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British Journal of Pharmacology

British Journal of Pharmacology (2016) •• ••–••

REVIEW ARTICLE THEMED ISSUE

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Received 3 October 2015; **Revised** 15 December 2015; **Accepted** 7 January 2016

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For many years, microglia, the resident CNS macrophages, have been considered only in the context of pathology, but microglia are also glial cells with important physiological functions. Microglia-derived oxidant production by NADPH oxidase (NOX2) is implicated in many CNS disorders. Oxidants do not stand alone, however, and are not always pernicious. We discuss in general terms, and where available in microglia, GSH synthesis and relation to cystine import and glutamate export, and the thioredoxin system as the most important antioxidative defence mechanism, and further, we discuss in the context of protein thiolation of target redox proteins the necessity for tightly localized, timed and confined oxidant production to work in concert with antioxidant proteins to promote redox signalling. NOX2-mediated redox signalling modulates the acquisition of the classical or alternative microglia activation phenotypes by regulating major transcriptional programs mediated through NF-κB and Nrf2, major regulators of the inflammatory and antioxidant response respectively. As both antioxidants and NOX-derived oxidants are co-secreted, in some instances redox signalling may extend to neighboring cells through modification of surface or cytosolic target proteins. We consider a role for microglia NOX-derived oxidants in paracrine modification of synaptic function through long term depression and in the communication with the adaptive immune system. There is little doubt that a continued foray into the functions of the antioxidant response in microglia will reveal antioxidant proteins as dynamic players in redox signalling, which in concert with NOX-derived oxidants fulfil important roles in the autocrine or paracrine regulation of essential enzymes or transcriptional programs.

Abbreviations

Aβ, amyloid-β peptide 1–42; DAMP, damage-associated molecular pattern molecules; GCL, glutamate cysteine ligase; GPx, GSH peroxidase; HNE, 4-hydroxy-2-nonenal; HO-1, haem oxygenase-1; KEAP1, Kelch-like ECH-associated protein; LTD, long-term depression; PLA_2, phospholipase A_2; Prx, peroxiredoxins; Trx, thioredoxin; TrxR, thioredoxin reductase; xCT, cystine–glutamate exchanger

Tables of Links

TARGETS	
Enzymes[a]	**Ligand-gated ion channels**[c]
ASK1	AMPA receptor
ERK	GluR1 (GluA1) receptor
HO-1, haem oxygenase 1	NMDA receptor
p38	**Other ion channels**[d]
PLA_2, phospholipase A_2	Aquaporin 3 (AQP3)
UCP2 (SLC25A8)	VRAC
Xanthine oxidase/ dehydrogenase	**Transporters**[e]
Catalytic receptors[b]	xCT, cystine/glutamate transporter; SLC7A11
TLR2	**Other proteins**[f]
TLR4	KEAP1

LIGANDS
Aβ, amyloid β peptide
DCPIB
GSH, glutathione
HNE, 4-hydroxy-2-nonenal
H_2O_2
IL-1β
IL-4
IL-6
IL-13
LPS
TGFβ
TNF-α

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,e,f] Alexander *et al.*, 2015a,b,c,d,e,f).

DOI:10.1111/bph.13426

Introduction

Microglia, the resident immune cells of the brain, are exquisitely sensitive cells, which through a large and unique repertoire of sensing cell surface receptors (Hickman *et al.*, 2013) responding to ligands of exogenous or endogenous nature (Kettenmann *et al.*, 2013) continuously survey the brain parenchyma for even the smallest deviation from homeostasis. When the fine, motile processes of microglia encounter a problem, microglia assume an activation phenotype adapted to the quick resolution of damage and return to homeostasis. If instigating insults cannot be resolved, chronic microglia activation ensues. The differing nature of insults that can be incurred is reflected in a continuum of microglial activation states, characterized by morphological and molecular changes (Town *et al.*, 2005). The prevailing terminology is based on that used for peripheral macrophages, which describes a resting state M0, and two activation states M1 (classical activation) and M2 (alternative activation) with further subdivisions (Cherry *et al.*, 2014; Hu *et al.*, 2015). We will refer to the activation states in the same way, although it should be realized that this reductionist approach seriously underestimates the true plasticity of microglia and other mononuclear phagocytes (Martinez and Gordon, 2014). The M0 state in microglia is characterized by expression of many genes related to neuronal function and development (Butovsky *et al.*, 2014), and these surveying microglia carry a molecular signature driven by TGFβ signalling, which sets them apart from other tissue macrophages (Hickman *et al.*, 2013; Butovsky *et al.*, 2014). Mixed, and temporally changing, M1 and M2 populations of microglia are most often induced by any CNS insult, and chronic pathological conditions are characterized by a lack of balance in the activation continuum. For instance, prolonged predominance of M2 activation hampers a sufficient immune response, whereas predominance of the M1 phenotype is thought to lead to excessive reactive oxygen species (ROS) production and neuroinflammation (Cherry *et al.*, 2014; Hu *et al.*, 2015).

