Front. Biol. 2013, 8(2): 175–188
DOI 10.1007/s11515-012-1250-y

REVIEW

The role of NADPH oxidase (NOX) enzymes in neurodegenerative disease

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Abstract **Recently, mounting evidence implicating reactive oxygen species (ROS) generated by NADPH oxidase (NOX) enzymes in the pathogenesis of several neurodegenerative diseases including Amyotrophic lateral sclerosis (ALS), Alzheimer's (AD), Parkinson's (PD) and polyglutamine disease, have arisen. NOX enzymes are transmembrane proteins and generate reactive oxygen species by transporting electrons across lipid membranes. Under normal healthy conditions, low levels of ROS produced by NOX enzymes have been shown to play a role in neuronal differentiation and synaptic plasticity. However, in chronic neurodegenerative diseases over-activation of NOX in neurons, as well as in astrocytes and microglia, has been linked to pathogenic processes such as oxidative stress, exitotoxicity and neuroinflammation. In this review, we summarize the current knowledge about NOX functions in the healthy central nervous system and especially the role of NOX enzymes in neurodegenerative disease processes.**

Keywords neurodegeneration, oxidative stress, NADPH oxidase, microglia, inflammation

Introduction

Reactive oxygen species (ROS) are small oxygen derived reactive molecules used as cell signaling intermediates at low concentrations. However, excess cellular ROS can cause oxidative stress, a state where oxidative damage to organelles and macromolecules like mitochondria, DNA, proteins and lipids occur. Oxidative stress has been implicated in the neuronal death in many neurodegenerative diseases including Amyotrophic lateral sclerosis (ALS), Alzheimer's (AD), Parkinson's (PD), and polyglutamine (PolyQ) disease, for review see (Halliwell, 2001; Grimm et al., 2011). ROS are produced as a byproduct in many reactions, for instance by oxidative phosphorylation in mitochondria and by cytochrome P-450, xanthine oxidase, lipooxygenase and cyclooxygenase (Fatokun et al., 2008). Mitochondria are a major source of ROS, as a number of electrons passing the mitochondria electron transport chain during oxidative phosphorylation escape and react with molecular oxygen to yield ROS. A vast number of studies have implicated mitochondrial ROS production as an underlying cause of neuronal death in neurodegenerative diseases (Moreira et al., 2010; Federico et al., 2012).

Another important source of ROS is NADPH oxidase (NOX) enzymes. These enzymes were first described in phagocytic leukocytes, in which bursts of ROS produced by NOX participate in the killing of invading microorganisms, see Bedard and Krause for a historical overview (Bedard and Krause, 2007). To date, seven NOX family members have been described and their ROS production has been implicated in a number of additional important functions (Bedard and Krause, 2007; Brown and Griendling, 2009). Furthermore, altered NOX activity has been linked to a number of pathological situations including ischemia, diabetic nephropathy and demyelinating diseases, for reviews see (Kahles and Brandes, 2012; Sedeek et al., 2012; Sorce et al., 2012). Recently, mounting evidence has also linked NOX enzymes to several neurodegenerative diseases like ALS, AD and PD. The focus of this review is to summarize the current understanding of NOX enzymes in pathological processes in these neurodegenerative diseases.

The NOX family members and their activation

The NADPH oxidase family consists of seven members,

Received September 1, 2012; accepted November 23, 2012
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NOX1, NOX2, NOX3, NOX4, NOX5, DUOX1 and DUOX2. All members of the family contain at least six transmembrane domains, plus a FAD and a NADPH binding domain in the cytosolic C-terminal, for extensive reviews see (Brown and Griendling, 2009; Katsuyama et al., 2012). The NOX enzymes generate ROS by transporting electrons from NADPH on the cytoplasmic side, via FAD and two hemes coordinated by the transmembrane helixes, to oxygen in the cell exterior or in intracellular compartments. NOX1, NOX2, NOX3 and NOX5 appear to produce mainly superoxide-anions (O_2^-), whereas NOX4 and DUOX1-2 produce mainly hydrogen peroxide (H_2O_2), for review see (Brown and Griendling, 2009).

A number of stimuli including angiotensin II, thrombin, platelet-derived growth factor (PDGF), transforming growth factor β (TGF-β) and inflammatory factors, like LPS and cytokines, have been reported to activate NOX enzymes in various cell types, see Fig. 1 and (Brown and Griendling, 2009; Jiang et al., 2011; Katsuyama et al., 2012) for extensive reviews.

NOX1 and NOX2 are expressed in a variety of cell types, with the highest expression of NOX1 in colon epithelia and NOX2 in cells of the myeloid linage (neutrophils, macrophages) (Bedard and Krause, 2007). NOX3 on the other hand is almost exclusively expressed in the inner ear. The activation mechanism for NOX1–3 is similar and involves a complex series of protein–protein interactions, see Fig. 1A.

Activation of NOX2, the NADPH oxidase first identified in phagocytic leukocytes, has been most extensively studied. In non-activated phagocytes, NOX2 is kept in a completely inactive state and for full activity the assembly of a multi-subunit complex consisting of NOX2, p22phox, p47phox, p67phox, rac and p40 phox takes place, for detailed reviews see (Nauseef, 2004; Sumimoto et al., 2005; Brown and Griendling, 2009; Katsuyama et al., 2012). The p22phox protein is constitutively associated with and stabilizes the NOX2 enzyme (Parkos et al., 1989; Huang et al., 1995; DeLeo et al., 2000). However, upon cell stimulation, the GTP binding protein rac and p47phox are independently redistributed from the cytosol to the plasma membrane and recruited to the NOX2 complex (Sumimoto et al., 1996; Koga et al., 1999; Lapouge et al., 2000; Diebold and Bokoch, 2001; Sarfstein et al., 2004; Groemping and Rittinger, 2005; Bäumer et al., 2008). The rac translocation is controlled by phosphorylation of GDP dissociation inhibitors (GDIs), which when phosphorylated by kinases such as Src no longer can interact with rac and thereby maintain rac in a non-membrane-associated state (Bokoch et al., 2009). The translocation of p47phox is controlled by stepwise phosphorylation of several p47phox serine residues by multiple kinases, including protein kinase C (PKC) family members, Akt (also known as protein kinase B), serine/threonine-protein kinase1 (PAK1) and mitogen activated protein kinases (MAPKs) ERK1/2 and p38 MAPK, for detailed review see (Bokoch et al., 2009). The translocation of p47phox brings with it two additional subunits, p67phox and p40phox. Upon recruitment the p67phox subunit binds to NOX2 and induces the enzymatic activity, whereas p40phox appears to modulate NOX2 activity by facilitating the membrane translocation of p47phox and p67phox (Han et al., 1998; DeLeo et al., 1999; Nisimoto et al., 1999; Cross, 2000; Lapouge et al., 2002).

