improved neurological function, reduced infarct volume, and reduced the incidence of cerebral hemorrhage	[150]
			NOT at higher doses of 3.75 and 5 mg/kg			
	2 h MCA occlusion with intraluminal filament followed by 24 h reperfusion	C57BL/6 J mice	2.5 mg/kg	i.v. at the onset of reperfusion	Reduced infarct volume, better neurological function, less BBB disruption and hemorrhage, decreased MMP-9 expression and prevention of tight junction protein loss	[165]
	1.5 h MCA occlusion with a nylon monofilament (4-0) followed by 2 h reperfusion	Sprague-Dawley rats	50 mg/kg	i.p. injection 30 min prior to reperfusion	Reduced NOX activity, superoxide levels and infarct size	[149]
	0.5 h MCA occlusion followed by 23.5 h reperfusion	C57BL/6 J mice	2.5 mg/kg	i.p. injection 0.5 h before ischaemia	Reduced total infarct volume, neurological impairment, superoxide production and mortality	[66]
	75 min MCA occlusion using a 6-0 coated suture followed by reperfusion	C57BL/6 J mice	4 mg/kg	i.p. injection 5 min before suture withdrawal	Reduced oxidative stress, 50% less brain infarction and 70% less cleaved spectrin	[27]
	90 min MCA occlusion with 22.5 h reperfusion	Sprague-Dawley rats	30 mg/kg	i.p. injection 1 h before the onset of MCAO	Reduced the MMP-9 increase, BBB damage brain edema, and NOX activity	[91]
	2 h MCA occlusion with a 4-0 nylon monofilament followed by 22 h reperfusion	Wistar rats	5 mg/kg	i.p. injection 5 min before reperfusion	Reduction in infarct size, inflammation and cellular death	[45]

Table 2 Apocynin use in vivo in experimental CNS disorder models

Disease	Model	Species	Dose	Way/duration admin	Therapeutic benefit	References
Hemorrhagic stroke	MCA occlusion with a silicone-coated 8-0 monofilament for 2 h followed by 22 h reperfusion	C57BL/6 mice	0.4, 4, and 40 mg/kg	i.v. 1 h before induction of MCAO	40 mg/kg prevented BBB disruption	[70]
	90 min MCA occlusion followed by 1, 4, 8, 24 h reperfusion	Sprague–Dawley rats (7 weeks old)	5 mg/kg	i.p. 30 min before induction of MCAO and 5 min after reperfusion	Attenuated protein carbonylation in the postsynaptic density proteins and cerebral infarct volume	[111]
	Occlusion of both common carotid arteries	Mongolian gerbils	5 mg/kg	i.p. 30 min before ischemia	Attenuated oxidative stress, neuronal degeneration and glia activation	[160]
	MCA occlusion with tissue plasminogen activator	Young (3–4 months) and aged (18–20 months) rats	5 mg/kg	i.p. 30 min before induction of MCAO	in aged rats increased mortality rate and failed to improve the functional outcome, total infarct volume, edema formation, and BBB permeability	[73]
	Transient global cerebral ischemia induced by bilateral common carotid artery occlusion for 22 min followed by 3, 6 and 72 h reperfusion	C57BL/6	2.5 mg/kg	i.v. 15 min before ischemia	Attenuated oxidative injury, microglial activation, and neuronal death	[167]
	1 h MCA followed by 24 h reperfusion	C57BL/6 mice	4 mg/kg	i.v. 1 h after MCAO	No reduction of infarct size	[77]
	Endovascular perforation of the right anterior cerebral artery	Rats	50 mg/kg	i.p. injection 3 times daily for 2 days	Enlarged basilar artery diameter, reduced neurological deficits, decreased NOX activity and superoxide production	[172]
	Intracerebral haemorrhage	Rats	3, 10 and 30 mg/kg	i.p. 2 h after haemorrhage	No neuroprotective effect	[151]
	Glycerol-induced intraventricular hemorrhage (IVH) in prematurely delivered (E29) rabbit pups by intraperitoneal infusion of 50% glycerol (6.5 g/kg) solution 2 h after birth	Rabbit	2.5 mg/kg per dose	i.v. injections at 3 and 12 h after IVH	Reduced ROS generation and cell death	[174]
	Ischemic brain injury associated with hyperglycemia	Transient bilateral common carotid artery occlusions and hyperglycemia	C57BL/6 J mice	15 mg/Kg	i.p. injection before reperfusion	Decreased neuronal superoxide production and death
Ischemic brain injury associated with chronic alcohol consumption	Liquid diet with alcohol (6.4% v/v) for 8 weeks, 2-h middle cerebral artery occlusion followed by 24-h reperfusion	Sprague–Dawley rats	Acute: 5 mg/kg chronic: 7.5 mg/kg/day	Acute: i.p. 30 min before MCAO Chronic: per os, 4 weeks prior to MCAO	Both treatments reduced infarct volume, improved neurological outcome, attenuated superoxide production in alcohol-fed rats	[171]
Ischemic brain injury associated with hypertension	Two renal arteries were constricted bilaterally with two ring-shaped silver clips	Stroke-prone renovascular hypertensive rats	1.5 mmol/L	Per os in the drinking water for 28 days beginning on the 5th week after the operation	Decrease in the expression of p22 ^{phox} protein and fibronectin levels in the cerebral vasculature	[32]
Ischemic brain injury associated with excess salt consumption	8% sodium diet from 11 weeks of age during 1, 2, or 4 weeks	Stroke-prone spontaneously hypertensive rats	0.6 mmol/kg per day	Per os in the drinking water for 4 weeks	Reduced oxidative stress, cell death and stroke incidence	[166]

