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REVIEW ARTICLE THEMED ISSUE

NADPH oxidases in oxidant production by microglia: activating receptors, pharmacology and association with disease

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Microglia are the resident immune cells of the CNS and constitute a self-sustaining population of CNS-adapted tissue macrophages. As mononuclear phagocytic cells, they express high levels of superoxide-producing NADPH oxidases (NOX). The sole function of the members of the NOX family is to generate reactive oxygen species (ROS) that are believed to be important in CNS host defence and in the redox signalling circuits that shape the different activation phenotypes of microglia. NOX are also important in pathological conditions, where over-generation of ROS contributes to neuronal loss via direct oxidative tissue damage or disruption of redox signalling circuits. In this review, we assess the evidence for involvement of NOX in CNS physiopathology, with particular emphasis on the most important surface receptors that lead to generation of NOX-derived ROS. We evaluate the potential significance of the subcellular distribution of NOX isoforms for redox signalling or release of ROS to the extracellular medium. Inhibitory mechanisms that have been reported to restrain NOX activity in microglia and macrophages *in vivo* are also discussed. We provide a critical appraisal of frequently used and recently developed NOX inhibitors. Finally, we review the recent literature on NOX and other sources of ROS that are involved in activation of the inflammasome and discuss the potential influence of microglia-derived oxidants on neurogenesis, neural differentiation and culling of surplus progenitor cells. The degree to which excessive, badly timed or misplaced NOX activation in microglia may affect neuronal homeostasis in physiological or pathological conditions certainly merits further investigation.

Abbreviations

CR3, complement receptor 3; DPI, diphenylene iodonium; HMGB1, high mobility group box 1; JAK, Janus kinase; NAC, N-acetylcysteine; NLRP3, NOD-like receptor family, pyrin domain containing 3; SVZ, subventricular zone; TLR, toll-like receptor

Tables of Links

TARGETS	
Catalytic receptors[a]	**Ligand-gated ion channels**[c]
CD11b (integrin αM)	NMDA receptor
CD18 (integrin β2)	P2X7 receptor
NLRP3, NOD-like receptor family, pyrin domain containing 3	**Enzymes**[d]
TLR2	Akt
TLR4	ERK1/2
GPCRs[b]	Haem oxygenase 1
$mGlu_3$ receptor	Inducible NO synthase
$mGlu_5$ receptor	Lyn
$P2Y_2$ receptor	p21-activated kinase 1 (PAK1)
$P2Y_4$ receptor	p38

LIGANDS
(RS)-2-chloro-5-hydroxyphenylglycine
ATP
Aβ, amyloid β
H_2O_2
IL-1β
IL-6
IL-10
IL-18
Rac1
TGFβ

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2015/16 ([a,b,c,d] Alexander *et al.*, 2015a,b,c,d).

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Introduction

Microglia are the tissue-specific macrophages of the CNS, and unlike other brain cells, they derive from yolk sac haematopoietic stem cells, which populate the mouse brain around embryonic day 9 (Alliot *et al.*, 1999; Ginhoux *et al.*, 2010; Kierdorf *et al.*, 2013). Once established in the brain parenchyma, the population of CNS microglia is maintained throughout life by proliferation when needed, which occurs independently of bone marrow-derived precursors (Prinz and Priller, 2014; Ajami *et al.*, 2007; Elmore *et al.*, 2014; Bruttger *et al.*, 2015).

Since they were first identified by Pio del Rio-Hortega in 1920, most research has concentrated on the neuropathological associations of microglia (Kettenmann and Verkhratsky, 2011). Many effector functions of microglia are potentially cytotoxic, and a substantial body of evidence links excessive activation of microglia to the neuroinflammation that accompanies many forms of acute or chronic neuropathology. Release of pro-inflammatory cytokines, arachidonic acid derivatives, excitatory neurotransmitters, proteinases and ROS may all contribute to neurodegenerative disease, if unchecked. In particular, ROS production by microglia is considered to be a major cause of neuronal dysfunction, damage and death (Block *et al.*, 2007; Gao *et al.*, 2012) through direct oxidative damage to neuronal macromolecules (Wu *et al.*, 2006; Rojo *et al.*, 2014) or derangement of neuronal redox signalling circuits.

On the other hand, microglia have a vital role in cell survival. In common with other tissue-specific macrophages, such as skin Langerhans cells and spleen red pulp macrophages (which microglia most resemble) (Butovsky *et al.*, 2014), they have the same basic functions as immune surveillance cells, namely, clearance capabilities and maintenance of local homeostasis (Casano and Peri, 2015). Indeed, microglia are now recognized as being extremely plastic, versatile and multifunctional cells that, importantly, play key roles in the healthy brain (Hanisch and Kettenmann, 2007; Saijo and Glass, 2011; Prinz and Priller, 2014; Casano and Peri, 2015). Microglial processes constantly survey the parenchyma for pathogens and debris from damaged or dying cells and remodel and maintain the local environment (Davalos *et al.*, 2005; Nimmerjahn *et al.*, 2005). In the developing brain, microglia are responsible for clearance of apoptotic cells, neurogenesis and axonal growth, synaptic pruning (synaptic refinement) and vessel patterning (Casano and Peri, 2015). It is also increasingly recognized that regulated oxidant generation by the family of NADPH oxidases (NOX; Sorce and Krause, 2009; Lambeth and Neish, 2014; Nayernia *et al.*, 2014) contributes to cell homeostasis through the regulation of key redox-dependent pathways (Holmstrom and Finkel, 2014). For example, H_2O_2 modulates transcription factor activity, cytoskeleton dynamics, ion channel activity, receptor activation and tyrosine kinase cascades through reversible oxidation of cysteines with low pKa values in target proteins (Go *et al.*, 2015; Holmstrom and Finkel, 2014). Regulated H_2O_2 release to the surroundings is conceivably also involved in the paracrine modulation of redox sensitive targets in neighboring neurons or glia cells.

In this review, we discuss the state of the art of oxidant production in microglia, emphasizing the critical role of NOX and the sensory receptors that regulate NOX activity, or are subjected to NOX-generated redox signalling, in the healthy or diseased brain. Because of the (still) limited literature on the subject in microglia, we rely in part on lessons learned from macrophages and, to a lesser extent, from mesenchymal cell types.

Oxidant production in microglia

With the exception of H_2O_2, which is not a free radical, ROS are small molecules or ions characterized by the presence of unpaired electrons (radicals). In biological settings, they are generated either in a regulated manner from specific enzymes, such as NOX, or as by-products or end-products of oxidative metabolism.

The discovery of NOX family members as enzymes with the sole purpose of generating superoxide ($O_2^{\cdot-}$) and H_2O_2, together with the recognition of widespread regulatory redox modification of target proteins (Herrmann and Dick, 2012; Go *et al.*, 2015), has led to the concept that ROS form part of a highly complex and sophisticated regulatory system, referred to as the Redox Code (Jones and Sies, 2015). Both $O_2^{\cdot-}$ and H_2O_2 play important roles in cell signalling as second messengers (see Holmstrom and Finkel, 2014). Antioxidants act as a counterbalance to ROS, and in microglia, they both regulate redox signalling and oppose the toxicity associated with free radical production. The accompanying review (Vilhardt *et al.*, this issue) provides a detailed analysis of the role of antioxidant systems in microglia.