The enzymatic activity of NOX1is controlled in a less stringent, but still similar manner, involving p22phox and rac. However, NOX1 is mostly activated by the p47phox and p67phox homologs NOXO1 and NOXA1, for review see (Sumimoto et al., 2005).The activity of NOX3 is even less stringently controlled. However, both p22phox and NOXO1 are essential for activation and at least under certain conditions NOXA1 is also required (Bánfi et al., 2004; Cheng et al., 2004; Kawahara et al., 2005; Ueno et al., 2005; Kiss et al., 2006).

NOX4 is fairly ubiquitously expressed, with the highest expression in renal cells. The enzyme exhibits high constitutive activity and is not dependent on any of the regulators that support NOX1–3, for review see (Brown and Griendling, 2009; Katsuyama et al., 2012). The p22phox protein can, however, interact with NOX4 and stimulate the enzymatic activity (Kawahara et al., 2005; Martyn et al., 2006). Based on these finding, NOX4 has been suggested to be an inducible NOX, where the activity is proportional to the NOX4 expression level (Brown and Griendling, 2009). Several stimuli, including TGF-β, insulin and insulin growth factor 1 (IGF-1), have been reported to induce NOX4 expression, see Fig. 1 and (Brown and Griendling, 2009) for an extensive review.

NOX5 is expressed in a variety of tissues including testis and spleen. DUOX1 and DUOX2 are most highly expressed on epithelial surfaces of mucosal tissues and several endocrine and exocrine glands, such as the thyroid (Bedard and Krause, 2007). Compared to the other NOX enzymes, NOX5, DUOX1 and DUOX2 have extended N-terminals containing Ca^{2+} binding EF-hand motifs, for review see (Brown and Griendling, 2009; Katsuyama et al., 2012). Upon Ca^{2+} binding these NOX family members undergo conformational changes which induce the enzymatic activity, see Fig. 1. The activity of these NOX enzymes is also further modified by phosphorylation, for review see (Bokoch et al., 2009). Phosphorylation of NOX5, by MAPKs and calcium/calmodulin-dependent kinase II, has been implicated in NOX5 activation (Pandey and Fulton, 2011; Pandey et al., 2011), whereas phosphorylation of DUOX1 by PKA and DUOX2 by PKC has been identified (Rigutto et al., 2009). The activity of DUOX1 and 2 are also controlled by DUOX maturation factors, DUOXA1 and DUOXA2, which not only enable endoplasmic reticulum (ER) exit, but also affect the type of ROS produced by DUOX enzymes (Grasberger and Refetoff, 2006; Morand et al., 2009).