The NOX family of NADPH oxidoreductases (NOX1-5 and DUOX1-2) is the main source of oxidants in most cells. Their regulated production of superoxide and hydrogen peroxide through the one electron reduction of molecular oxygen (Bedard and Krause, 2007) has been implicated as essential in different types of acute and chronic brain disease (Nayernia *et al.*, 2014). We refer to the accompanying review for a more detailed description of the NOX family in microglia (Haslund-Vinding *et al.*, 2016). First recognized for the role of the classical phagocyte NADPH oxidase (NOX2) in production of superoxide and derived oxidants in neutrophil-pathogen combat, NOX-produced hydrogen peroxide is now known to participate in redox regulation of a great number of different proteins through the transient and reversible oxidation of target protein cysteines to produce typically sulphenic acid or S-glutathionylated protein adducts (Winterbourn, 2013; Holmstrom and Finkel, 2014). Therefore, in addition to direct oxidative damage to CNS cell constituents, the concept of oxidative stress has been expanded to incorporate that unbalanced or inappropriately localized oxidant production can derange redox signalling mechanisms in an autocrine or paracrine manner (Sies, 2014; Jones and Sies, 2015). Although probably all cell types of the CNS express one or more NADPH oxidase isoforms (Nayernia *et al.*, 2014), microglia by far express the highest levels of NOX, in particular NOX2, the classical phagocyte NADPH oxidase (Table 1). Whether the NOX-generated oxidants are used for pathogen eradication or redox signalling purposes, it is important for microglia to have a sophisticated battery of antioxidant proteins (Table 1), which serve to keep the redox homeostasis of the cells, and to avoid excessive oxidative damage to constituent macromolecules. Also, astrocytes express many antioxidant proteins at high levels, and astrocytes are essential for the maintenance of the global redox balance in the CNS under normal (Schreiner *et al.*, 2015) or pathological conditions (Gan *et al.*, 2012).

Several non-cell-autonomous brain diseases of the neuropsychiatric spectrum have been identified, where genetic defects in microglia confer aberrant neuronal function, suggesting that derangement of the close reciprocal interactions between microglia and neurons is sufficient to precipitate disease based on perturbation of neuronal fine circuitry (Chen *et al.*, 2010; Maezawa and Lee-Way, 2010; Derecki *et al.*, 2012). Recent years have provided evidence for the direct molecular and even structural modification of neuronal synapses in the developing and mature CNS by microglia (Schafer *et al.*, 2012; Zhang *et al.*, 2014a), and microglia may also guide differentiation and axon outgrowth of maturing neurons (Squarzoni *et al.*, 2014). Reciprocal interactions between T-cells and different phagocyte compartments of the brain including microglia have been shown to modulate cognitive processes on a more global scale (Ziv *et al.*, 2006; Derecki *et al.*, 2010).

In the following, we will address how microglial oxidant production and associated antioxidants function physiologically to maintain and support the neuronal circuitry and communication with other CNS cell types and pathologically to cause oxidative damage and derangement of autocrine and paracrine redox signalling. In combination with the accompanying review (Haslund-Vinding *et al.*, 2016), we place the emphasis on the family of NADPH oxidases (NOX family) in oxidant production, and the GSH-thioredoxin antioxidant system required for the transient thiol modification of target proteins for signalling purposes. We refer the interested reader to a detailed review for discussion of other antioxidant systems in microglia (Dringen, 2005).

Microglial redox status

GSH in microglial cells

The habitual exposure of microglial cells to high ROS levels dictates that they have highly effective antioxidant defence systems to protect against oxidative damage. GSH (γ-glutamylcysteinyl glycine) is highly expressed in microglial cells (Chatterjee *et al.*, 1999; Hirrlinger *et al.*, 2000; Hollensworth *et al.*, 2000). The cytosolic concentration of GSH is typically in the region of 3 mM (Cooper and Kristal, 1997), of which less than 10% exists as oxidized GSH and equates to a GSH/GSSG redox potential of −230 mV. Extracellularly, the GSH/GSSG redox potential averages at the more oxidized value of −140 mV. Mitochondrial GSH accounts for 10–15% of the total cellular GSH pool, but the concentration is similar to that in the cytosol (Griffith and Meister, 1985).

Table 1

Expression of selected oxidant and anti-oxidant related genes from a (healthy) mouse cerebral cortex RNA transcriptome database (Zhang *et al.*, 2014b).