NADPH oxidase (NOX)

NOX is by far the most important source of oxidants in cells, but mitochondria may also produce oxidants under certain conditions. Discussion of other cellular sources of oxidants is beyond the scope of this review, but we refer to a recent review for details (Casas *et al.*, 2015).

The NOX family consists of seven enzymes, NOX1–5 and DUOX1–2, all of which generate $O_2^{\cdot-}$, or in the case of NOX4, H_2O_2 (Lambeth, 2004; Bedard and Krause, 2007). NOX2 (also known as gp91phox) is highly expressed in microglia of both humans and rodents (Sorce *et al.*, 2014) and, to a differing degree, in other CNS cell types including neurons and neuronal stem cells (Nayernia *et al.*, 2014). Expression of NOX1 and NOX4 has been documented in microglia, but the results are less clear-cut, because of a lack of specific antibodies. NOX1, NOX2 and NOX4 are similar in size and domain structure (Lambeth, 2004). They consist of a short cytosolic N-terminal domain followed by six transmembrane domains, where two haem prosthetic groups are contained, and a long C-terminus that has flavin adenine dinucleotide (FAD) and NADPH binding sites. A membrane-bound flavocytochrome b_{558} complex containing subunits p22phox and NOX2 (gp91phox) together with cytosolic subunits constitutes the phagocyte NADPH oxidase.

When activated, gp91phox abstracts electrons from cytosolic NADPH and shuttles them through the membrane for the one-electron reduction of molecular oxygen on the other (luminal or extracellular) side of the membrane to generate $O_2^{\cdot-}$. The NOX2 complex consists of additional cytosolic regulatory proteins p40phox, p47phox and p67phox and the

small GTPase Rac1 (Figure 1). Translocation of these subunits to flavocytochrome b_{558} in the membrane is necessary for ROS-generating NOX activation, as p67phox and Rac1 in tandem regulate the electron flow from NADPH to FAD (Diebold and Bokoch, 2001). Rac1/2 is mobilized separately from the cytosolic phox proteins but is available at the membrane following release from Rho-GDI (DerMardirossian *et al.*, 2004) and interaction with GDP/GTP exchange factors such as VAV1 (Roepstorff *et al.*, 2008). In microglia, the rate-limiting step in NOX2 activation is the critical serine (and threonine) phosphorylation of p47phox (Roepstorff *et al.*, 2008). This is catalysed by kinases that are activated downstream of cell surface receptor stimulation and include PKC isoforms, p21-activated kinase 1, Akt, p38MAP kinase and ERK1/2 (El-Benna *et al.*, 2009).

In general, mitochondrial production of ROS (complexes I and III of the electron transport chain are the main sites of O_2^- production) is orders of magnitude smaller than the oxidant output generated by NOX enzymes and is not associated with the extracellular release of ROS (Brown and Borutaite, 2012). Nonetheless, it has been hypothesized that mitochondrial and NOX-mediated ROS production may be functionally linked, particularly in the stimulation of microglial activation by pro-inflammatory mediators. A discussion of the association is the subject of an excellent critical review by Bordt and Polster (2014).

Subcellular localization of NOX in microglia

The site of oxidant production is important because of the differing membrane permeability of the various ROS, coupled with their varying susceptibility to neutralization by the cytosolic reducing environment. Thus, the precise subcellular localization, timing and nature of the ROS generated are essential for biological function. The chemical reactivity of the different ROS also has an important bearing on their potency. For example, in the presence of transition state metals such as iron, O_2^- and H_2O_2 form the highly reactive and destructive hydroxyl radical ($^{\cdot}OH$) in the well-known Haber–Weiss reaction (Figure 1B). By this mechanism, O_2^- reduces free iron to the Fe^{2+} form (Fenton reaction) that, in turn, oxidises H_2O_2 to $^{\cdot}OH$. This chain reaction may have marked effects in specific brain regions with high concentrations of iron, such as the substantia nigra (Youdim *et al.*, 1991), and may explain the particular vulnerability of dopaminergic neurons to oxidative stress (Qin *et al.*, 2004). The reaction product of O_2^- and NO, peroxynitrite anion ($ONOO^-$) is also highly reactive, and in many instances, the oxidative toxicity experienced by neurons and glia cells depends on the combined production of NO and O_2^- (Wang *et al.*, 2004; Li *et al.*, 2005).

It is generally assumed that H_2O_2 and $ONOO^-$ generated in the cytosol can cross the plasma membrane by diffusion