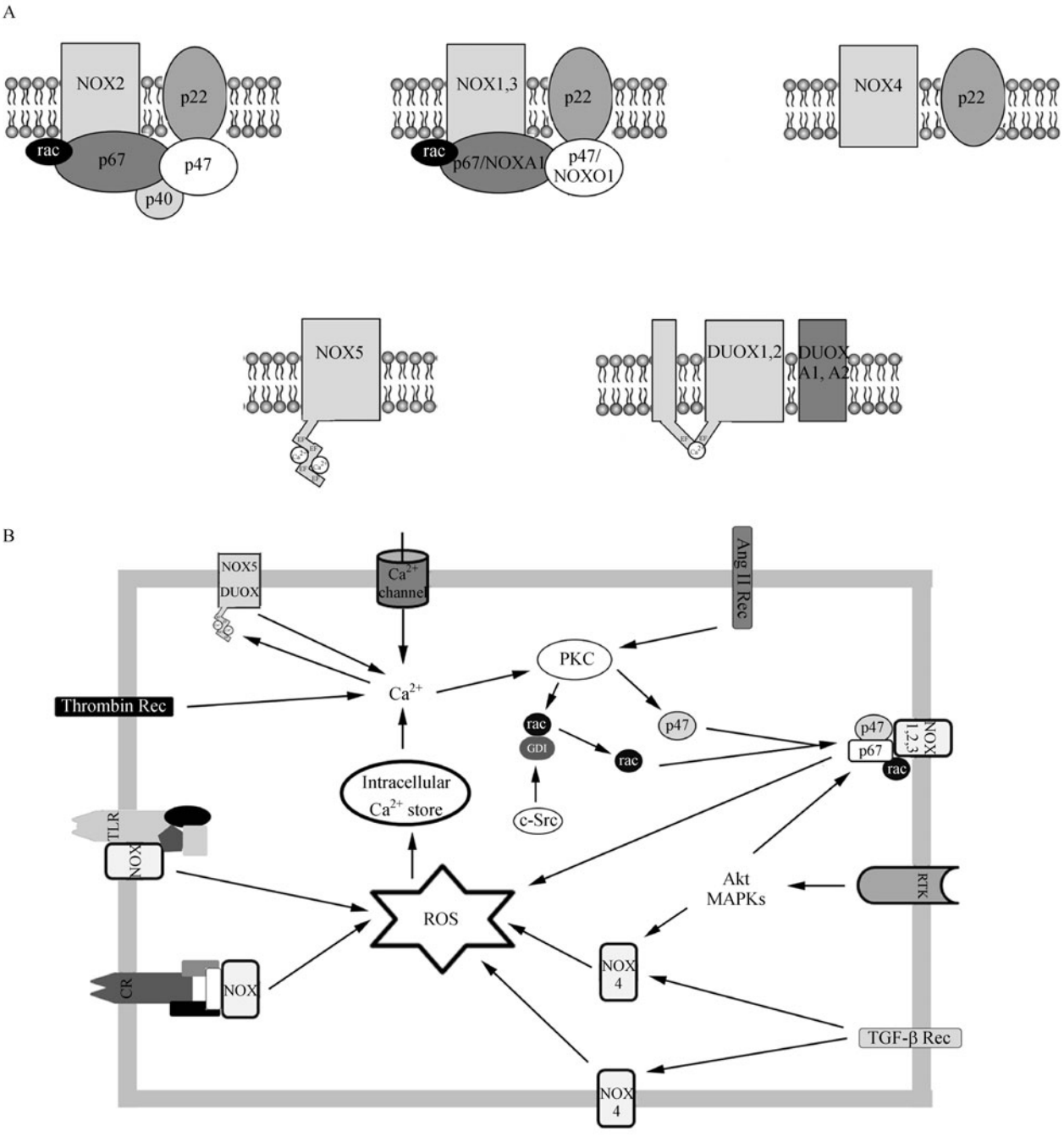

Figure 1 The NOX family members and their activation. (A) Schematic diagrams of the NADPH oxidases and their regulatory subunits. The p22phox subunit interacts with NOX1–4. NOX2 activation also involves association with rac, p47phox, p67phox and p40phox. NOX1 activity is believed to primarily involve association with rac, NOXO1 and NOXA1. However, the p47phox and p67phox can replace NOXO1 and NOXA1, respectively. NOX3 subunit dependency is less characterized, but activity is believed to involve rac, p47phox and NOXA1. NOX4 is constitutively active, but the activity is stimulated by p22phox. NOX5 and DUOX1/2 contain EF-hands (EF) and are activated by Ca^{2+} binding. DUOX 1/2 also requires the association with DUOX maturation factors A1/2, respectively. (B) Summary of some key stimuli and pathways known to activate NOX enzymes in various cell types. Translocation of cytosolic regulatory subunits play an essential role in activation of NOX1–3. During NOX2 activation, the translocation of p47phox is controlled by a stepwise phosphorylation of p47phox. PKC, which can be activated by multiple pathways, including increased cytosolic Ca^{2+} levels, is one important kinase responsible for p47phox phosphorylation. Other kinases involved include Akt and MAPKs (ERK1/2 and p38), which can be activated by signaling from receptor tyrosine kinases (RTKs) including the insulin, Trk, PDGF and VEGF receptors. NOX2 activation also involves Rac translocation, and this step is controlled by phosphorylation of GDP dissociation inhibitors (GDIs) by src and possibly PKC. Elevated cytoplasmic Ca^{2+} concentrations, through opening of membrane ion channels or intracellular stores, are a key in NOX5 and DUOX1/2 activation. TGF-β receptor signaling has been shown to induceexpression of NOX4 on both the endoplasmic reticulum and the plasma membrane. Transcription of NOX4 can also be induced by RTK signaling. NOX enzymes have also been reported to interact with and be activated by adaptor proteins involved in Toll-like receptor (TLR) and cytokine receptor (CR) signaling.

Cellular signaling by NOX enzymes

Studies in various cell types have shown that NOX enzymes can regulate many essential physiological processes, including cell survival, differentiation, migration, apoptosis and inflammation, for extensive review see (Gough and Cotter, 2011; Jiang et al., 2011). NOX-produced ROS mediate these functions by affecting a number of redox-sensitive intracellular signaling molecules, including phosphatases, transcription factors and ion channels, see Fig. 2.

Both protein tyrosine phosphatases (PTPs) and dual-specificity phosphatases (DSPs) are inactivated by ROS through reversible oxidation of cysteine residues (Chen et al., 2009; Jiang et al., 2011). PTPs remove the phosphate group from proteins phosphorylated on tyrosine residues and inhibition of these enzymes hence promote tyrosine phosphorylation by protein tyrosine kinases (Ostman et al., 2011). DSPs are important regulators of MAPK pathways, as they can dephosphorylate MAPKs on both phospho-threonine and phospho-tyrosine residues (Caunt and Keyse, 2012). Together PTPs and DSPs hence control the activation of various important kinases, such as Akt, ASK-1, ERK1/2, p38MAPK and JNK MAPK, used in regulation of survival, differentiation, stress response, inflammation and apoptosis, see Fig. 1.

Redox-sensitive transcription factors regulated by NOX include NF-κB, p53, NFAT and activator protein 1 (AP-1) (Chen et al., 2009; Jiang et al., 2011). NOX can also regulate intracellular and plasma membrane ion channels, including K^+ channels, L-type plasma membrane Ca^{2+} channels and intracellular ryanodine Ca^{2+} receptors regulating the intracellular Ca^{2+} storage, for review see (Bedard and Krause, 2007). A single NOX member can hence modulate multiple pathways and to date little is known about how NOX can be controlled to transduce one signal pathway over another.

The roles of NOX enzymes in the healthy nervous system

The function of the nervous system is not only dependent on neurons, but also a number of other cell types, including oligodendrocytes, astrocytes and microglia. NOX enzymes have been identified and implicated in a number of functions in all these cell types. However, much still remains to be discovered about the exact roles of NOX enzymes in the nervous system.