Table 2 continued

Disease	Model	Species	Dose	Way/duration admin	Therapeutic benefit	References
Neonatal hypoxic-ischemic injury	On postnatal day 7, left common carotid artery was cauterized over a length of 5 mm. 2 to 4 h after surgery, the rats were exposed to 100% oxygen at 37°C for 2 h	Wistar rats	4 mg/kg	i.p. injection, first dose following hyperoxia and again the following morning	Increased NO and reduced O ₂ ⁻	[37]
Huntington disease	Intraatrial injection of quinolinic acid (QUIN) 240 nmol/ul	Wistar rats	5 mg/kg	i.p. 30 min before and 1 h after QUIN injection or only 30 min after QUIN injection	Decreased lipid peroxidation, circling behavior, and histological damage	[96]
Insulin-induced hypoglycemia	Hypoglycemia followed by glucose reperfusion (infusion of 50% glucose in Krebs-Henseleit buffer)	Sprague-Dawley rats	15 mg/kg	i.p. injection before the start of glucose reperfusion	Reduced neuronal death	[147]
NMDA receptor-dependent excitotoxicity	Injections of 6 nmol of NMDA in the right hippocampus	C57BL/6 mice	15 mg/kg	i.p. injection 20–30 min before the NMDA injections	Prevented superoxide production and neuronal death	[20]
Epilepsy	Pilocarpine injections in hippocampus	Wistar rats	10 mg/kg	In the drinking water 7 days prior to induction of status epilepticus	Decreased ROS production and neurodegeneration	[122]
Anxiety	l-buthionine-(S,R)-sulfoximine (BSO, 300 mg/kg i.p.) 48 and 24 h before behavioral testing	ICR mice	3 mg/kg	i.p. injection 30 min before each BSO treatment	Reduced anxiety-like behavioral	[101]
Obstructive sleep apnea	Long-term intermittent hypoxia	C57BL/6 J mice	3 mg/kg/day	Subcutaneously by way of microosmotic pump for 8 weeks	Conferred resistance to hypersomnolence, reduced oxidative damage and catecholaminergic neuron death in wake-active brain regions	[169, 173]
	Long-term intermittent hypoxia	Sprague-Dawley rats	3 mg/kg/day in 50 ul of 0.9% saline	Intragastrically 3 days before	Amelioration of spatial learning deficits, decreased oxidative stress and apoptosis	[60]

impairment associated with the ischemic insult. Another approach is to prevent the occurrence of stroke in subjects at risk [134].