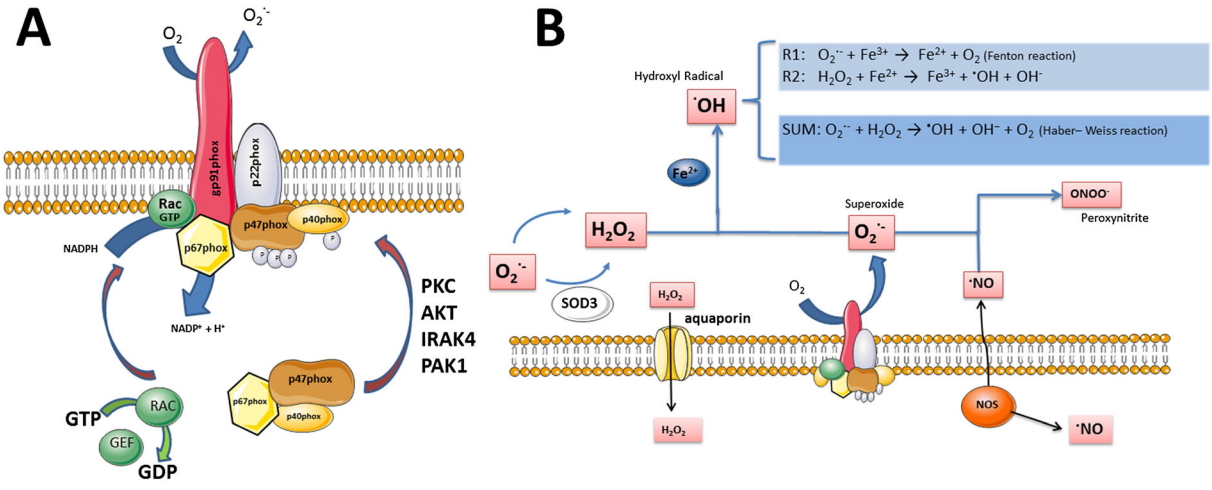

Figure 1

The phagocyte NADPH oxidase (NOX2). (A) Assembly of the phagocyte NADPH oxidase consisting of the integral flavocytochrome b_{558} complex [cyt b_{558}; composed of gp91phox (NOX2) and p22phox subunits] and cytosolic subunits p40, p47 and p67phox and the small GTPase Rac1. Of the cytosolic subunits, p67phox and Rac1 are catalytic, while p40phox and p47phox serve to guide, position and retain p67phox interactions with cyt b_{558} in the membrane. In resting cells, binding motifs contained within p47phox (and p40phox) for interaction with PIP_3 in the membrane and p22phox are shielded by the so-called auto-inhibitory region. However, upon multiple serine and threonine phosphorylations by activating kinases, the auto-inhibition is released, and either p47phox or p40phox in a ligand/receptor-specific manner transports p67phox to cyt b_{558} in the membrane. Rac1 is activated by release from RhoGDI and subsequent nucleotide exchange by a GTP/GDP exchange factor (GEF). Rac1 in concert with p67phox mediates electron transfer from NADPH to the redox centers of gp91phox (FAD and haem) and finally to molecular oxygen on the extracellular side of the membrane to form O_2^-. (B) NOX2-derived oxidant reactions. The membrane permeability of O_2^- is low, and release of oxidants to the extracellular environment therefore requires that NOX2 is localized to the cell surface. Released O_2^- quickly dismutates to H_2O_2 either spontaneously or through the action of extracellular SOD (SOD3). In the presence of free iron, H_2O_2 and O_2^- can react to form the highly reactive hydroxyl radical $^{\cdot}OH$, while O_2^- in the presence of NO can form the similarly neurotoxic peroxynitrite ($ONOO^-$). A small fraction of extracellularly produced H_2O_2 can diffuse into the cytosol either directly through the membrane or via aquaporin channels, to alter the activity of redox targets.

through aquaporins (Bienert and Chaumont, 2014) or anion channels. The membrane permeability of O_2^- and ·OH is low, and a substantial release to the surroundings requires that NOX is localized to the cell surface.

In resting macrophages and microglia, NOX2 is present on the plasma membrane, but following cell activation, it is redistributed (partly or fully depending on species) by clathrin-mediated endocytosis to an intracellular, agonist-regulated storage compartment consisting of numerous small (<100 nm) vesicles (Figure 2) (Ejlerskov *et al.*, 2012). When resident on the cell surface, NOX2 associates with lipid rafts (glycosphingolipid and cholesterol-enriched microdomains) in microglia and other cell types (Vilhardt and van Deurs, 2004). These domains may form a platform for NOX2 mobility and signalling. In contrast, NOX1 in microglia is contained in intracellular vacuoles of undefined, but perhaps lysosomal, nature (Cheret *et al.*, 2008). There are very few reports of NOX4 expression in microglia, and all refer to identification of mRNA, rather than the protein (Harrigan *et al.*, 2008; Li *et al.*, 2009a; Mead *et al.*, 2012). However, in monocyte-derived macrophages, NOX4 has been detected at the protein level and appears to occupy an intracellular compartment of small vesicles (Lee *et al.*, 2013b). NOX4 does not seem to be required for microglia activation *in vivo* (Kallenborn-Gerhardt *et al.*, 2012) or for expression of most pro-inflammatory genes *in vitro* (Li *et al.*, 2009a).

Knowledge of the cellular sorting machinery that governs localization and agonist-regulated distribution of the NOX is fragmentary. A hierarchy of undefined sorting signals is presumed to regulate NOX trafficking (Helmcke *et al.*, 2009; von Lohneysen *et al.*, 2010). In microglia, together with neutrophils, dendritic cells and macrophages, the small GTPase Rab27A/B determines trafficking of NOX2-associated organelles to phagosomes containing IgG-opsonized targets (Jancic *et al.*, 2007; Anderson *et al.*, 2011; Ejlerskov *et al.*, 2012). Additionally, in microglia, Rab27 is required to sequester the NOX2 complex in intracellular vesicles away from the cell surface (Ejlerskov *et al.*, 2012). In other cell types, Ras GTPase-activating-like protein (IQGAP1) is a tethering factor for NOX2 at the plasma membrane (Ikeda *et al.*, 2005). In many instances, stimulation of phagocytes does not lead to overt NOX activation, but rather primes NOX for enhanced oxidant production following stimuli that directly activate the oxidase. A part of this priming derives from the shuttling of NOX2 from internal stores to the cell surface (Ward *et al.*, 2000; Uriarte *et al.*, 2011; Ejlerskov *et al.*, 2012).

Receptor-mediated NOX activation in microglia

Microglia are endowed with an enormous array of sensing receptors (Hanisch and Kettenmann, 2007; Hickman *et al.*, 2013) that guide each cell to assume an activation status that is finely tuned to the environment. In this section, selected examples of microglial surface receptors are described, in which NOX expression and ROS generation have been implicated in signalling and/or disease. For more detailed information on signalling pathways in microglia, the reader is referred to a recent review on immune cell receptor expression (Hu *et al.*, 2014).

Toll-like receptors (TLR)

The TLR family of pattern recognition receptors binds a variety of endogenous ligands that are relevant in innate immune responses. The TLRs mediate their effect through two arms of signal transduction, depending on the requirement for myeloid differentiation factor 88 (MyD88), leading to activation of MAPK and NF-kB and transcription of IFNβ genes. Microglia express most TLRs, and their expression levels are altered by microglia activation (Olson and Miller, 2004; Jack *et al.*, 2005; McKimmie and Fazakerley, 2005). TLRs contribute to neuropathology, because of association with disease-related molecules that include amyloid β (Aβ; Jana *et al.*, 2008), α-synuclein (Fellner *et al.*, 2012; Codolo *et al.*, 2013; Kim *et al.*, 2013), mutant SOD1 (Liu *et al.*, 2009; Zhao *et al.*, 2010), oxidized high-mobility group box protein 1 (Agalave *et al.*, 2014), oxidized phospholipids (Imai *et al.*, 2008), galectin-3 (Burguillos *et al.*, 2015) and gangliosides (Jou *et al.*, 2006). In many cases, NOX expression and oxidant