Neuronal NOX under non-pathological conditions

In neurons, expression studies have reported the presence of NOX2 in all regions of the forebrain, midbrain and hindbrain, with particularly high levels of NOX2 in neurons in the hippocampus (CA1 and CA3 areas), cortex, amygdala,

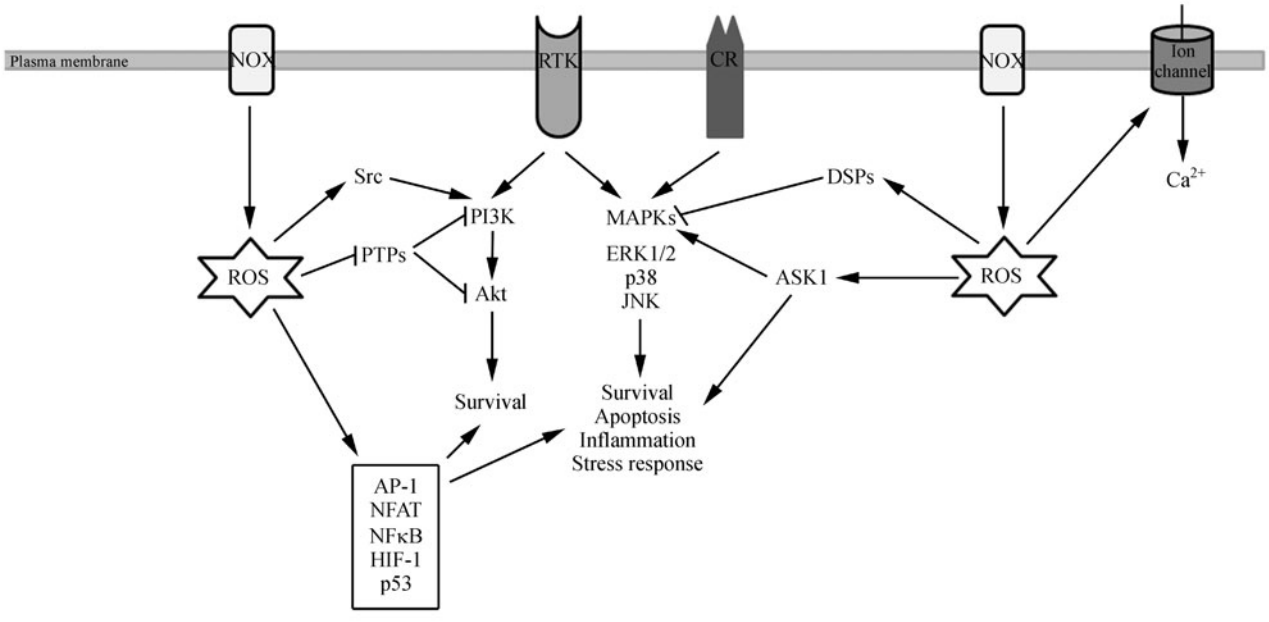

Figure 2 Cellular signaling by NOX enzymes. The ROS produced by NOX enzymes affect a number of redox-sensitive molecules, including protein tyrosine phosphatases (PTPs), dual-specificity phosphatases (DSPs), and transcription factors such as AP-1, NFAT, NFκB, HIF-1 and p53. Other redox sensitive targets include the apoptosis signal-regulating kinase 1 (ASK1) and the tyrosine-protein kinasesrc. Src and PTPs control signaling in the PI3K-Akt survival pathway. DSPs and ASK1 are important regulators of ERK1/2, p38 and JNK mitogen activated kinase (MAPK) pathways, which are important for a number of functions including survival, apoptosis, inflammation and stress responses. NOX enzymes have also been shown to regulate intracellular or plasma membrane ion channels. RTK = receptor tyrosin kinase, CR = cytokine receptor.

striatum and thalamus (Noh and Koh, 2000; Serrano et al., 2003; Tejada-Simon et al., 2005). Moreover, NOX2 have been reported in sensory neurons, petrosal ganglion neurons, dorsal root ganglia, sympathetic primary neurons and in Purkinje cells in the cerebellum (Mizuki et al., 1998; Dvorakova et al., 1999; Tammariello et al., 2000). Expression of other NOX family members have also been reported and include NOX4, DUOX1 and DUOX2 in photoreceptor neurons (Bhatt et al., 2010), NOX1 and NOX4 in cerebellar granule neurons (Coyoy et al., 2008) and NOX3 in vestibular and cochlear sensory epithelia and in the spiral ganglions (Bánfi et al., 2004).

Under normal physiological conditions neuronal NOX-mediated ROS production has mainly been implicated in regulation of neural stem cell behavior, neuronal differentiation, regulation of angiotensin II effects, and NMDA-mediated synaptic plasticity (Gao et al., 2012; Katsuyama et al., 2012). Dickinson et al. (2011) recently reported that oxidation and deactivation of PTEN, a phosphatase that inhibits PI3K-AKT signaling, by NOX2-mediated ROS is important to stimulate normal growth and proliferation of hippocampal neural stem cells. Similarly, NOX-mediated stimulation of PI3K-Akt signaling was shown to stimulate retinal ganglion cell survival (Mackey et al., 2008; Groeger et al., 2009). Several studies have also implicated NOX family members in regulation of neuronal differentiation (Suzukawa et al., 2000; Ibi et al., 2006; Munnamalai and Suter, 2009; Nitti et al., 2010). In aplysia bag neurons, NOX-mediated ROS production is critical for maintaining a dynamic F-actin cytoskeleton required for growth cone motility and neurite outgrowth (Munnamalai and Suter, 2009). NOX have also been shown to promote nerve growth factor (NGF) induced differentiation of PC12 cells. NGF induces PC12 differentiation by activation of the tyrosin kinase receptor TrkA and the p38 MAPK pathway. TrkA activation has also been shown to induce NOX via rac and PKC stimulation. In the early stage of differentiation, the resulting ROS has been shown to mediate proper p38MAPK activation and neurite sprouting (Suzukawa et al., 2000; Puntambekar et al., 2005; Ibi et al., 2006). However, at later stages of PC12 cell differentiation, NOX-mediated ROS production seems to suppress NGF-induced neurite outgrowth via stimulation of the PI3K-Akt pathway (Ibi et al., 2006).