Considerable variation in the outcome of ischemic stroke using transient middle cerebral artery occlusion (tMCAO) in NOX-deficient animals was observed. In an early study, Walder et al. [157] described that the infarct volume was reduced by approximately 50 % in NOX2-deficient mice after 2 h transient ischemia followed by 22 h reperfusion. These observations were further confirmed in different studies using similar ischemia–reperfusion approaches ranging from 30 to 75 to 120 min ischemia followed by 22–24 h reperfusion [27, 66, 70]. A recent study repeated exactly the study by Walder et al. [157] confirming the protective effect of NOX2 deficiency [165]. For NOX1-deficient mice, the time of ischemia appears to be critical for the switch between a protective or a deleterious effect of NOX1 activity. NOX1 deficiency decreased infarct size when 60 min ischemia was applied, while no differences were observed with occlusion time was 2 h or more [70]. In a model of 30 min ischemia followed by 24 h reperfusion, NOX1-deficient mice showed no difference in neurological score, total or subcortical cerebral infarct volume, or edema volume as compared to WT. However, cortical infarct volume was approximately fourfold greater in brains of NOX1-KO versus WT mice [66]. Moreover, a recent study using 60 min ischemia followed by 24 h reperfusion showed impressive protection in NOX4-deficient mice, but no difference in either NOX1- or NOX2-deficient mice. Substantial decrease of infarct size was also observed in NOX4-deficient mice when no reperfusion was applied 24 h after ischemia, while no data are available for NOX1- or NOX2-deficient mice for this model [77]. Such discrepancies are unfortunately not rare in rodent models of ischemia–reperfusion, as experimental details can affect the lesion in either beneficial or detrimental way, because tiny differences in the cerebral vasculature between mouse strains and surgical technique can account for important changes in infarct severity, such as body temperature control, blood pressure and blood monitoring, anesthetic used, and surgery time [36, 88]. In order to reconcile and understand these divergent data, some approaches can be proposed: (1) genotypic homogeneity of the strains could be verified, (2) a single operator could perform blindly both transient and permanent ischemia, (3) different times of ischemia could be tested for different knock-outs, and (4) permanent occlusion models could be used and neuronal cell death followed up at different time points as a relevant model of stroke. Although a direct neurotoxic role of ROS is possible, the possible mechanism played by NOX in stroke is still unclear, and a role in the BBB integrity and cerebrovascular permeability is emerging [41].

Obstructive sleep apnea syndrome (OSAS)

Obstructive sleep apnea is a respiratory disturbance characterized by recurrent occlusions of the upper airways and reduction in oxygen availability during sleep followed by sudden awakening and reoxygenation. The underlying neuropathological events of obstructive sleep apnea are still unclear, but it is known that intermittent hypoxia episodes cause behavioral alterations and cognitive impairments [9].

OSAS often affects obese patients, due to fat deposition in the parapharyngeal space, in the tongue, and under the mandible, reducing the upper airway caliber and predisposing them to breathing disorders during sleep [51].

The main remedy for OSAS consists of the application of devices to induce a nocturnal continuous positive airway pressure (CPAP), but this is poorly accepted by patients. Surgery can also be considered in certain cases to definitively remove the obstruction of upper airways. Otherwise, weight loss in obese patients or avoidance of risk factors, e.g., alcohol consumption, can be helpful to reduce the occurrence of this respiratory disturbance [51].

The animal model of OSAS is called long-term intermittent hypoxia (LTIH). It consists of the use of a particular chamber with an oxygen/nitrogen delivery system which automatically decreases the content of oxygen from 21 to 10 % at certain intervals for few seconds, inducing arterial oxyhemoglobin saturation [169]. Excessive production of ROS, neuronal death, and tissue damage are associated with the cognitive dysfunctions in this model [161].

In animals submitted to LTIH, genetic depletion of NOX2 (knock-out mice) or pharmacological (apocynin) inhibition of NOX2 reduced hypersomnolence and prevented oxidative damage in the wake-active regions of the brain [169, 173]. A recent report similarly shows that cognitive deficits induced by recurrent hypoxia events during sleep are mediated by excessive NOX2 activity [112].

In patients affected by OSAS, markers of inflammation (interleukin-6) and oxidative stress (8-isoprostane) can be detected in exhaled breath condensate [23]. Interestingly, neutrophils (which mainly express NOX2) of OSAS patients also show an enhanced production of superoxide [137]. The presence of these signs of oxidative stress has been mainly associated with the cardiovascular consequences of the disease. However, in light of the results obtained from animal models, it is possible that increase of NOX2 activity also contributes to the cognitive decline. Increased mRNA expression of *p22phox* was detected in peripheral blood mononuclear cells of 107 subjects affected by OSAS. In addition, in these patients, a significantly higher frequency of the C242T polymorphism in *CYBB*, the gene coding for *p22phox*, a NOX subunit necessary for the function of 4 NOX isoforms (NOX1-4), was detected as

compared to 69 healthy subjects [90]. Altogether, these findings suggest a potential for a NOX2-based therapy to treat the neurological consequences of sleep-associated breathing disorders.

Beneficial effect of NOX enhancement: potential CNS indications

Neurological autoimmune diseases

Multiple sclerosis (MS) is an inflammatory disease leading to myelin damage, which progresses to physical and cognitive disabilities. Disease onset usually occurs in young adults and affects 3 times more females than males. MS prevalence ranges between 2 and 150 per 100,000, but MS is much more common in northern Europe. MS takes several forms, with new symptoms occurring either in discrete attacks (relapsing forms) or slowly accumulating over time (progressive forms). There is no known cure for MS. Available treatments attempt to return function after an attack, prevent new attacks, and prevent disability. Administration of β -interferon 1a (Rebif[®]) shows some beneficial effects in a subset of patients, but, despite claims to the contrary, their ability to modify disease course has not been clearly established [39]. Antibodies to α 4-integrin (Natalizumab[®]) suppress the extravasation of lymphocytes into the CNS, but may trigger progressive multifocal leukoencephalopathy. Inhibition of the sphingosine-1-phosphate receptor (Fingolimod[®]) results in the sequestration of lymphocytes in lymph nodes. Use of drugs which non-specifically suppress the immune system (glucocorticoids and the antineoplastic agent mitoxantrone) slows the progression of the disease but are associated with harmful side effects.