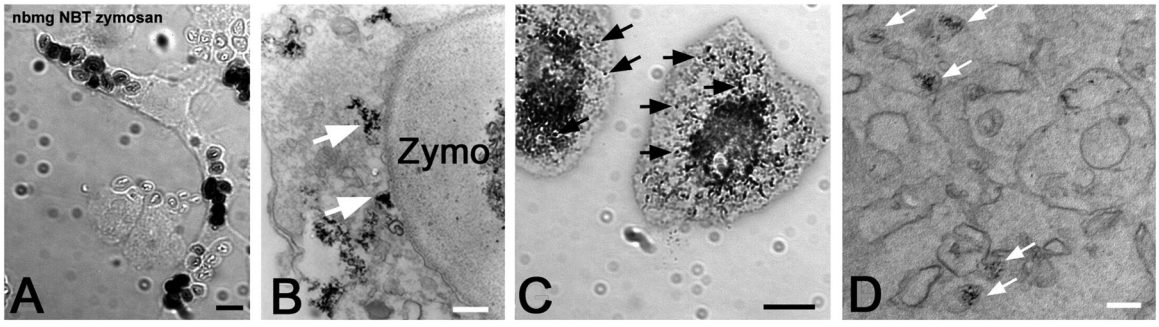

Figure 2

Microglia oxidant production in culture. Cytochemical reactions of primary rat microglia exposed to zymosan (A and B) or phorbol 12-myristate 13-acetate (C and D) in the presence of either NBT to measure O_2^- production (A and C) for light microscopy or the H_2O_2-sensitive $CeCl_3$ (B and D) for electron microscopy. Notice that oxidant production is mainly intracellular and resides with a population of small vesicles (arrows in C and D), which become mobilized for fusion with the phagosome containing zymosan (arrows in B). Bars A and C, 10 μm; B and D, 100 nm (unpublished results; F. Vilhardt).

production are up-regulated by these ligands (Imai *et al.*, 2008; Liu *et al.*, 2009; Zhao *et al.*, 2010; Fellner *et al.*, 2012; Codolo *et al.*, 2013). However, in some neuropathological conditions where genetic evidence points to a role for TLRs and oxidant production, the endogenous ligands remain unknown, for example, in cerebral ischaemia–reperfusion injury (Abe *et al.*, 2010; Suzuki *et al.*, 2012).

While TLR signalling is believed to set the level of oxidant production as a component of a general phagocyte activation programme, TLR activation induces a small, acute oxidant burst. It has been found that TLRs rely on NOX activity to initiate signalling. For instance, NOX-derived ROS directly regulate the partitioning of TLRs to lipid rafts in the membrane (Nakahira *et al.*, 2006; Wong *et al.*, 2009) or promote the assembly of signalling complexes (Matsuzawa *et al.*, 2005; Yang *et al.*, 2008), which are required for efficient signalling. In this respect, the direct physical interaction between TLR2 and either NOX1 or NOX2, and TLR4 and NOX4 in phagocyte and mesenchymal cell types may be important (Park *et al.*, 2004; Yang *et al.*, 2009; Suzuki *et al.*, 2012; Lee *et al.*, 2013a). Additionally, signalling through TLRs is important for priming (hyperresponsiveness) of NOX2. Thus, bacterial LPS induce the recruitment of cytosolic phox proteins to the membrane (DeLeo *et al.*, 1998) by the phosphorylation of Ser^{345} in p47phox (Dang *et al.*, 2006). Priming of NOX2 downstream of other cell surface receptors is often dependent on p38MAPK and ERK (El Benna *et al.*, 1996; Forsberg *et al.*, 2001); however, TLR signalling also activates IL-1 receptor-associated kinase 4 (IRAK4) in the MyD88-dependent TLR signalling axis. IRAK4 not only primes NOX but also phosphorylates p47phox on several residues to activate the oxidase directly (Pacquelet *et al.*, 2007), which correlates with the inability of IRAK4-deficient neutrophils to prime and activate NOX2 (Picard *et al.*, 2003), and the dependency of TLR-mediated NOX activation on the MyD88 signalling arm of TLRs (Laroux *et al.*, 2005). TLR engagement also leads to activation of GTP/GDP exchange factor VAV, which mediates nucleotide exchange on Rac1, a catalytic subunit of the NOX complex (Miletic *et al.*, 2007). In addition, Rac1 activated downstream of TLRs also activates p38MAPK, which may participate in mobilization of p47phox (Miletic *et al.*, 2007).

Complement receptor 3 (CR3; CD11b/CD18)

LPS-mediated activation of microglia has been used as a model in numerous studies of neuroinflammation. Infusion of LPS into the mouse brain causes wide-spread microglia activation, increased oxidant production and development of a Parkinsonian-like brain disease with loss of (selectively vulnerable) dopaminergic neurons (Gao *et al.*, 2002; Gao *et al.*, 2003a). Similarly, microglial activation and ROS production are features of *in vitro* models of Parkinson's disease (Gao *et al.*, 2003a; Kim *et al.*, 2007b; Rodriguez-Pallares *et al.*, 2008). Significantly, disease progression is inhibited in NOX2-deficient animals (Gao *et al.*, 2003b; Wu *et al.*, 2003; Hernandes *et al.*, 2013), which underlines the importance of ROS as a contributing factor in Parkinson's disease.

LPS is mostly a ligand for TLR4, acting in concert with CD14 and LPS binding protein. However, LPS stimulation of NOX2 activity in microglia mainly occurs through binding of LPS to complement receptor 3 (CR3) (Qin *et al.*, 2005b; Pei *et al.*, 2007). CR3 is composed of CD11b (integrin α_M) and CD18 (integrin β2) subunits. CD11b is a frequently used histopathological marker for microglia activation, and its expression is partly regulated by NOX2 and inducible NO synthase (Roy *et al.*, 2008). CR3 also acts as a phagocytic receptor for C3b/iC3b-opsonized targets including endogenous targets such as synapses (Schafer *et al.*, 2012) and neurites (Linnartz *et al.*, 2012). The correlation between CR3 ligation and NOX2 activation in neutrophils and other phagocytes, even in the absence of phagocytosis, is well established (Lofgren *et al.*, 1999; Serrander *et al.*, 1999). Integrins can signal on their own, but leukocyte integrins (β2 integrins) pair up with receptors such as DAP12 or FcγRs (Zhou and Brown, 1994) containing an immunoreceptor tyrosine-based activation motif, which direct Src kinase-mediated signal transduction (Linnartz and Neumann, 2013). Proximal signal transduction arms involve Syk, Vav and PLC, the latter supporting PKC activation through inositol trisphosphate and DAG release.

Furthermore, as in the case of TLR signalling, NOX2-derived oxidants are implicated in the redox regulation of signalling pathways downstream of CR3 ligation in macrophages, for example, MAPK and NF-kB activation (Zhou *et al.*, 2013).

In terms of pathology, an interaction between CR3 and fibrinogen is important in axonal damage and demyelination (Adams *et al.*, 2007). Part of this pathology is likely to derive from the CR3-induced and fibrinogen-induced activation of microglial ROS production (as measured by fluorescent probes by two-photon microscopy) (Davalos *et al.*, 2012). The high-mobility group box 1 protein (HMGB1) is normally not present in the CNS extracellular space but acts as a damage-associated molecular pattern protein in neuropathological states and binds to CR3 to elicit oxidant production from microglia (Gao *et al.*, 2011), leading to death of neurons. Binding of aggregated α-synuclein to CR3 also induces NOX-mediated oxidant release from microglia (Zhang *et al.*, 2005) (Figure 3), leading to a redox-mediated regulation of the non-receptor tyrosine kinase Lyn to evoke chemotaxis (Wang *et al.*, 2015c). It is worth noting that zebrafish phagocytes chemotactically respond to gradients of H_2O_2 released from transformed or damaged cells in living tissues (Niethammer *et al.*, 2009; Feng *et al.*, 2010). Interestingly, Lyn is an important redox sensor in this context and may be activated by both exogenous (Yoo *et al.*, 2011) or endogenously produced ROS (Wang *et al.*, 2015c) to effect chemotaxis. As yet, no direct correlate for this phenomenon has been discovered in the mammalian CNS.