In the healthy adult brain, NOX2 has also been linked to N-methyl-D-aspartate (NMDA) receptor signaling. NMDA receptors are ionotropic glutamate receptors essential for brain function and participate in synaptic transmission and triggering of synaptic plasticity (Rebola et al., 2010; Traynelis et al., 2010). Synaptic plasticity, the ability of a synapse between two neurons to change in strength, is thought to underlie cognition, memory and learning. Long-term potentiation (LTP), whereby brief periods of synaptic activity can produce a long-lasting increase in synaptic strength, is one of the most studied forms of synaptic plasticity and requires activation of NMDA receptors in post-synaptic neurons (Rebola et al., 2010; Traynelis et al., 2010). Upon activation, NMDA receptors permit entry of Ca^{2+} into the neuron and this results in synaptic remodeling through activation of multiple Ca^{2+} sensitive signaling pathways, including the ERK1/2 MAPK pathway (Rebola et al., 2010; Traynelis et al., 2010). In both cortical and hippocampal neurons, NOX2 is expressed in synapses and co-localization with the NMDA receptor subunit NR1 have been reported (Tejada-Simon et al., 2005; Girouard et al., 2009). Stimulation of NMDA receptors have been shown to activate NOX2 through a NO, cGMP and PKG pathway (Brennan et al., 2009; Girouard et al., 2009). In turn, the physiological level of superoxide produced from NOX2, is suggested to be essential for proper NMDA receptor-dependent activation of ERK1/2 (Kishida et al., 2005; Brennan et al., 2009; Girouard et al., 2009). Furthermore, the NOX-produced ROS, together with ERK1/2, seem essential for induction of plasticity related synaptic changes, including upregulation of NR2B and NR1 NDMA receptor subunits (Di Maio et al., 2011). The produced ROS have also been suggested to diffuse from the activated neuron into surrounding oligodendrocytes and induce changes in myelination (Atkins and Sweatt, 1999). Together these data suggest a crucial role for NOX-mediated ROS in NMDA receptor signaling and synaptic plasticity. In accordance with this, both humans (Pao et al., 2004) and mice (Kishida et al., 2006) that lack functional NOX2 have deficits in synaptic plasticity, learning and memory.

NOX in oligodendrocytes

Myelination of neuronal axons speeds up neurotransmission and is vital for proper function of the nervous system. In the peripheral nervous system the myelination is performed by Schwann cells, while the myelination is done by oligodendrocytes in the central nervous system (CNS). Very little is known about the presence and functions of NOX family members in myelinating cells (Bedard and Krause, 2007). Recently, DUOX proteins where, however, shown to be expressed in a human oligodendocyte cell line (Damiano et al., 2012) and oligodendrocytic NOX activity was suggested to promote maturation of oligodendrocytes and favors myelination, possibly via regulation of the PI3K/Akt pathway (Cavaliere et al., 2012).

NOX in astrocytes under non-pathological conditions

Astrocytes are the predominant glial cell type in the CNS and play important roles in the maintenance of brain homeostasis, for review see (Benarroch, 2005; Verkhratsky and Parpura, 2010). Important astrocytic functions include regulation of the blood-brain barrier, providing metabolic support to neurons and regulation of synaptic transmission via re-uptake of neurotransmitters from the synaptic cleft (Benarroch, 2005; Verkhratsky and Parpura, 2010). NOX1, NOX2, as well as NOX4 have been reported in astrocytes, however, NOX2

appears to be the most predominant form (Abramov et al., 2005). Under normal physiological conditions, the levels of NOX2 are low (Noh and Koh, 2000), but upregulation of NOX levels via PKC and Ca^{2+} have been reported (Abramov et al., 2005; Zhu et al., 2009; Hsieh et al., 2012). Under non-pathological conditions NOX can stimulate astrocyte survival (Liu et al., 2005). NOX-mediated ROS production can also regulate actin remodeling and MMP-9 expression in astrocytes via activation of MAPK pathways (Zhu et al., 2009; Hsieh et al., 2012).

The role of NOX in normal microglial behavior

Microglia are innate immune cells essential for normal healthy CNS function. Besides being important for the immune defense in the brain, microglia also remove cellular debris and participate in synaptic plasticity (Kettenmann et al., 2011). Microglia typically exist in a resting state, however, in response to injury, infection or inflammation microglia are activated (Block et al., 2007; Kettenmann et al., 2011). Activated microglia have high proliferative, migratory and phagocytic capacity and can perform a diverse set of functions supporting neuronal survival during challenges and regeneration. Microglia can for instance stimulate neuronal survival by release of neurotrophic factors, stimulate neurogenesis and guide migrating stem cells (Block et al., 2007; Kettenmann et al., 2011).

Microglia have been shown to express three NOX isoforms, NOX1 (Chéret et al., 2008; Harrigan et al., 2008), NOX2 (Sankarapandi et al., 1998; Lavigne et al., 2001; Harrigan et al., 2008) and NOX4 (Harrigan et al., 2008; Li et al., 2009). In non-activated microglia, low levels of NOX have been reported (Noh and Koh, 2000; Serrano et al., 2003). However, during microglial activation, NOX enzymes are upregulated and the subsequent ROS production can regulate the activation process and the microglial response (Pawate et al., 2004; Mander et al., 2006; Roepstorff et al., 2008; Huo et al., 2011; Savchenko, 2012). Many endogenous or exogenous signals that alert the microglia to nearby danger, for instance the bacterial endotoxin LPS or inflammatory mediators like prostanoids or cytokines have been shown to activate NOX, see Fig. 1.