Although autoimmunity is a primary trigger in MS lesion formation, it is now widely accepted that immune-mediated inflammation contributes to MS pathogenesis. Thus, the role of inflammatory auto-reactive CD4-positive T helper (Th) cells has been extensively proven in animal models of MS [57, 168]. However, although oxidative damage is a known feature of MS [153], ROS produced by NOX2 have been shown to be anti-inflammatory in autoimmune diseases. Indeed, low ROS generation by NOX2 appears to prevent autoimmune responses in the chronic EAE model of MS [62]. NOX2-dependent ROS of antigen presenting cells are a key regulator of T cell activation. Interestingly, leukocyte ROS production correlates inversely with disease severity in MS [110] and recurrent Guillain Barré syndrome (GBS) [108, 109]. Recurrent GBS is also mediated by autoimmunity and is caused by damage to the myelin sheet of the peripheral nerves. Current treatments of recurrent GBS consist of corticosteroids, plasmapheresis, and intravenous immunoglobulins.

Due to the critical role played by NOX enzymes in CNS pathological states, it is almost certain that other pathologies where oxidative stress is known to regulate disease progression will show beneficial effects of NOX inhibition (e.g., epilepsy, HIV-mediated dementia, or Huntington among others) or NOX enhancement in autoimmune diseases, such as leukodystrophies or progressive multifocal leukoencephalopathy.

Therapeutics targeting ROS and NOX enzymes

Antioxidants

In the case of schizophrenia, several clinical trials have recently been made with Vitamin C, E, omega-3 fatty acids, or N-acetyl cysteine, suggesting a certain efficacy of improving the antioxidant defence as adjunctive to a primary antipsychotic treatment [131]. Of particular note, parallel administration of N-acetyl cysteine could also moderately diminish negative symptoms, such as akathisia [16]. However, as for cardiovascular diseases, the use of antioxidant therapies has led to contradictory and mostly disappointing outcomes in clinical trials for CNS diseases [82], in spite of promising results obtained in *in vitro* studies and in animal models for AD [141], and other neuropathologies, such as stroke [134] or ALS [10]. Failure of antioxidant treatments should not necessarily preclude the search of therapies targeting oxidative stress to treat neuropathologies because there are numerous possible reasons for the apparent failure of antioxidant therapies, including lack of specificity, potency, and bioavailability of antioxidant drugs, poor trial design, or lack of relevant biomarkers of oxidation. Such issues are discussed in details elsewhere [19, 47]. Nevertheless, as of today, the medical use of such an approach still awaits solid evidence of therapeutic benefit. On the other hand, the concerted search and discovery of NOX inhibitors is only emerging. Neuroprotective action of several compounds have been described as acting on the NOX pathway, but they are probably acting upstream of NOX and, therefore, are blocking other pharmacological targets [29]. However, recently, systematic screenings of chemical libraries were performed and have identified new chemical entities targeting NOX enzymes [18, 46, 80]. Although peptidic and siRNAs have been designed to target NOX enzymes, we will only describe small molecules that are currently described as NOX inhibitors.

Natural compounds

Apocynin

Apocynin or acetovanillone (MW 166.174) is a natural organic compound widely used as a NOX inhibitor in