Ionotropic and metabotropic purinergic receptors

Ionotropic P2X and metabotropic P2Y purinergic receptors are important for regulation of the microglial actin cytoskeleton, which controls various cellular functions, such as process motility, migration, pinocytosis and phagocytosis (Madry and Attwell, 2015). In microglia, the expression pattern of purinergic receptors differs significantly from that of peripheral macrophages (Hickman *et al.*, 2013). This suggests that microglia have adapted for specific perception of cell damage or messenger molecules secreted by neurons or

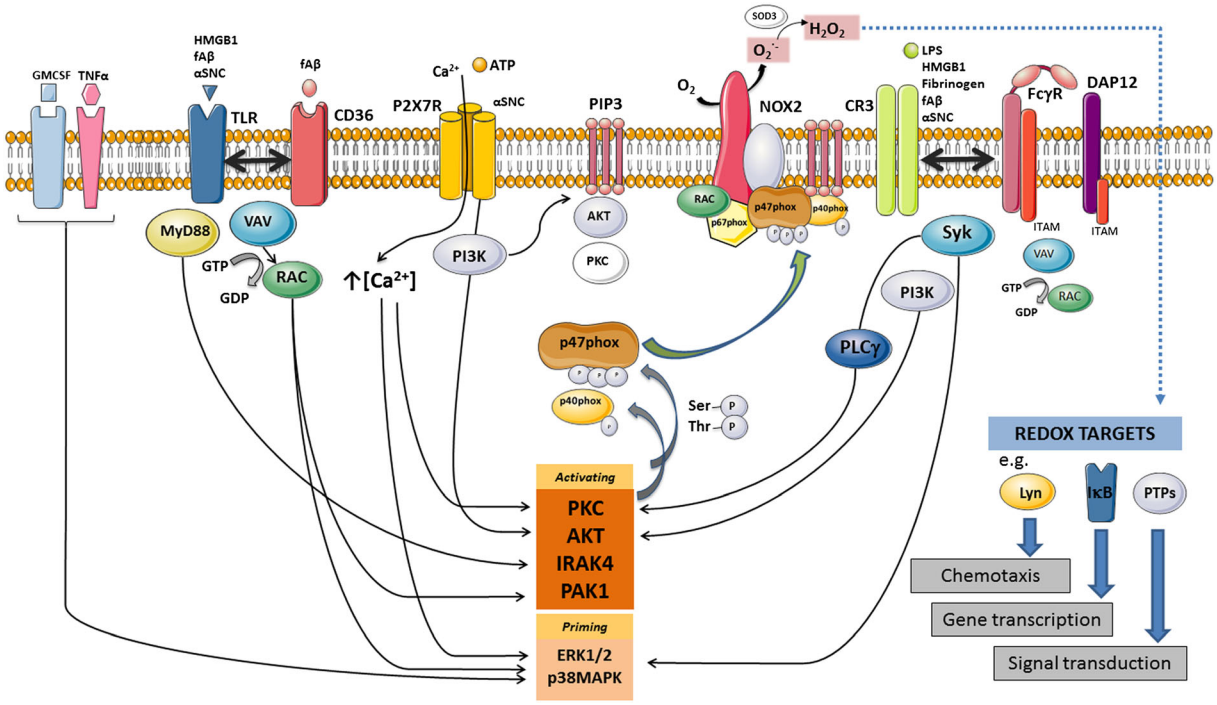

Figure 3

Activating microglia cell surface receptors, proximal signalling proteins and NOX2 activation by p47phox phosphorylation and Rac1 nucleotide exchange. Note that the figure is meant to organize activating surface receptors, their ligands and second messengers discussed in the review and that several important regulators of NOX activity have been omitted for clarity. The rate-limiting step for NOX2 activation in microglia is activation of cytosolic subunit p47phox by phosphorylation of a number or serine and threonine residues in an auto-inhibitory region of p47phox. Depending on the specific residues, phosphorylated p47phox can become 'primed' by different kinases including IRAK4, p38MAPK and ERK1/2, while full activation requires a number of residues to become phosphorylated by kinases such as PKC, Akt, IRAK4 or p21-activated kinase 1 (PAK1). In some instances, for example, following FcγR signalling, p40phox rather than p47phox phosphorylation is required for mobilization of p67phox to the membrane. PI3K phosphorylates PI lipids in the membrane to produce PIP_3, which serves as a recruitment factor of both regulatory proteins such as AKT and PKC isoforms, and certain GTP/GDP exchange factors, in addition to retaining mobilized p40phox and p47phox at the membrane through interactions with their PX domains. PLC contributes to activation by producing inositol trisphosphate, which increases cytosolic calcium levels, and DAG, which activates several PKC isoforms. In macrophages, GTP/GDP nucleotide exchange on Rac1 is performed by VAV isoforms. Released superoxide dismutates to H_2O_2 either spontaneously or through the catalytic activity of extracellular superoxide dismutase (SOD3) but can diffuse into the cytosol through the membrane either directly or through aquaporin channels (Hara-Chikuma *et al.*, 2015) to alter activity of cytosolic redox targets. In this kind of autocrine redox modification, extracellular catalase, but not SOD, deactivates the response (Pawate *et al.*, 2004; Mander *et al.*, 2006; Wang *et al.*, 2015c).

astrocytes. There is firm evidence that stimulation of the ionotropic P2X7 receptor by ATP induces ROS production and release in microglia. However, formal proof of an association between P2X7 and NOX activity has yet to be provided (Parvathenani *et al.*, 2003; Mead *et al.*, 2012).

Calcium entry is required for signalling and ROS production to occur following ATP stimulation in microglia and macrophages (Kim *et al.*, 2007a; Martel-Gallegos *et al.*, 2013). An increase in intracellular calcium also increases NOX activity through ERK1/2-dependent (Apolloni *et al.*, 2013; Martel-Gallegos *et al.*, 2013), p38MAPK-dependent and PI3K-dependent (Parvathenani *et al.*, 2003) pathways. Signalling through these kinases in macrophages is reinforced by NOX-derived H_2O_2 (Cruz *et al.*, 2007). *In vivo*, the source of ATP that stimulates microglial activation and ROS production could be derived from neurons or other glia but may also be provided in an autocrine fashion, because it is known that microglia secrete ATP in response to certain stimuli (Kim *et al.*, 2007a; Higashi *et al.*, 2011; Pascual *et al.*, 2012).