Scavenger receptors and pattern-recognition receptors (PRRs) expressed on the microglial surface are essential for microglial recognition of invading microorganism or host-derived stress signals. Several scavenger receptors, including SR-A, SR-B1 and CD36, as well as several PPRs including toll-like receptor (TLR) 2, TLR4 and Mac1, have been implicated in microglial NOX2 activation. Furthermore, activation of TNFα, IL-1 and interferon gamma cytokine receptors, have been shown to stimulate NOX activity, for detailed review see (Jiang et al., 2011).The intracellular signaling pathways by which these scavenger, pattern-recognition and cytokine receptors activate microglial NOX is still mostly unclear. JNK and p38 MAPK pathways, as well as PAK1 mediated phosphorylation of p47phox, has however been implicated (Roepstorff et al., 2008; Huo et al., 2011).

The role of NOX enzymes in neurodegenerative disease processes

Neurodegenerative diseases are characterized by the progressive loss of subpopulations of neurons in different areas of the CNS. Alzheimer's disease (AD), the leading cause of dementia in the elderly, is characterized by loss of neurons in hippocampus and cerebral cortex. While Parkinson's disease (PD) is characterized by loss of dopaminergic neurons in the substantia nigra and loss of motor neurons is a hallmark of Amyotrophic lateral sclerosis (ALS). Oxidative stress has been suggested as an important mechanism in these neurodegenerative diseases and findings pointing toward miss-regulation of NOX-mediated ROS production as a contributing factor to the oxidative stress, is now emerging. Increased expression of NADPH oxidases have for instance been shown in post-mortem brains from both PD and AD patients (Shimohama et al., 2000; Wu et al., 2003; Ansari and Scheff, 2011). Moreover, in AD transgenic mice, as well as in dementia patients, the level of NOX activity has been shown to correlate with the individual's cognitive status (Bruce-Keller et al., 2010; Ansari and Scheff, 2011; Bruce-Keller et al., 2011). Furthermore, deletion of NOX2 reduced neuronal oxidative stress, cerebrovascular dysfunctions and behavioral deficits in an AD mouse model (Park et al., 2008). Similarly, deletion of NOX2 was shown to slow disease progression and extended the survival of an ALS mice model (Wu et al., 2006; Marden et al., 2007). A close link between NOX4 and ALS disease was also found in genome wide association studies (Dunckley et al., 2007). The exact role of NOX enzymes in the pathological processes in these different neurodegenerative diseases is still, however, not completely understood. Increased NOX activation in neurons, as well as in astrocytes and in microglia is most likely involved, see Fig. 3.

The role of neuronal NOX in neurodegenerative diseases

Neurons are highly sensitive to oxidative stress due to an abundance of oxidative-sensitive lipids, low anti-oxidant defense capacity, a restricted renewal and regenerative capacity, as well as high usage of mitochondrial respiration. Oxidative damage to neuronal proteins, lipids and DNA is a hallmark of many neurodegenerative diseases (Barnham et al., 2004). Increased neuronal NOX levels have been reported in AD, PD, as well as several polyglutamine disease models and could contribute to the oxidative damage and death of neurons in these disorders (Jana and Pahan, 2004; Anantharam et al., 2007; Bertoni et al., 2011; Ajayi et al., 2012). Extensive oxidative damage of DNA can for instance result in widespread PARP-1 activation, which in turn results in mitochondria depolarization and activation of an apoptosis

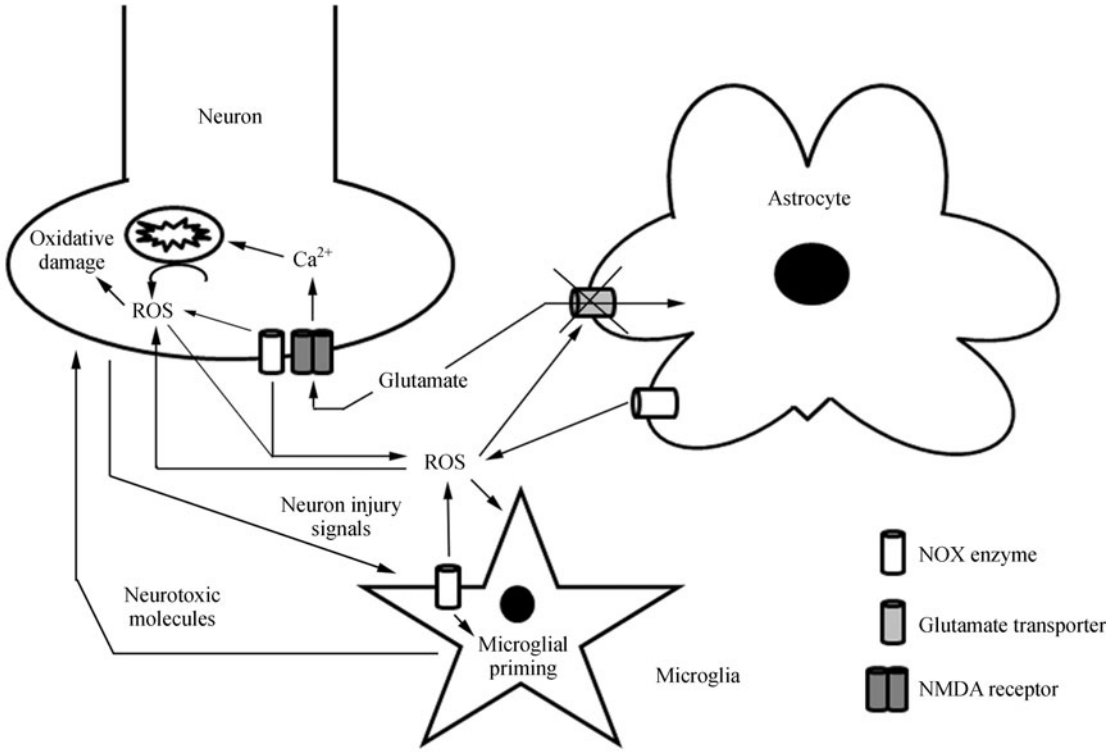

Figure 3 The roles of NOX in neurodegenerative disease. NOX enzymes are expressed in neurons, astrocytes and microglia. Over-activation of NOX in neurons can lead to neuronal damage and release of injury signals which activatesurrounding astrocytes and microglia. Activated astrocytes and microglia in turn upregulate their NOX activity resulting in release of ROS and further oxidative damage to neurons. Chronic activation of NOX in microglia can also result in microglial priming and release of additional neurotoxic molecules. In astrocytes, the ROS can damage glutamate transporters resulting in decreased clearance of glutamate from the synaptic cleft. This in turn can result in over-activation of NMDA receptors, increased NOX activationand excitotoxicity in neurons. Excitotoxicity results in increased Ca^{2+} levels, mitochondrial damage and increased mitochondrial ROS production in the neuron.