models involving NOX enzymes [144], and has been shown to be beneficial in numerous models of CNS diseases at different doses and types of administration (summarized in Table 2). However, the use of this molecule as a NOX inhibitor remains controversial as its mode of action is thought to be mostly through oxidant scavenging activity, although formation of an apocynin dimer (diapocynin) accounts for NOX2 inhibition through the activity of a peroxidase, such as the myeloperoxidase of leukocytes [145, 56]. Surprisingly, for such a compound which seems to represent a *panacea* for a large panel of diseases, few studies of its bioavailability in vivo are available. One study showed that, following single intraperitoneal injection (5 mg/kg), apocynin was detected in the brain, but in a glycosylated form, while no diapocynin could be detected [159]. However, another study showed a contrary result following chronic oral treatment (150 and 300 mg/kg/day for above 100 days): no glycosylation, but conversion of apocynin into diapocynin was detected in the brain and spinal cord [152]. In almost all studies described in Table 2, the compound has been administered (in concentrations ranging from 0.4 to 300 mg/kg) as preventive treatment ranging from more than 2 months before disease onset in the case of ALS [54] to a few minutes before ischemic stroke [27]. It is clear that a curative approach would be more relevant, although the outcome of such studies is sometimes disappointing, as for hemorrhagic stroke [151]. In the case of ALS, chronic treatment with apocynin in the drinking water led to extremely variable results: a first study described an increase of survival (113 days) in SOD^{G93A} mice, a real hope for a potential treatment in patients with ALS [54]. However, this impressive outcome could not be repeated. A study following exactly the same treatment protocol showed no benefit at all [152], while another study using a similar protocol showed a modest increase of survival (5 days) [89] (see Table 2). Interestingly, direct administration of diapocynin (150 mg/kg/day) after disease onset showed an 8-day increase in mean survival of SOD^{G93A} mice [152]. As diapocynin is considered the active form of apocynin [56], the rationale for NOX inhibition as a treatment for ALS remains valid.

Discrepancies have also been described in studies with chronic administration of apocynin (over 4 months) in transgenic models of AD (see Table 2) [34, 95]. Differences in the dose, mouse model, and the age of the mice at the beginning of the treatment may account for these contrasting results. Future comprehensive PK/PD studies for apocynin would greatly help researchers in the choice of a dose and mode of administration. Due to such discrepancies, lack of clear mechanism of action, lack of specificity, high metabolism, previous failure of antioxidant drugs, low potency on NOX enzymes, and extensive

patenting status, apocynin has a low potential for development by pharmaceutical companies [1]. Nevertheless, apocynin shows several advantages, which could potentially make it a therapeutic agent for CNS disorders: low toxicity, oral bioavailability, high potency in neuropathologies, and impact on surrogate markers of oxidative stress in phase I clinical studies following aerosol administration [123, 144]. Therefore, future studies associating apocynin bioavailability (PK/PD) with real therapeutic benefit in controlled preclinical trials after disease onset would provide proof of concept for a possible clinical development as therapeutics for intractable CNS diseases.

Celastrol

Celastrol (MW 450.6) is a natural compound extracted from the medicinal plant *Tripterygium wilfordii*, which has recently been shown to be a bone fide NOX inhibitor as it blocks within minutes both the increase of superoxide and hydrogen peroxide (the product of the reaction catalysed by NOX enzymes) and the decrease of its substrate, i.e. oxygen [68]. Although celastrol has only recently been identified as a NOX inhibitor, its neuroprotective properties have been established several years ago. Injection of celastrol (2 and 8 mg/kg/day) improved survival of SOD^{G93A} mice by 9.4 and 13 %, respectively [74], improved dopaminergic neuron survival in the MPTP model of PD (3 mg/kg, i.p. before and after MPTP injection) and with a dose of 3 mg/kg twice a day for 5 days significantly decreased the striatal lesion volume induced by 3-nitropropionic acid, a neurotoxin used to model Huntington disease in rats [30]. In a transgenic model of AD (Tg PS1/APPsw), celastrol (1 mg/kg i.p. for 4 days) and chronic treatment (32 days) with celastrol (2.5 mg/kg/day s.c. in a matrix-driven delivery pellet system) reduced the levels of both soluble and insoluble amyloid beta peptides, microglial activation, and amyloid beta plaque deposition [118]. Recently, intraperitoneal administration of celastrol improved the cognitive decline following major surgery in old mice and reduced β -amyloid accumulation and τ phosphorylation in the brain [158]. However, celastrol is a complex molecule with numerous targets [72], and its use as therapeutic awaits further study about its tolerability and possible efficacy for CNS pathologies.

Phytol

In contrast to previous examples, phytol (3,7,11,15-tetramethyl-2-hexadecene-1-ol, MW 296.53) is a compound which enhances NOX activity. Phytol has impressive effects in vivo as it completely blunts autoimmune inflammation. In the case of autoimmune disorders, phytol (i.p., but mostly s.c.) therapeutic effect was first described in arthritis-prone Ncf1 (DA) rats, which have a decreased NOX2-dependent

oxidative burst. This compound has been shown to act by increasing the phagocyte oxidative burst in vivo [61]. In an acute model of Guillain Barré, phytol treatment led to a strong reduction in experimental allergic neuritis disease severity and a lower number of IFN- γ -secreting cells in late disease stage [58]. The fact that phytol is an oil makes it an unlikely candidate for development by the pharmaceutical industry because of challenging SAR, pharmacokinetics, and metabolism. Nevertheless, it represents a proof of concept for therapies aiming at enhancing NOX activity.