Genes	Microglia	Neuron	Astrocyte	Oligodendro.	Endothelium
Oxidant producers					
Cybb (NOX2)	◑	◔	○	○	○
Ncf1 (Neutrophil cytosolic factor 1)	●	◔	◔	◔	◔
Ncf2 (Neutrophil cytosolic factor 2)	◕	◔	○	◔	◔
Ncf4 (Neutrophil cytosolic factor 4)	◕	○	◔	◔	◔
Cyba (Cytochrome b-245)	●	◔	◑	◑	●
NOX1	○	○	○	○	○
Noxa1 (NADPH oxidase activator 1)	○	○	○	○	○
Noxo1 (NADPH oxidase organizer 1)	◑	◔	◔	◔	◑
NOX4	○	◔	○	○	◕
NOX3	○	○	○	○	○
Duox1 (Dual oxidase 1)	○	◔	○	○	○
Duoxa1 (DUOX maturation factor 1)	◑	◔	◔	◔	○
Duox2 (Dual oxidase 2)	○	○	○	○	◔
Duoxa2 (DUOX maturation factor 2)	○	◔	○	○	○
MPO (Myeloperoxidase)	◔	○	○	○	○
Xdh (Xanthine Oxidase)	◔	○	○	○	○
NOS1 (neuronal)	○	◕	○	○	◔
NOS2 (inducible)	○	○	○	○	◑
NOS3 (endothelial)	◔	◔	◔	○	◕
Transcription factors					
NFkβ1	◕	◑	◑	◔	◕
NFkβ2	◕	◔	◑	◑	◕
Nrf2 (Nfe2l2)	◕	◑	◕	◑	◕
Anti-oxidants					
SOD1 (Superoxide dismutase 1)	◕	◕	◕	◕	◕
SOD2	◕	◕	◕	◕	◑
SOD3	◔	◔	◑	◔	◕
Hmox1 (Haem oxygenase 1)	●	◑	◕	◑	◕
Hmox2	◕	◕	◑	◕	◕
Cat (Catalase)	◕	◕	◕	◕	◕
Prdx1 (Peroxiredoxin 1)	◕	◕	◕	●	●
Prdx2	◕	●	◕	◕	●
Prdx3	◕	◕	◕	◕	◕
Prdx4	◕	◕	◕	◕	◕
Prdx5	●	◕	◕	◕	◕
Prdx6	◕	◕	●	◕	◕
Txn1 (Thioredoxin 1)	◕	◕	◕	◕	◕
Txn2	◕	◕	◕	◕	◕
Gpx1 (Glutathione peroxidase 1)	●	◕	◕	◕	●
Gpx3	◑	◑	◑	◔	◔
Gpx4	◕	◕	◕	◕	◕
Gpx7	◔	◑	◕	◑	◕
Slc7a11 (Xc-transporter)	◔	◔	◕	◔	◑
Level of expression:	○	◔	◑	◕	●
FMPK-unit cut-off values	<0,1	>0,1-1	>1-10	>10-100	>100

The data are presented as FMPK-units (fragments per kilobase of exon per million fragments mapped), which is an expression of mRNA transcript abundance normalized for transcript length (the FMPK value indicates the number of expected fragments for each thousand bases for every $N/10^6$ fragments sequenced).

The GSH content of microglia decreases with increasing age, thus promoting an age-dependent increase in the vulnerability of microglia to oxidative stress (Njie *et al.*, 2012). On the other hand, docosahexaenoic acid, a natural anti-inflammatory agent, increases the total GSH content of microglial cells and enhances their antioxidant capacity to limit production of the pro-inflammatory cytokines, TNF-α and IL-6 (Pettit *et al.*, 2013).

GSH scavenges superoxide anion (O_2^-) and other ROS either directly, by coupling to oxidation to GSSG, or more rapidly, via enzyme-catalysed reactions. Such reactions include the GSH peroxidase (GPx)-catalysed oxidation of GSH and glutaredoxin (GRx)-mediated reduction of oxidized cysteine residues in proteins (Figure 1). Microglia have the highest activity of GPx amongst brain cells in both rat (Lindenau *et al.*, 1998) and human (Power and Blumbergs, 2009), and expression of the enzyme increases directly in response to oxidative stress or excitotoxin-mediated cell damage (Wang *et al.*, 2015).

Cysteine and GSH synthesis in microglia

Cysteine is the precursor to GSH, hydrogen sulphide (H_2S) and taurine, each of which has significant antioxidant, neuromodulatory or neuroprotective properties. Free cysteine readily oxidizes to its corresponding disulphide, cystine, but the intracellular reducing conditions favour cysteine. In the more oxidized extracellular environment, there is a 5:1 surplus of cystine over cysteine. Consequently, transport systems that import cystine, as opposed to cysteine, have particular significance in fuelling GSH synthesis in the brain. In microglia and astrocytes, cystine is substrate for the x_c^- cystine–glutamate exchanger (xCT; SLC7A11) (Bannai, 1984; Sato *et al.*, 1999; Bassi *et al.*, 2001; McBean, 2002).

GSH is consumed in the process of scavenging O_2^- and O_2^--derived ROS. GSH depletion may therefore be either a cause or consequence of oxidative stress-related brain disorders. The reciprocal relationship between NOX-derived O_2^- and GSH was explored by Won *et al.* using an *in vitro* rat model of ischaemia–reperfusion injury (Won *et al.*, 2015). A time-dependent decrease in GSH was prevented by blocking O_2^- production during reperfusion. Conversely, boosting GSH levels by supplying N-acetyl cysteine protects against oxidative stress and neuronal degeneration in this condition. The close relationship between NOX and GSH is reinforced by the observation that suppression of O_2^- formation following apocynin treatment led to a corresponding increase in GSH content. Furthermore, p47phox(−/−) mice, which cannot assemble a functional NOX2 complex, show no change in either ROS or GSH content in response to ischaemia–reperfusion (Won *et al.*, 2015). Mechanistically, it is not known whether NOX interacts directly with GSH in microglia, nor is there information on whether NOX regulates GSH synthesis at the level of glutamate cysteine ligase