Activation of microglial purinergic receptors has also been implicated in neurodegenerative disease (Parvathenani *et al.*, 2003). Indeed, Aβ-mediated and α-synuclein-mediated activation of microglia is reduced in P2X7 (Parvathenani *et al.*, 2003; Kim *et al.*, 2007a; Jiang *et al.*, 2015) or P2Y receptor-deficient cells (Kim *et al.*, 2012). A role for NOX in this activation pattern has been proposed in several cases (Parvathenani *et al.*, 2003; Kim *et al.*, 2007a; Jiang *et al.*, 2015). For example, a direct association between α-synuclein and P2X7 receptor stimulation is thought to involve downstream signalling through PI3K, leading to NOX activation (Jiang *et al.*, 2015).

Activating neurotransmitter receptors

Microglia express a large number of neurotransmitter receptors in their surveying and activated states (Pocock and Kettenmann, 2007; Kettenmann *et al.*, 2013). However, the first studies that established NOX2 as the primary source of NMDA receptor-induced O_2^- production were performed on

cultured neurons and mouse hippocampus and neocortex (Girouard *et al.*, 2009; Brennan *et al.*, 2009). Later, it was observed that NOX was activated by agonists of glutamate metabotropic (mGlu3 and group III), $GABA_A$ and purinergic P2X7 or $mGlu_5$ receptors in the rodent BV2 microglial cell line (Mead *et al.*, 2012). A neuroprotective microglial phenotype was promoted by GABA and purinergic receptor stimulation, whereas activation of NOX by glutamate receptor agonists had the opposite effect. The results from a complementary study revealed that activation of microglia *in vitro*, following NMDA receptor stimulation, was accompanied by secretion of ROS (and pro-inflammatory cytokines) that was toxic to neurons (Kaindl *et al.*, 2012). It has also been shown that, in mesencephalic mixed cultures, substance P exerts neurotoxicity, mediated via microglial NOX activation (Block *et al.*, 2006). Moreover, dynorphin-derived peptides inhibit NOX2-mediated intracellular and extracellular ROS production following LPS stimulation of microglia (Qin *et al.*, 2005a). Overall, it is evident that disease-associated disruptions to neurotransmitter systems have a significant effect on the activation state of microglia and, consequently, neuronal integrity. Information on receptors that couple negatively to NOX is provided in the following section on NOX inhibition.

CD36

CD36 is a class B scavenger receptor that is essential for NOX-mediated oxidant production in microglia activated with Aβ (Bianca *et al.*, 1999; Coraci *et al.*, 2002). Further work revealed that CD36 on microglia engages Aβ in conjunction with cell surface receptors CD47 and integrin α6β1, which are all required for binding, signalling and oxidant production (Bamberger *et al.*, 2003). Downstream signalling involves tyrosine kinase activation (Bamberger *et al.*, 2003; Wilkinson *et al.*, 2006) of Rac1 GDP/GTP exchange factor VAV1 (Wilkinson *et al.*, 2006) to elicit NOX activation. However, CD36 also induces VAV–Rac1 signalling (and MAPK38 activation) and oxidant production following stimulation with Aβ in concert with TLR2 and TLR4 (Reed-Geaghan *et al.*, 2009). CD36 also acts as a co-receptor for TLR ligands in the setting of CNS ischaemic insults (Abe *et al.*, 2010; Park *et al.*, 2011) where CD36 is required for development of vascular oxidative stress (Cho *et al.*, 2005) although endogenous TLR ligands have not been defined.

NOX inhibition

Endogenous mechanisms

There are a number of physiological mechanisms that decrease NOX activity. TGFβ signalling opposes expression of inflammatory mediators in microglia (Paglinawan *et al.*, 2003) and induces a quiescent phenotype in microglia or recruited myeloid cells in organotypic hippocampal cultures (Abutbul *et al.*, 2012). However, only recently has the importance of TGFβ as a major determinant of both mouse and human microglial gene expression and physiology been appreciated (Butovsky *et al.*, 2014). To date, the correlation between the anti-inflammatory activity of TGFβ and NOX activity in microglia (Qian *et al.*, 2008) has not been explored. TGFβ mediates NOX2 inhibition through inhibition of Ser^{345} phosphorylation of p47phox (Qian *et al.*, 2008), a phosphorylation event, which is known to prime the NOX2 system and greatly increase ROS release to subsequent stronger stimuli (Dang *et al.*, 2006). IL-10, another anti-inflammatory compound, affords neuroprotection and reduces NOX2 activity by impeding JAK activation and mobilization of p47phox to the membrane following LPS challenge (Qian *et al.*, 2006).

An important antioxidative protein that is induced by alternative (IL-4) activation of microglia and macrophages is haem oxygenase 1, which degrades haem to free iron, biliverdin and CO. Carbon monoxide thus produced in turn inhibits NOX2 in macrophages, maybe by binding to gp91phox, and blunts LPS-induced inflammatory responses and other TLR signalling events, by preventing lipid raft recruitment of TLRs required for signalling (Nakahira *et al.*, 2006). Bilirubin formed from biliverdin in an additional step also inhibits NOX2 activity required for induction of inducible NO synthase expression in macrophages (Lanone *et al.*, 2005), while the haem degradation in itself is limiting for the incorporation into NOX (Taille *et al.*, 2004).

Recently, the membrane-bound factor Slamf8 was found to negatively regulate NOX2 activity in response to a large range of stimuli in murine macrophages (Wang *et al.*, 2012b). Slamf8 could be identical to the NOX2 repressive membrane factor previously described in dendritic cells (Elsen *et al.*, 2004). In Slamf8(−/−) macrophages, excessive phosphorylation of cytosolic subunit p40phox was observed, which correlates with an enhanced O_2^- production (Wang *et al.*, 2012b). On the other hand, Slamf1, through interaction with Beclin and PI3K, may enhance NOX2 activity in response to certain stimuli (Ma *et al.*, 2012). Reciprocal signalling through these two receptors to differentially regulate NOX activity was found to affect migration of myeloid cells (monocytes, macrophages and dendritic cells) *in vivo*. Moreover, in Slamf8 (−/−) cells, the accelerated migration rate was abolished in diphenyleneiodonium (DPI)-treated animals (Wang *et al.*, 2015a).

A number of studies highlight the association between glutamate stimulation of microglia and decreased NOX activity (Loane *et al.*, 2009). In microglia, $mGlu_3$ and $mGlu_5$ are the dominant metabotropic glutamate receptors expressed (Pocock and Kettenmann, 2007; Berger *et al.*, 2012), and of these, the $mGlu_5$ receptor is emerging as a potential target to modify microglial activation and prevent neuronal apoptosis in a number of experimental models of microglial cell activation. Studies of traumatic brain injury in mice or subarachnoid haemorrhage in rats have provided evidence that pharmacological activation of $mGlu_5$ receptors reduces microglial activation and promotes neuronal survival (Loane *et al.*, 2013; Zhang *et al.*, 2015). The results have been replicated using brain-permeable, positive allosteric modulators of $mGlu_5$ receptors with greater potency (Loane *et al.*, 2014; Xue *et al.*, 2014). Data suggest that the benefits of mGlu receptor activation derive from NOX inhibition (Loane *et al.*, 2013), because the selective mGlu receptor agonist, (RS)-2-chloro-5-hydroxyphenylglycine, reduced NF-κB activity and nitrite production in LPS-stimulated microglia but was ineffective in NOX2 deficient (gp91(phox−/−)) cultures. In complementary work, Chantong and colleagues conversely showed that blockade of $mGlu_5$ receptors using 2-methyl-

6-(phenylethynyl)-pyridine in BV-2 microglial cells induces a stress response characterized by increased intracellular ROS, mitochondrial ROS production and enhanced expression of inducible NOS and IL-6 (Chantong *et al.*, 2014).