Chemically synthesized molecules

Triazolopyrimidines

Several compounds developed by Vasopharm have been described as NOX inhibitors (for review, see [69, 75, 162]). Recently, VAS2870I, a low-molecular-weight pharmacological NADPH oxidase inhibitor was shown to inhibit NOX1, NOX2, and NOX4, but this may be through an indirect mode of action, at least for NOX2, because it does not block NOX2 activity in a semi-recombinant membrane assay [44]. When 2 mg VAS2870I solubilized in 10 % DMSO was injected intrathecally 2 and 12 h after ischemia, brain infarct volumes were reduced by 75 % compared to vehicle-treated mice thereby improving neurological outcome and mice viability [77]. However, this study failed to demonstrate efficacy of apocynin (100 μ g, i.v. 1 h before occlusion), thereby challenging other studies (Table 2). It was also the first report that provides evidence for a potential use of NOX inhibitors in the clinic for CNS diseases and suggests that investigation of other compounds targeting NOX should be similarly performed. Nevertheless, low solubility of this compound and mode of administration (intrathecal) makes it an unlikely candidate CNS drug for use in humans.

2-Acetylphenothiazine

Although they have not yet been used in CNS disorders, it can be expected that the compound 2-acetylphenothiazine (ML171), which shows potent inhibitory activity (0.25 μ M in a cellular assay) on NOX1, but also NOX2, NOX3, and NOX4 in the low micromolar range [46], can be active in the CNS because it belongs to the phenothiazine family of compounds, which are known to cross the BBB and to act as antipsychotics, but also act on other targets and are potent antioxidants [114].

3-Pyrazolopyridines

Recently, orally bioavailable small molecules developed by Genkyotex show high potency against NOX1 and NOX4 [43, 80] have shown high efficacy in animal models [21, 138, 154]. However, at this stage, no information

about CNS permeability and efficacy in CNS disorders has been documented.

4-Perhexiline

Perhexiline is primarily considered as a carnitine palmitoyltransferase inhibitor, and is used in patients to treat angina pectoris [4]. Perhexiline has been known for a long time to have NOX inhibitory activity [69], but a direct action on the NOX2 isoform ($IC_{50} = 13.2 \mu$ M in a semi-recombinant assay) was recently demonstrated [44]. Because of oral bioavailability, perhexiline might be recommended as a NOX inhibitor to treat CNS diseases. However, such an indication should, unfortunately, not be recommended for this compound because in many cases it induces drug-induced neuropathies [49, 133].

NOX inhibitors are currently developed by both academic [18] and industrial laboratories, such as Shionogi or Mitsubishi, for which only patents have been published (for review, see [69, 75], but, unfortunately, BBB penetration or efficacy in CNS indications has to our knowledge not been published.

Discussion and conclusion

Research and development of drugs for CNS disorders are particularly difficult and characterized by several challenges: (1) in the majority of the cases, the etiology of the disease is unknown, (2) mimicking CNS disorders in animal models offers a limited predictive value, (3) direct analysis of human brain samples can in most cases only be performed post mortem, (4) most CNS pathologies have a slow progression and can be diagnosed only when the disease is already at an advanced stage of development, making difficult both the identification of pathogenic events and the possible therapeutic intervention, (5) the absence or low availability of validated biomarkers to study disease progression, and (6) the presence of the blood-brain barrier, which limits access of compounds to the CNS, and requires complex additional assays to measure the concentration of drug that can effectively reach the target [117].

In this review, we have summarized the present knowledge showing that NOX enzymes represent new therapeutic targets with high potential for treatment of a large panel of CNS disorders. However, today, most of our knowledge is based on the protective effect of congenital absence of NOX2 (stable knock-out mice) or pretreatment with the antioxidant/NOX inhibitor apocynin. Therefore, alterations or adaptive responses due to the lack of NOX2 activity during the development of the brain cannot be excluded. As for future directions, use of conditional knock-outs as well as patient studies should be performed to measure NOX expression and activation. It is still unclear, for example,

whether increased activity of NOX2 in neurons or microglia is responsible for diseases such as schizophrenia (with low or no inflammation) or ALS (with a strong neuroinflammation). In addition, the role of other NOX isoforms especially NOX1 (Parkinson) and NOX4 (stroke) is only just emerging. Nevertheless, it is striking how consistently a key role of NOX activity is found in CNS pathologies and may in the future represent either a therapeutic target or a biomarker of CNS disease progression. However, many more studies are required to understand the exact impact of NOX enzymes in the different human CNS pathologies.

What would be the advantages of developing compounds to target NOX2?