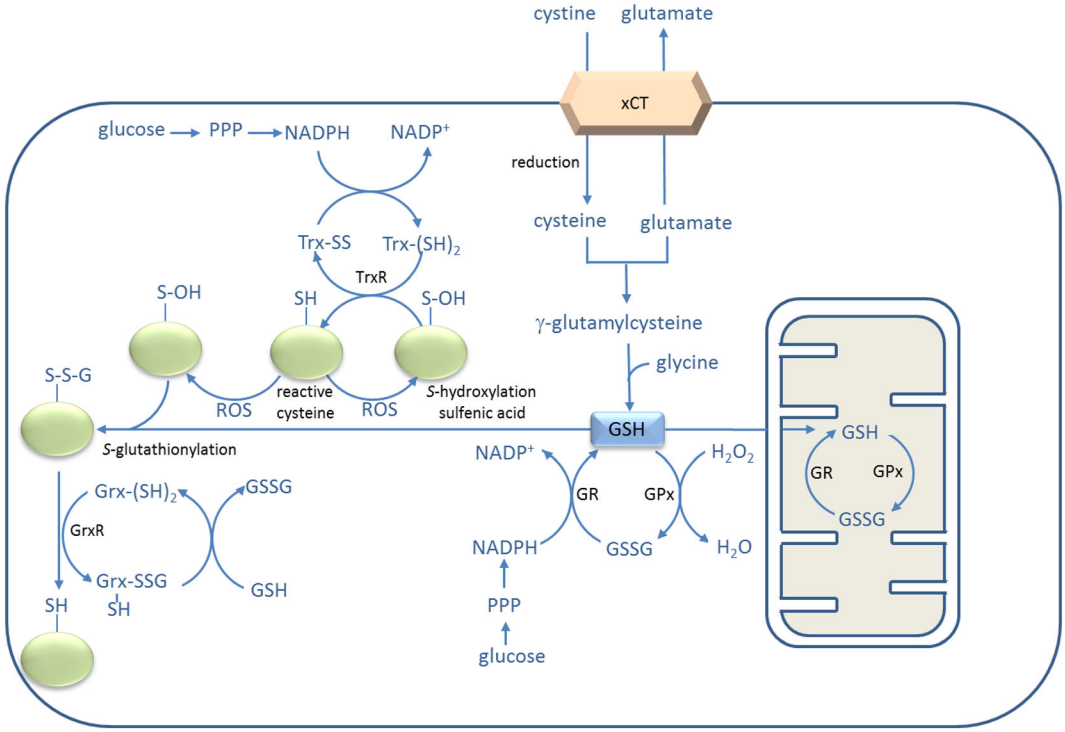

Figure 1

Thiol redox homeostasis in microglia. Cysteine for GSH is imported as cystine via the plasma membrane xCT exchanger. GSH is oxidized to GSSG during GSH peroxidase (GPx)-catalysed reduction of H_2O_2 in cytosolic and mitochondrial compartments. GSSG is substrate for GSH reductase (GR) that replenishes the GSH pool. Thioredoxin reductase (TrxR) reduces sulphenic acid derivatives of free thiol groups in proteins by coupling to oxidation of thioredoxin (Trx-$(SH)_2$). Alternatively, oxidized protein thiols may be S-glutathionylated by GSH, followed by reduction by glutaredoxin reductase (GrxR). The pentose phosphate pathway (PPP) supplies NADPH for reduction of GSSG and oxidized thioredoxin (Trx-SS). Grx-$(SH)_2$, reduced glutaredoxin; Grx-SSG-SH, oxidized glutaredoxin.

(GCL). However, there is evidence of a NOX4/PI3 kinase pathway regulating GCL activity and GSH production in endothelial cells (Pattillo *et al.*, 2010), and it remains to be determined whether microglial GCL is similarly regulated.

Cystine and glutamate

Transport of cystine by the xCT exchanger is matched by the outward flow of glutamate down its concentration gradient that provides the driving force for cystine import (Bannai, 1984; Sato *et al.*, 1999; McBean, 2002). Piani and Fontana were some of the first to identify xCT as a source of potentially toxic glutamate during macrophage activation (Piani and Fontana, 1994). More recently, the neurotoxic capacity of glutamate released via xCT has been verified *in vivo* (Kigerl *et al.*, 2012). Using a model of non-traumatic microinjection of a low dose of LPS into spinal cord grey matter, it was observed that neurotoxicity only occurs if cystine is co-injected with LPS. It was concluded that redox balance is controlled by induction of xCT and that a high GSH:GSSG ratio predicts the neurotoxic potential of activated brain macrophages/microglial cells.