inducing factor (AIF) dependent cell death program (Kauppinen and Swanson, 2007; Chaitanya et al., 2010). Activation of this pathway has been reported in both AD models and patient brains (Abeti et al., 2011; Strosznajder et al., 2012). Alternatively oxidative damage to DNA could induce p53 mediated apoptosis involving bax and caspases (Amaral et al., 2010; Reinhardt and Schumacher, 2012). Increased NOX activity in neurons could also contribute to the neuronal cell death by direct effects of ROS on redox-sensitive intracellular messengers controlling survival or apoptotic pathways. NOX-produced ROS is for instance known to influence signaling pathways controlling neuronal cell death after TNFα stimulation, potassium deprivation, staurosporine treatment and neurotrophic factor withdrawal (Tammariello et al., 2000; Coyoy et al., 2008).

The mechanism(s) by which neuronal NOX activity is elevated to toxic levels in different neurodegenerative diseases is still largely unclear. However, direct effect on NOX by some disease related proteins, as well as over-activation of pathways normally regulating NOX activity has been suggested. In models of polyglutamine diseases, the mutant disease causing protein has been shown to co-immunoprecipitate with NOX2 and based on this, the disease protein was suggested to directly stabilize NOX and thereby increase the activity (Bertoni et al., 2011). Rotenone, a chemical which causes PD-like disease in mice, has also been shown to directly bind NOX2 and thereby trigger NOX activation and subsequent neuronal cell death (Zhou et al., 2012). However, other PD inducing toxins have been suggested to activate NOX by causing mitochondrial damage, increased mitochondrial ROS production and activation of the Lyn tyrosine kinase, which in turn activates NOX (Zawada et al., 2011).

In ALS disease, miss-regulation of rac has been suggested as an underlying cause of NOX over-activation, as two proteins, SOD1 and Alsin, in which mutations can cause ALS disease, have been shown to normally regulate rac and thereby influence NOX activation (Harraz et al., 2008; Li et al., 2011). Rac mediated activation has also been described in paraquat induced PD (Cristóvão et al., 2009).

Aß, the neurotoxic peptide in Alzheimer's disease, has been shown to activate NOX in a NMDA receptor dependent manner (Shelat et al., 2008; He et al., 2011). Aß was shown to potentiate the Ca^{2+} influx through NMDA receptors and thereby result in sustained NOX-mediated ROS production (Shelat et al., 2008; He et al., 2011). Blockage of NMDA

receptor subunits could prevent Aß induced hippocampal dysfunction (Costa et al., 2012). As discussed above in section 4, NMDA receptor mediated activation of ROS production via NOX is important for synaptic plasticity. However, chronic exposure of neurons to low ROS concentrations, or brief exposure to high ROS levels have been shown to impair LTP, for review see (Knapp and Klann, 2002; Massaad and Klann, 2011). A sustained NMDA-mediated activation of NOX enzymes might hence inhibit LTP and cause oxidative damage in neurons. In addition, excess NMDA-mediated Ca^{2+} influx is associated with a form of toxicity known as excitotoxcity. During excitotoxicity, mitochondria are damaged by the excess Ca^{2+} levels and mitochondrial ROS production is subsequently increased (Markowitz et al., 2007; Barber and Shaw, 2010). In addition, nitric oxide synthase is activated resulting in production of nitric oxide (NO), which when reacting with ROS generates the highly damaging peroxynitrite radical (Markowitz et al., 2007; Barber and Shaw, 2010). Taken together, the Aß peptide's ability to cause sustained NOX activation and excitotoxicity via NMDA receptors results in a highly oxidative environment, where important neuronal components including DNA, proteins and lipids are damaged and LTP is reduced. In addition, NOX-mediated activation of endoplasmic reticulum stress and activation of apoptosis via a redox sensitive cPLA2-dependent sphingomyelinase-ceramide pathway could contribute to the Aß induce toxicity (Jana and Pahan, 2004; Malaplate-Armand et al., 2006; Shelat et al., 2008; Costa et al., 2012).

Glial NOX and chronic neuroinflammation in neurodegenerative diseases

Inflammation is a cardinal defense response to injury or infectious agents and can be beneficial to the host. However, chronic inflammation is a prominent feature shared by all neurodegenerative diseases and is believed to contribute to the disease pathology. Microglia and to some extent astrocytes play a key role in CNS inflammation, i.e. neuroinflammation (Block et al., 2007; Verkhratsky and Parpura, 2010). In response to damage, neurons can generate inflammatory mediators which activate microglia and astrocytes. As mentioned previously, see the section "The roles of NOX enzymes in the healthy nervous system", NOX enzymes regulate the activation of microglia and several of the microglial functions supporting neuronal survival during stress (Block et al., 2007; Verkhratsky and Parpura, 2010). However, if the stress persists over a long time period, microglia can be over-activated or primed, and rather than support neurons cause additional neuronal damage by release of large amounts of neurotoxic substances (Block et al., 2007; Kettenmann et al., 2011). NOX1, NOX2, as well as NOX4 activity has been implicated in shifting the microglia into a primed state (Li et al., 2009; Choi et al., 2012).

These NOX enzymes have also been implicated in controlling the production and release of many of the neurotoxic substances including nitric oxide (NO), glutamate and pro-inflammatory cytokines, like TNFα, by primed microglia (Pawate et al., 2004; Barger et al., 2007; Chéret et al., 2008; Harrigan et al., 2008; Li et al., 2009). Miss-regulation of NOX enzymes in microglia, could hence not only result in continuous NOX activation and release of high levels of ROS, but also release of many other neurotoxic molecules resulting in propagation of the neurotoxicity, see Fig. 1.