If antioxidant treatments have shown no efficacy in clinical trials, why should it be worthwhile developing compounds to target NOX enzymes in the CNS? There are several reasons to sustain such an effort:

1. *Inhibition of ROS production at the source:* antioxidants act as scavengers and they are not able to prevent the actual generation of ROS. In contrast, a molecule designed to target NOX enzymes will be able to block directly the source, with the possibility of having a curative effect. Indeed, blocking NOX2-dependent ROS production, for example in microglia, would also allow for diminishing its activation and subsequent production of neuroinflammatory mediators [17].
2. *Specificity:* antioxidant molecules do not have a specific target and, as a consequence, their molecular structure cannot be systematically improved. In contrast, developing NOX targeting molecules allow for studying the effects of structural modifications on potency and isoform selectivity. Structure–activity relationships can be developed and molecules can be improved to target the desired protein.
3. *Modulation:* decreasing ROS is not always the therapeutic solution. It has been demonstrated that in certain pathological situations diminishing ROS can even be deleterious, such as in autoimmune CNS diseases [63]. In those cases, targeting the NOX enzyme can allow the design of compounds able to increase or restore NOX activity.

How to measure NOX activity in vivo? Use of oxidation biomarkers

Although NOX genetic deletion shows efficacy in pre-clinical models, a key requirement for a successful development of NOX targeted approach in humans would be the in vivo demonstration that the targeted NOX isoforms are blocked. This can only be achieved by the

identification of specific biomarkers as molecular signatures of excessive NOX activity. Such biomarkers would provide extremely useful information for in vivo demonstration and for the organization of clinical trials. Increased concentration of oxidized molecules in biological fluids as biomarkers of neuropathologies is extensively documented. As an example, increased markers of lipid peroxidation (isoprostanes) and nucleic acid oxidation (8-oxodeoxyguanosine) were detected in ALS sporadic patients [106] and PD patients [139]. However, the reliability of the correlation between the presence of oxidized molecules and disease has been questioned because of numerous unresolved experimental and technical flaws (for a detailed discussion of these specific issues, see the excellent critical review in [47]). The causes involved are the fact that biomarkers of oxidative stress are often chemically unstable molecules, which require complicated and expensive detection methods for reliable quantification, such as mass spectrometry and radioimmunoassay, but also that the source of these oxidized molecules remains unresolved.

However, levels of isoprostane, a marker of lipid peroxidation, and 8-hydroxy-2'-deoxyguanosine, a marker of nucleic acid oxidation, were markedly decreased in urine of patients with hereditary deficiency in NOX2 (CGD patients) [155, 156]. NOX2 in CNS pathologies show a positive relationship of isoprostanes with AD [126] and oxidized nucleic acids in psychosis [136, 143] as well as other CNS pathologies [112]. Such biomarkers could prove invaluable for CNS pathologies, such as Alzheimer disease: currently the only way to tell whether a patient is affected by AD in a conclusive manner is post mortem histological analysis of amyloid plaques deposition. Whether NOX enzymes are a main cause of the formation of oxidized biomarkers in CNS disease awaits further confirmation. This could be done by correlating the presence of isoprostanes and oxidized nucleic acids during the progression of different neuropathological animal models using available NOX knock-outs (i.e. NOX1, NOX2, and NOX4). Validated oxidized biomarkers for NOX activity would be extremely useful in drug development, such as to assess the effect of a NOX inhibitor on disease progression, help select drug candidates, define dose effects, and facilitate the selection of populations for clinical trials, and therefore optimize chances of the successful development of a drug, and drastically decrease development costs. It would also help in comparing therapies (NOX inhibitors versus antioxidant or drugs with another mode of action).

For which pathologies would it be useful to develop NOX targeting drugs?

As mentioned above, the involvement of the NOX2 enzyme has emerged from experimental animal models of

several incurable CNS diseases (Fig. 1). Because of the absence of treatment, severity, and strong rationale for NOX involvement, ALS could be a primary clinical indication for NOX inhibitors. Although ALS is quite rare, it is still possible to recruit a sufficient number of patients to organize placebo versus treatment clinical trials, while for recurrent Guillain Barré, for example, its incidence is so low that only a few patients can be included, generating data of poor statistical significance. In the case of therapeutic benefit, this would pave the way to other intractable CNS pathologies, such as Huntington disease or other more frequent neurodegenerative diseases, including AD and PD. A large effort is being put into the identification of novel therapeutic options for AD with promising targets [24]. The therapeutic benefit of NOX inhibition requires more proof of concept animal models. Similarly to AD, the potential for NOX inhibitors for PD and stroke as well as activators for MS are high, but therapeutic options are available and new drugs are emerging, so more studies will be necessary to demonstrate an added benefit compared to existing drugs. However, the future of drug development for neurodegenerative disease probably relies on complex therapies with several drugs that target various pharmaceutical targets, such as other anti-inflammatory drugs, glutamate excitotoxicity, and drugs targeting neurotransmitters [92, 107].