Expression of the regulatory subunit of xCT is regulated by Nrf-2 and increases in response to oxidative stress (Sasaki *et al.*, 2002). Equally, Nrf-2 overexpression causes up-regulation of xCT and enzymes (GCL and GSH synthase) that catalyse formation of GSH (Shih *et al.*, 2003). On the down-side, up-regulation of xCT is linked to pathological release of microglia-derived glutamate that may be relevant to a number of neurological diseases. For instance, the HIV protein, Tat, increases xCT-mediated glutamate release from primary microglial cultures (Gupta *et al.*, 2010) in a process which is partly dependent on NOX-mediated oxidant production that increases following production of Tat protein (Turchan-Cholewo *et al.*, 2009). Likewise, incubation of rat cortical microglia with the amyloid peptide Aβ enhances xCT gene expression (Savchenko, 2013). These findings concur with the earlier observations of Barger and Goodwin (Barger *et al.*, 2007), who showed that microglial glutamate release occurs as a result of the oxidative burst and is viewed as a point of conversion of oxidative stress to excitotoxic stress. This concept has been strengthened by the fact that the Tat-induced release of glutamate was sensitive to inhibition by both apocynin and inhibitors of xCT (Gupta *et al.*, 2010). Additionally, microglial expression of xCT and glutamate export was recently found to be required for the CNS recruitment and infiltration of autoreactive T-cells in experimental autoimmune encephalitis (EAE) a widely used model of multiple sclerosis. Indeed, in xCT-deficient mice EAE did not develop (Evonuk *et al.*, 2015), indicating that the xCT system in microglia is important for the involvement of the adaptive arm of the immune system in CNS pathology.

Whilst the xCT is arguably the best documented mechanism of glutamate export from microglia, there are other avenues of glutamate release that may also be relevant in a pathological context, which include disruption to glutamine metabolism (Maezawa and Lee-Way, 2010; Jin *et al.*, 2015), volume-regulated anion channels (Harrigan *et al.*, 2008) and gap junction hemi-channels (Maezawa and Lee-Way, 2010) that offer potential targets for drug action. For example, DCPIB (4-(2-butyl-6, 7-dichloro-2-cyclopentyl-indan-1-on-5-yl) oxobutyric acid), a potent volume-regulated anion channel inhibitor, restricts microglial activation-related glutamate release, thus limiting neuronal injury during ischaemic conditions, both *in vivo* and *in vitro* (Han *et al.*, 2014).

Another effector of glutamate release from microglia is 4-hydroxy-2-nonenal (HNE), which is an end product of lipid peroxidation and one of the most abundant cytotoxic aldehydes (Zarkovic, 2003). As a strong electrophile, HNE forms adducts with proteins and, to a lesser extent, nucleic acids and phospholipids. Although toxic at high concentrations, HNE is also a reactive aldehyde that functions as a second messenger to regulate redox-sensitive proteins (Forman, 2010). The release of glutamate from LPS-activated microglia is stimulated by HNE and acrolein (another product of lipid peroxidation), thus increasing the likelihood of glutamate-mediated neurodegeneration (Barger *et al.*, 2007). In fact, elevated HNE has been identified in glia and neurons of the spinal cord of amyotrophic lateral sclerosis patients (Shibata *et al.*, 2001). Other actions of HNE include stimulation of phospholipase A2 in Ra2 microglia (Shibata *et al.*, 2011) and up-regulation of GCL, leading to increased GSH in response to oxidative stress in bronchial epithelial cells (Dickinson *et al.*, 2002).

The thioredoxin system

While the concentration of GSH may reach the millimolar range in the cytosol, the reaction with disulphides is slow, and thioredoxins and GSH peroxidases are the most important catalytic means of controlling GSH reactions. Thioredoxins (Trx) are proteins whose principal action is to reduce protein disulphides to free thiols. Trx1 is mostly cytoplasmic, but may also be found in the nucleus, plasma membrane and as a secreted protein. Trx2 is confined to the mitochondria. As an important regulator of thiol redox balance, Trx protects proteins by reducing oxidized cysteine residues. Trx is coupled to two thioredoxin reductases - TrxR1 which is cytoplasmic and and TrxR2, found in mitochondria - that use electrons provided by NADPH to recycle oxidized cysteines on Trx back to the reduced form. Other substrates for TrxRs include peroxides, notably hydrogen peroxide and protein disulphide isomerases. Expression of Trx and TrxR is increased in response to oxidative stress as a 'downstream' target of the binding of the transcription factor Nrf2 to the nuclear antioxidant response element in target cells. Trx operates in conjunction with peroxiredoxins (Prx) in reduction of H_2O_2 to H_2O. Prx, Trx and TrxR together form the so-called mammalian thioredoxin system that is essential for antioxidant defence and in preventing oxidative stress-related injury.

Little is known about the specific actions of Trx in microglial cells. However, several reports describe Trx activation in response to conditions that are known to cause microglial activation. These include LPS (Wang *et al.*, 2007), Aβ peptide (Zhang *et al.*, 2010) and IL-1β (Sharma *et al.*, 2007). In addition, it is known that Trx1 negatively regulates the HIV-encoded transcriptional activator, Tat, in macrophages (Kalantari *et al.*, 2008). siRNA knockdown of Trx1 increases HIV replication independently of NF-κB activation through targeting the two disulphide bonds in Tat that are

actively involved in transactivation. In other work, organoselenium compounds (including benzeneselenol, dibenzylic selenide, diphenyl diselenide and ebselen) promote a more reducing extracellular microenvironment by increasing cysteine efflux from macrophages as well as enhancing expression of extracellular Trx1 in murine RAW264.7 peritoneal macrophage cells (Sahaf *et al.*, 2005).