Pharmacological inhibitors

Because of the involvement of NOX activity in a large array of diseases, there is considerable interest in developing suitable NOX inhibitors, in particular, for microglial NOX2 activity. A large number of potentially therapeutic molecules have been reported to act through NOX inhibition in microglia, including ligands that bind μ-opioid receptor (Liu *et al.*, 2000; Qin *et al.*, 2005a; Qian *et al.*, 2007b; Qian *et al.*, 2007c; Wang *et al.*, 2012c; Yang *et al.*, 2014), neurotransmitter receptors (Moon *et al.*, 2008; Hu *et al.*, 2012), other receptors (Liu *et al.*, 2003; Zhou *et al.*, 2008; Chechneva *et al.*, 2011; Chung *et al.*, 2012), ion channels (Li *et al.*, 2009b; Liu *et al.*, 2011) and general anti-inflammatory drugs (Colton and Chernyshev, 1996; Choi *et al.*, 2005; Huo *et al.*, 2011; Wang *et al.*, 2012a).

However, careful evaluation through a stringent flow chart involving the use of ROS-measuring probes, NOX-associated oxygen consumption and semi-recombinant assays (Jaquet *et al.*, 2009; Zielonka *et al.*, 2014; Hirano *et al.*, 2015) has so far validated only a handful of molecules with unequivocal NOX inhibitory activity. DPI inhibits all forms of NOX *in vitro* in the low micromolar range (Jaquet *et al.*, 2011), but its use *in vivo* is limited by high toxicity (Cooper *et al.*, 1988). DPI is a potent flavoprotein inhibitor, which non-specifically and irreversibly inhibits FAD-mediated electron transfer. It therefore inhibits other flavin-containing enzymes, such as NO synthase and mitochondrial cytochromes. However, recent studies indicate that almost homeopathic doses (subpicomolar concentrations) of DPI can exert neuroprotective effects in mixed neuron-glia cultures (Qian *et al.*, 2007a; Wang *et al.*, 2014) or in animal models of Parkinson's disease (Wang *et al.*, 2015b). DPI at these concentrations did not affect several cytosolic or mitochondrial flavoproteins (Wang *et al.*, 2014; Wang *et al.*, 2015b) but did reduce phorbol 12-myristate 13-acetate-induced and LPS-induced NOX2 activity and oxidative stress, as assessed by 4-hydroxynonenal staining. However, no stringent proof of a direct effect of DPI on NOX at these concentrations was provided.

Celastrol inhibits NOX activity *in vitro* (Jaquet *et al.*, 2011) but appeared inactive on NOX activity following *in vivo* administration (Hirano *et al.*, 2015). N-substituted phenothiazines show NOX inhibitory activity in the low micromolar range *in vitro* (Seredenina *et al.*, 2015) and cross the blood–brain barrier; however, similarly to celastrol, they contain highly promiscuous chemical structures and thus interfere with many pharmacological targets, making it difficult to distinguish NOX inhibitory activity from other pharmacological effects, such as dopamine receptor antagonism. The thioxo-dihydroquinazolin-one compound 43 has been forwarded as a validated potent NOX2 inhibitor (Zielonka *et al.*, 2014) but is more likely a myeloperoxidase inhibitor (Li *et al.*, 2015). Recently, a novel specific NOX2 inhibitor, GSK2795039, has been described (Hirano *et al.*, 2015). It shows efficacy *in vivo* and can penetrate into the brain. It remains to be determined whether GSK2795039 or other newly discovered NOX inhibitors have therapeutic utility in CNS disease through microglial NOX2 inhibition.

Apocynin is often referred to as an NOX inhibitor. It penetrates the CNS and has shown therapeutic benefit in numerous CNS disorders, possibly by inhibiting microglia activation (Sorce and Krause, 2009). However, although it may prove useful as a therapeutic agent, evidence that it acts as a *bona fide* NOX inhibitor is lacking (Gatto *et al.*, 2013). Apocynin more likely acts as an anti-inflammatory drug or an antioxidant (Heumuller *et al.*, 2008). Although antioxidant supplements have not reached therapeutic expectations (Schmidt *et al.*, 2015), novel therapeutic antioxidant molecules are emerging for CNS disease, with a mode of action possibly involving dampening of microglial oxidant generation. Treatment with the potent antioxidant edaravone in patients with stroke and cerebral infarction is used in some countries (Isahaya *et al.*, 2012; Kikuchi *et al.*, 2013) and may be useful in treatment of Parkinson's disease as well (Yuan *et al.*, 2008). With edaravone's effect attributed to its antioxidant properties, it seems strange that the effects on microglia NOX have not been directly investigated. N-acetylcysteine (NAC) is another antioxidant with documented experimental neuroprotective effects but is currently only in clinical use for acetaminophen intoxication and as a mucolytic agent. N-acetylcysteine amide is a modified compound with higher bioavailability, being able to cross the blood–brain barrier and the mitochondrial membrane and with a higher radical scavenging ability than NAC itself, that shows promising results and decreased neuronal degeneration and apoptosis following experimental penetrating traumatic brain injury (Gunther *et al.*, 2015), but again, this compound has not been tested directly on NOX activation in microglia.

NOX in inflammasome activation and neurogenesis

The involvement of NOX activity in microglia in chronic neurodegenerative diseases or acute brain disorders such as ischaemia–reperfusion syndrome has recently been carefully reviewed (Saijo and Glass, 2011; Gao *et al.*, 2012; Nayernia *et al.*, 2014). Here, we discuss the recent implication of NOX-derived oxidants in mechanisms relevant to neurological disease, namely, inflammasome activation and suppression of neurogenesis in the inflamed brain.