Since the discovery of chlorpromazine in 1952, little progress has been made for the therapy of schizophrenia. Indeed, available drugs to treat schizophrenia are not curative and cause considerable side effects. They all target the same pathway (dopaminergic neurotransmission), and they primarily decrease positive symptoms, thereby inhibiting behaviors not well tolerated by the society (Fig. 2a). Excessive dopaminergic release may not be the primary event, but rather the result of alteration of GABAergic-glutamatergic neurotransmission [38]. However, these findings have not provided new opportunities for treatment. Generation of ROS by NOX2 is emerging as a possible novel mechanism, which (1) can first elicit the abnormal release of glutamate and dopamine and (2) promote neurochemical adaptive responses [11, 12, 136, 143]. NOX2 would represent an upstream target to this cascade of events, leading to the manifestation of behavioral alterations (Fig. 2b). If the complete protection seen with NOX2 inhibition in animal models translates into schizophrenic patients, the potential for the design of novel treatment for schizophrenia is huge and would provide a new paradigm in the fields of psychiatric diseases.

Possible side effects of NOX therapeutics

As most CNS diseases are generally chronic diseases, intake of NOX-based therapeutics would possibly last for

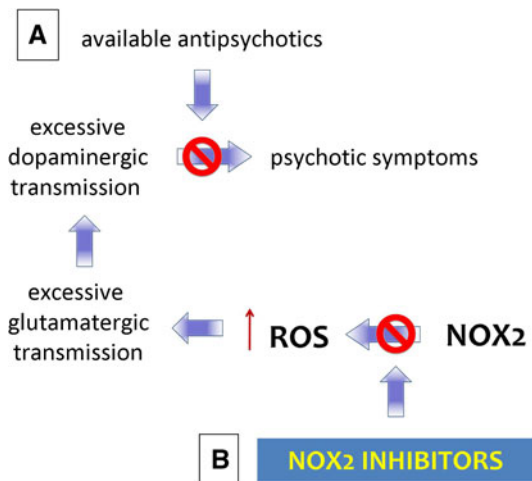


Fig. 2 Schematic representation of possible use of NOX2 inhibitors in schizophrenia. **a** Available antipsychotic compounds inhibit excessive dopaminergic transmission, but not curative and are associated with considerable side effects. **b** From experimental data, it emerges that NOX2 activation could be the primary source of neurotransmission alterations, which lead to psychotic symptoms. Therefore, blocking NOX2 activation could represent a novel therapeutic approach

years. Therefore, side effects represent a serious concern. Although off-targets effects are difficult to predict and can be determined by systemic assessment of toxicity, possible on-target side effects may result in clinical manifestations similar to what is known from animals and humans carrying genetic mutations in NOX genes [63, 67, 115]. Safety concerns regarding NOX inhibition include (1) NOX2 inhibition on microglial killing and development of hyperinflammatory states, (2) inhibition of NOX3 could lead to balance disorders as mice affected by mutations in NOX3 genes and its regulatory subunits show impaired otoconia formation and balance disorders, and (3) mutations affecting DUOX2 function lead to impaired thyroid hormone synthesis and congenital hypothyroidism. The effect of enhancing NOX2 activity, although potentially beneficial in autoimmune diseases, might result in phagocyte-mediated tissue damage. Therefore, to ensure proper monitoring of those effects, preclinical toxicity studies should include evaluation of balance disorders, measurements of circulating thyroid hormones, and inflammatory autoimmune manifestations.

Perspectives

Today, most treatments for CNS diseases are palliative or symptomatic, rather than curative or disease-modifying, and hence provide only slight relief instead of a cure. Also, they are generally associated with numerous side effects. With the increasing understanding of the biological basis of CNS diseases, common pathological mediators have been

identified [65]. These include glutamate, ROS, aggregated misfolded proteins, and inflammation, and, now, NOX enzymes are emerging as key upstream regulators of at least some of the above-mentioned features. NOX enzymes represent promising CNS therapeutic targets as (1) they are major ROS generators in the CNS, (2) they regulate microglia and/or astrocyte activation, and (3) they are key modulators of T lymphocyte activation in autoimmune diseases.

These remarkable features show that, although NOX enzymes are not likely to be responsible for the etiology of CNS diseases, they represent a novel and extremely promising therapeutic area for CNS pathologies.

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