Redox signalling in microglia

Protein thiolation in redox signalling and protection with GSH

The redox-based post-translational modification of protein thiols is increasingly recognized as a significant component of both normal cellular responses and oxidative stress-associated pathological conditions (Winterbourn, 2013; Holmstrom and Finkel, 2014). In particular, H_2O_2 that is derived from O_2^- following NOX2 stimulation in macrophages and other phagocytes (and by inference, microglial cells), is required for cellular responses to stimuli including insulin, angiotensin and growth factors (Forman and Torres, 2002). In many cases, the mechanism is unknown, but increasingly, evidence points to S-glutathionylation (see Figure 1 for details) of target proteins as being an essential part of the process. In particular, several signalling proteins undergo S-glutathionylation, including enzymes such as protein tyrosine kinases and phosphatases, as well as transcription factors. Other spheres of cellular activity particularly associated with S-glutathionylation are microglial activation and recruitment, cytoskeletal rearrangements, protein trafficking and regulation of transcription. For example, stimulation of the respiratory burst and release of superoxide in macrophages promotes S-thiolation of several proteins as protection against auto-oxidation (Rokutan *et al.*, 1991). It is noted that individual proteins undergo thiolation and de-thiolation at differing rates, which implies selectivity in the process.

Hydroquinone, a benzene derivative that is an industrial by-product as well as a natural component of certain fruits, vegetables and dairy products, is a modulator of the inflammatory response. Experiments using mouse peritoneal macrophages demonstrated that hydroquinone acts by thiolation of cysteine residues in Src kinase, thereby up-regulating the phase 2 detoxification enzyme, haem oxygenase-1 (HO-1), producing immunosuppressive and anti-inflammatory actions (Byeon *et al.*, 2013). In a more physiological context, H_2O_2 production following stimulation of the respiratory burst in the rat alveolar NR8383 cell line causes reversible S-glutathionylation of protein tyrosine phosphatase 1B (detected using S-glutathioylated-specific antibodies) that affects downstream signalling pathways. The transience of the effect demonstrates the potential of a molecular switch mechanism that can temporally initiate a signalling pathway in response to H_2O_2 production. Its selectivity and physiological significance is illustrated by the fact that two other protein tyrosine kinases, SHP-1 and SHP-2, were not S-glutathionylated unless non-physiological levels of GSSG were included in the assay (Rinna *et al.*, 2006). In monocytes, stimulation of NOX4 promotes S-glutathionylation of actin and acceleration of chemotaxis (Lee *et al.*, 2013). Moreover, induction of NOX4 is the rate-limiting step in monocyte adhesion and migration, illustrating the central role played by the enzyme in actin dynamics in response to stress. Although cysteine oxidation and S-glutathionylation primarily serve to deliver a transient and reversible signal to target redox proteins, in some cases, it leads to the degradation of the target protein in question, for example, the down-regulation of MAPK phosphatase in monocytes which directly impinges on (hyper)activation of ERK and p38MAPK and increased monocyte adhesion and chemotaxis in response to the chemokine CCL2 (Kim *et al.*, 2012). Further instances of S-glutathionylation that contribute to macrophage function/dysfunction can be found in the excellent review of the subject by Ullevig *et al.* (2013).

A possible reason for the apparent redundancy of antioxidant proteins is that their function is not just tied to oxidant scavenging or recycling of reduced cysteine residues for signalling purposes. Rather, antioxidants also take direct part in redox signalling pathways as relayers of the redox signal through redox-dependent interactions with other proteins, which confers an additional level of specificity through protein–protein interactions, apart from the typical spatio-temporal confinement in which redox targets, oxidants and antioxidants operate, (Winterbourn, 2013). Thus, the Prx act as relayers of the redox signal in different cell types (Jarvis *et al.*, 2012; Sobotta *et al.*, 2015; Yan *et al.*, 2015). Prx 1 receives, transduces and transmits the peroxide signal to oxidize cysteines in ASK1, which then phosphorylates and activates downstream kinase p38MAPK (Jarvis *et al.*, 2012). Yet another layer of redox regulation is imposed on ASK1, as this important kinase in steady state is kept inactive through binding to thioredoxin. However, oxidation of thioredoxin releases ASK to fulfil its signalling function (Saitoh *et al.*, 1998).

Expression of antioxidant proteins is not always correlated with a decreased oxidative stress. In this respect, Prx 6 occupies a special position, as it is a bifunctional enzyme containing both GSH peroxidase and phospholipase A_2 (PLA_2) activities. Prx 6 is primarily expressed in astrocytes (Table 1); (Power *et al.*, 2008) but is up-regulated in activated microglia and macrophages. This correlates with increased expression levels in Alzheimer's disease, and in Prx 6-overexpressing mice, the progression of memory impairment and CNS pathology of Alzheimer disease models is accelerated due to increased oxidative stress (Yun *et al.*, 2013). The mechanism is still under elucidation, but it is interesting to note that the PLA_2 activity of Prx 6 enhances NOX2 activity, which depends on arachidonic acid for p47phox mobilization (Dana *et al.*, 1998), in alveolar macrophages (Chatterjee *et al.*, 2011) and other cell types (Ellison *et al.*, 2012).