Inflammasome activation

The inflammasome is a multimeric protein conglomerate consisting of NOD-like receptor family, pyrin domain containing 3 (NLRP3), ASC/PYCARD, and caspase-1, which, when activated by diverse insults of both foreign and endogenous nature (Cruz *et al.*, 2007; Tschopp and Schroder, 2010; Mead *et al.*, 2012), cleaves the pro-form of IL-1β and IL-18 to their active form for release. Regardless of stimulus, inflammasome activation requires ROS generation (Tschopp and Schroder, 2010), calcium mobilization (Murakami *et al.*, 2012) and a decreased cytosolic K^+ concentration. α-synuclein aggregates trigger NLRP3-dependent activation and release of IL-1β in a ROS-dependent manner in microglia (Codolo *et al.*, 2013). Also extracellular challenge of microglia with amyotrophic lateral sclerosis mutants of 43 kDa TAR DNA binding protein up-regulates the expression of gp91phox concurrent

with inflammasome induction and IL-1β secretion, but no mechanistic coupling was attempted (Zhao *et al.*, 2015). The source of ROS for inflammasome activation has been debated for some time after the initial report of p22phox dependency (Dostert *et al.*, 2008). However, later and more thorough studies making use of macrophages derived from chronic granulomatous disease patients with different forms of NOX deficiency (including p22phox required for NOX1–NOX4) found IL-1β secretion to be undisturbed, or even enhanced (Meissner *et al.*, 2010; van Bruggen *et al.*, 2009; van de Veerdonk *et al.*, 2010). None of the calcium-activated NOX - NOX5 and DUOX1 and 2 - play a role in inflammasome activation (Rada *et al.*, 2014). Therefore, mitochondria, or other systems of oxidant generation, are likely to be the source of ROS for inflammasome activation (Zhou *et al.*, 2011). The exact role of ROS in the signalling cascade leading to inflammasome activation is unclear (Bauernfeind *et al.*, 2011), although the recent identification of specific ROS targets is an important step forward in this respect (Zhou *et al.*, 2010). Thioredoxin-interacting protein normally associates with thioredoxin, but following oxidative modification, it is released and binds to and activates NLRP3, thereby providing a link between ROS and inflammasome activation (Zhou *et al.*, 2010).

Neurogenesis

Microglia are present in the subventricular zone (SVZ) and the subgranular zone of the dentate gyrus of the hippocampus (Sato, 2015) where new neurons are born in the postnatal brain. Indeed, the specific recruitment of microglia to these zones by neuronal factors (Lelli *et al.*, 2013; Arno *et al.*, 2014) seems to indicate that microglia fulfil important roles (Ziv *et al.*, 2006; Ueno *et al.*, 2013; Shigemoto-Mogami *et al.*, 2014; Kizil *et al.*, 2015). Here, NOX2 activity is important in mediating the microglial chemotactic response downstream of colony stimulating factor-1 and VEGF receptor stimulation and in ensuring correct migration of microglia from lateral ventricles to SVZ (Lelli *et al.*, 2013).

Alluringly, although not uncontested (Forsberg *et al.*, 2013), it is assumed that neural progenitor cells maintain their potential for self-renewal and neurogenesis by oxidant production. This occurs in part by signalling through the PI3K/Akt pathway that is sustained by NOX2-mediated oxidant production (Le Belle *et al.*, 2011). This takes place potentially downstream of vascular cell adhesion molecule (VCAM) ligation (necessary to maintain the neurogenic niche in the SVZ), which increases NOX2 expression and oxidant production (Kokovay *et al.*, 2012).

Forebrain neurons that are still developing and maturing (relative to terminally differentiated forms) have a very low antioxidant defence, because of epigenetic down-regulation of neuronal Nrf2 (Bell *et al.*, 2015). In some cases, exogenous addition of H_2O_2 is sufficient to bring about alterations in self-renewal and differentiation of progenitors (Le Belle *et al.*, 2011). This naturally opens the question of whether activated microglia, via release of oxidants, could modulate either the maintenance of the neural stem cell population (Le Belle *et al.*, 2011) or the differentiation of progenitors (Forsberg *et al.*, 2013). In the $MPTP^+$-induced model of Parkinson's disease, reduced proliferation of neural progenitor cells correlates with the phase of maximal microglial activation. Moreover, *in vitro* experiments using co-cultures of progenitors with microglia suggested that (apocynin-sensitive) NOX-derived ROS is important in the inhibition of progenitor proliferation through activation of neuronal glycogen synthase kinase β3, which affects the wnt/β-catenin signalling pathway that is integral to neurogenesis (L'Episcopo *et al.*, 2012). However, the correlation between neurogenesis and microglial activation is far from clear-cut. Studies have shown that neurogenesis can be depressed (Ekdahl *et al.*, 2003; Monje *et al.*, 2003; Butovsky *et al.*, 2005; Su *et al.*, 2014; Kizil *et al.*, 2015), enhanced (Cacci *et al.*, 2008; Deierborg *et al.*, 2010; Kizil *et al.*, 2015) or unaltered (Heldmann *et al.*, 2011; Ng *et al.*, 2012) by microglia activation and neuroinflammation. Therefore, at present, the best defined role for microglia-derived ROS in neurogenesis is the physiological culling of brain Purkinje cells (followed by phagocytosis) (Marin-Teva *et al.*, 2004) or surplus neurons in the hippocampus (Wakselman *et al.*, 2008) during development, both processes being dependent on microglial NOX activity.

Open questions and conclusions

In the current literature, there are few attempts to separate the microglial NOX from that of neurons. There is no doubt that NOX2 is expressed at much higher levels in microglia than in nerve cells, with a correspondingly higher oxidant production when activated. However, the expression of NOX 2 has also been described in different neuronal populations, and its activation may cause cellular dysfunction and pathology that does not involve microglia (Nayernia *et al.*, 2014). Neuronal NOX should thus be given serious consideration as a source of oxidative stress. Because of potential overlapping expression patterns of NOX1, NOX2 and NOX4 in microglia, other glia cells and different neuronal populations (Nayernia *et al.*, 2014), it has not been possible by the use of currently existing global NOX knock-out mouse strains to precisely identify the cellular sources of oxidants, their exact physiological role and involvement in pathology. Moreover, in neuropathological states, it is impossible to know, from the current evidence, whether a 'surge' of microglial oxidants would be more detrimental to a nerve cell than a smaller, but misplaced or badly timed, production of oxidants from within the nerve cell itself. Generation of mouse models with either inducible expression of NOX2 on a p22phox(−/−) background or conditional knock out of NOX2 specifically in microglia could aid in the elucidation of these questions. Further, the use of biologically encoded ROS sensors specifically expressed in neurons or microglia by transgenics or viral transduction would allow (i) separation of the cellular sources of ROS, (ii) separation of microglia-mediated oxidative stress from other pro-inflammatory functions of microglia on neuronal function and survival (to the extent that ROS generation in the absence of other activating signals would not induce an M1 phenotype) and (iii) analysis of the potential of microglia-derived ROS to affect redox targets in the neuronal cytosol. Finally, as discussed at length in the accompanying review (Vilhardt *et al.*, this issue), it will be important to resolve microglia-fuelled oxidative stress into its components of direct oxidative damage to crucial neuronal proteins or

deranged redox signalling (Jones and Sies, 2015). Perturbation of redox signalling circuits could either take place in microglia themselves to forward a pro-inflammatory state or work in a paracrine mode to affect neuronal or cerebrovascular signalling networks either globally or locally.

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Conflict of interest

The authors declare no conflicts of interest.

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