Besides causing oxidative damage in neurons, the ROS released by the primed microglia could also cause neurotoxicity via oxidative damage to astrocytes (Markowitz et al., 2007; Barber and Shaw, 2010). Astrocytic glutamate transporters remove glutamate from the synaptic cleft and are hence essential regulators of glutamatergic neurotransmission. These glutamate transporters are, however, highly sensitive to oxidative damage and reduced levels have been reported in several neurodegenerative diseases, for review see (Markowitz et al., 2007; Barber and Shaw, 2010). This reduction resulting in elevated levels of synaptic glutamate have been suggested to cause glutamate receptor over-activation and excitotoxicity in neurons (Markowitz et al., 2007; Barber and Shaw, 2010). Altogether, this creates a vicious cycle resulting in a progressive worsening of the neurodegeneration, see Fig. 3.

The involvement of microglial NOX and this cycle in different neurodegenerative diseases have been shown by multiple studies. Increased microglia NOX activity has, for instance, been shown in brains of AD, PD as well as ALS patients (Shimohama et al., 2000; Wu 2003; 2006). Furthermore, neuronal toxicity by activated microglia and astrocytes has been shown in coculture models of both AD and PD (Abramov et al., 2004; Zhang et al., 2005; Qin et al., 2006; Zhu et al., 2006). Moreover, deletion of NOX2 was shown to abate the microglial activation and killing of neurons in both AD and PD models (Wu et al., 2003; Qin et al., 2006). Several mutant proteins/peptides, associated with ALS, PD and AD, have also been shown to increase microglial NOX levels and activity. For instance, both extracellular α-synuclein, (a protein mutated in certain familial forms of PD), and Aß peptide, (the prime pathogenic mediator of AD), have been shown to activate microglia and microglial NOX (Bianca et al., 1999; Zhang et al., 2005). In microglia, Aß has been shown to activate NOX via the scavenger receptor CD36 (Coraci et al., 2002) and stimulation of cPLA (Szaingurten-Solodkin et al., 2009). Aß has also been suggested to activate the MAC1 receptor resulting in increased PI3K phosphorylation of p47phox and NOX2 activation (Zhang et al., 2011). In models of ALS, expression of ALS-causing mutant SOD1 protein in microglia has been shown to accelerates disease progression (Boillée et al., 2006) and increase the neurotoxic potential of microglia via increased NOX-mediated ROS production (Liu et al., 2009). Taken together these studies clearly indicate an

important role of microglial NOX in neurodegenerative diseases.

Inhibition of NOX as a therapeutic strategy in neurodegenerative disease

Deletion of NOX activity by genetic manipulations in animal models of ALS and AD has indicated a great promise for reduction of NOX activity as a therapeutic strategy in neurodegenerative diseases (Wu et al., 2006; Marden et al., 2007; Park et al., 2008). However, usage of chemical NOX inhibitors in animal disease models has so far given variable results, for extensive review see (Gao et al., 2012; Sorce et al., 2012). Currently, most studies have used apocynin, a natural organic compound, reported to inhibit the activity of NOX2 by preventing p47phox and p67phox translocation (Stolk et al., 1994). Positive effects of this compound have been reported in a quinolinic acid model of Huntington's disease (Maldonado et al., 2010), a paraquat-induced model of PD (Cristóvão et al., 2009) and in one study of G93A ALS mice (Harraz et al., 2008). However, another study using the same ALS mouse model and protocol could not reproduce the positive effect of apocynin (Trumbull et al., 2012). In that study they could, however, see a mild effect of diapocynin, which is considered to be the active form of apocynin (Trumbull et al., 2012). Apocynin treatment has also failed to improve behavioral, learning and memory deficits in several AD mice models (Dumont et al., 2011; Lull et al., 2011). Ibuprofen, suggested to inhibit NOX activation, did however lead to reduction in oxidative damage and plaque burden in one AD model (Wilkinson et al., 2012).The failure of apocynin to show as beneficial effects as genetic deletion of NOX is most likely due to issues of specificity. Recently, apocynin was shown to act more like an anti-oxidant than a NOX inhibitor (Heumüller et al., 2008). As mounting evidence is also implicating other NOX isoforms, like NOX1 and NOX4 in neurodegeneration, the weak results of apocynin could also be due to the lack of inhibition of these NOX isoforms. Hence, future studies to identify and test more specific NOX isoform inhibitors are clearly needed.

Concluding remarks and future perspectives

Taken together accumulating evidence indicate an important role of NADPH oxidase enzymes in neurodegenerative diseases, with miss-regulation and over-activation of NOX enzymes in neurons, as well as microglia, contributing to pathogenic processes. Over-activation of NOX enzymes in neurons have been linked to oxidative damage and apoptosis in neurons. Whereas sustained NOX activation in microglia have been linked to neuroinflammation and release of toxic molecules which accelerates and propagate the neuronal damage. However, the mechanisms by which NOX activity is dys-regulated in these different cell types in neurodegenerative diseases are still largely unclear. Furthermore, most studies have, so far, focused on NOX2, and very little is known about the role of other NOX enzymes reported to be present in cells of the nervous system. Especially more studies of NOX4 would be of interest, as NOX4 rather than NOX2 was shown to be increased in some AD models (Bruce-Keller et al., 2011) and genome wide association studies found a close link between NOX4 and ALS disease (Dunckley et al., 2007). Future efforts should also focus on development of more specific NOX inhibitor as these would not only be valuable research tools, but might also potentially be used as therapeutics.

Compliance with ethics guidelines

Abiodun Ajayi, Xin Yu and Anna-Lena Ström declare that they have no conflict of interest. This article does not contain any studies with human or animal subjects performed by any of the authors.

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