Redox control of microglia transcription programs and activation states

Activation of the M1 phenotype is the innate immune-inflammatory and pro-inflammatory response to many different stimuli, including many pathogen constituents (e.g. LPS) and tissue-derived damage-associated molecular pattern molecules (DAMPs) and is characterized by high levels of oxidant production, secretion of proinflammatory mediators, pathogen combat mechanisms and interaction with the Th1

arm of the adaptive immune defence. The M2 phenotype, classically brought about by IL-4 or IL-13 activation, is primed for tissue repair, phagocytosis, lysosomal degradation, chemokine expression and resolution of inflammation (Cherry *et al.*, 2014; Hu *et al.*, 2015).

Stimuli inducing the M1 phenotype generally up-regulate expression levels of NOX subunits, and at the same time, oxidant production by NOX2 is a prerequisite for expression of many proinflammatory cytokines and molecules associated with the M1 response including iNOS (Pawate *et al.*, 2004; Won *et al.*, 2009; Nam *et al.*, 2012; Kim *et al.*, 2013). In contrast, expression and secretion of IL-1β, also an important proinflammatory cytokine, is a property that seems to reside with NOX1 activity at least following some stimuli (Cheret *et al.*, 2008; Choi *et al.*, 2012). Later studies making use of NOX-deficient mouse models have confirmed the role of NOX2 for the expression of proinflammatory cytokines following induction of neuroinflammation with LPS or Aβ (Choi *et al.*, 2012). In fact, in the absence of a functional NOX2 complex, microglia up-regulate M2 markers when confronted with M1 stimuli such as LPS, indicating that NOX2 activity promotes the M1 phenotype while repressing the M2 phenotype (Choi *et al.*, 2012), and the same phenomenon is observed in peripheral tissue macrophages (Padgett *et al.*, 2015). Conversely, NADPH oxidase activity is subdued in M2-activated macrophages, in part due to down-regulation of NOX genes (Balce *et al.*, 2011). For example, in human and pig macrophages, the mRNA of gp91phox (NOX2) is down-regulated by 70% after 8 h following treatment with IL-4 (Zhou *et al.*, 1995), and similar results have been obtained for rat microglia (Savchenko, 2013), where also NOX1 mRNA was down-regulated. A few studies have found increased oxidant production and cytotoxicity in different settings of alternative microglia activation (Park *et al.*, 2008; Nam *et al.*, 2012; Won *et al.*, 2013). The changes in NOX activity and cytosolic oxidant production associated with M1 and M2 states are mirrored in mitochondrial oxidant production. Here, the mitochondrial anion carrier protein UCP2, whose level of expression is modulated by activation and regulates mitochondrial membrane potential and ROS release (Kizaki *et al.*, 2002), is differentially regulated by M1 and M2 stimuli and plays a functional role in the acquisition of these activation states (De Simone *et al.*, 2015).

NF-κB

Transcriptional activity of the NF-κB p65/p50 dimer appears pivotal in the switch to a proinflammatory phenotype and is activated together with the typical MAP kinases downstream of NOX activation and oxidant production in M1-activated microglia (Pawate *et al.*, 2004). The mere overexpression of a constitutively active NF-κB specifically in microglia is sufficient to drive these cells into a proinflammatory M1 state where they cause motor neuron death in models of amyotrophic lateral sclerosis (Frakes *et al.*, 2014), but NF-κB is not essential for the acquisition of the M1 phenotype (Taetzsch *et al.*, 2015). NF-κB is normally inactive in the cytosol by interaction with IkB, but oxidants, by reaction with IkB directly or via redox activation of upstream kinases, release the catalytic NF-κB subunits from their inhibitory binding with IkB and allow translocation of NF-κB into the nucleus, required for the expression of many proinflammatory genes in microglia (Rojo *et al.*, 2014). In addition, the NOX2-dependent inactivation of NF-κB p50/p50 dimers, which binds DNA but lacks a transactivation domain and therefore repress gene expression, seems to be a crucial hub in the redox regulation of the M1 phenotype in microglia (Taetzsch *et al.*, 2015). Deficiency of p50 prolongs the M1 response with exaggerated TNFα secretion, while interfering with the induction of M2 traits and resolution of inflammation (Porta *et al.*, 2009; Rolova *et al.*, 2014; Taetzsch *et al.*, 2015). C-terminal-binding protein 1 also known as CtBP1 is another important repressor of proinflammatory gene expression in microglia (Saijo *et al.*, 2011) and is redox regulated by the ratio of $NADH/NAD^+$ (Zhang *et al.*, 2002).

Nrf2

The Nrf2 transcription factor is the master regulator of antioxidant responses. Upon translocation into the nucleus, Nrf2 binds to genes containing regulatory antixodant response elements sequences to enhance transcription of a subset of genes involved in detoxification and antioxidant responses including HO-1 and other antioxidant proteins. Nrf2 is retained in the cytosol by interaction with Kelch-like ECH-associated protein (KEAP1), which indirectly mediates the proteasomal degradation of Nrf2 (Zhang and Hannink, 2003; Furukawa and Xiong, 2005). However, oxidation of cysteines in KEAP1 relieves binding and allows Nrf2 to accumulate, enter the nucleus and exert its activity (Zhang and Hannink, 2003; Zhang *et al.*, 2004; Yamamoto *et al.*, 2008). It therefore also follows that inherent to M1 activation and increased NOX2 activity, there is a certain level of Nrf2 activation and induction of antioxidant proteins (Kim *et al.*, 2010; Kong *et al.*, 2010), which again opposes NF-kB activity (Kong *et al.*, 2010; Cuadrado *et al.*, 2014) and oxidant levels to uphold redox homeostasis.

Although timing is crucial, there is a broad assumption that acquisition of the M2 phenotype is beneficial in acute or chronic brain disease (Cherry *et al.*, 2014; Hu *et al.*, 2015). Nrf2-deficient macrophages have an increased NOX2-mediated respiratory burst, which greatly increases TLR4 signalling, IkB inactivation and the resulting proinflammatory response (Kong *et al.*, 2010). In Parkinson's disease models, Nrf2 deficiency results in increased microgliosis and a skewed balance of M1 versus M2 gene expression, ultimately increasing neuronal death in the basal ganglia compared with wild-type mice (Chen *et al.*, 2009; Innamorato *et al.*, 2010; Rojo *et al.*, 2010; Gan *et al.*, 2012). Overexpression of Nrf2 specifically in astrocytes is sufficient to give neuroprotective effects in animal models of Parkinson's disease, and part of this may stem from the export of GSH from astrocytes (Shih *et al.*, 2003) or alternatively, astrocytes may act as a sink for released ROS.

Activation of α7-nAchR not only inhibits NADPH oxidase activity in microglia cultures (Moon *et al.*, 2008) but also leads to HO-1 expression in an Nrf2-dependent fashion after oxygen and glucose deprivation in organotypic cultures (Parada *et al.*, 2013). In this regime, α7-nAchR agonist-driven HO-1 expression in microglia was found to lessen tissue damage (Parada *et al.*, 2013); however, in the MPTP model of Parkinson's disease,

HO-1 was found to play no role in the neuroprotective Nrf2-dependent response (Innamorato *et al.*, 2010).

Some agents have been identified that modulate the conversion to either M1 or M2 activation states in ways that may depend on NOX activity. A novel mGluR5 positive allosteric modulator with good CNS penetrance decreases NOX2 activation and shifts the microglia phenotype towards the M2 pro-repair activation state, a phenomenon abolished in gp91phox (−/−) mice (Loane *et al.*, 2014). The antibiotic minocycline selectively inhibits development of the M1, but not the M2, activation response (Kobayashi *et al.*, 2013). Neurotoxin-induced dopaminergic degeneration in the substantia nigra depends on expression of NOX2 (Gao *et al.*, 2003; Wu *et al.*, 2003; Hernandes *et al.*, 2013). However, curiously, treatment of gp91phox(−/−) mice with minocycline results in enhanced microglia activation and neuronal loss relative to wild-type mice also treated with minocycline (Hernandes *et al.*, 2013). Disregarding non-cell-autonomous effects, these data, together with the observations of Block *et al.* (Taetzsch *et al.*, 2015), suggest that NOX2 activity can be required for anti-inflammatory effects in M2-activated microglia.

Paracrine effects of ROS and antioxidants

As a consequence of the recognition of widespread redox signalling to support the everyday life of cells, the notion that oxidative stress is 'a (global) unbalance between oxidants and anti-oxidants in favor of oxidants', (Sies, 2014) and that neurodegeneration arises due to direct destruction of macromachinery by ROS, is slowly changing to encompass that individual redox pathways may operate in a spatiotemporal confinement (specificity) not necessarily tied to major changes in global redox status (Go *et al.*, 2004; Jones, 2006; Jones and Sies, 2015). Oxidative stress can therefore also be defined as the 'disruption of redox signalling and control' (Jones, 2006; Sies, 2014). There is no doubt that unbalanced oxidant production in the brain can lead to excessive and unwarranted oxidation of macromolecules leaving the telltale histopathological signs of lipid peroxidation and breakdown and protein carbonylation. The question is how disturbing and deleterious such oxidation events are compared with the derangement of autocrine or paracrine redox signalling, which would require only low, but misplaced or badly timed, oxidant production for effect.

The membrane permeability of H_2O_2 is many times higher than that of $O_2^{\cdot-}$, but nevertheless, transport of H_2O_2 across the cell membrane is facilitated via passage (both ways according to gradient) through certain members of the aquaporin family (Bienert *et al.*, 2007; Bienert and Chaumont, 2014). In mammalian cells, aquaporin 3 (Miller *et al.*, 2010; Hara-Chikuma *et al.*, 2012) and 8 (Bertolotti *et al.*, 2013; Vieceli Dalla Sega *et al.*, 2014) have been implicated in transport of H_2O_2 from the external space into cells, whether supplied exogenously or following cell surface receptor activation of endogenous ROS production through NOX activation.

H_2O_2 or $O_2^{\cdot-}$ released to the surroundings from either cytosolic or cell surface-localized NOX can be assigned either a physiological function or a pathological role in oxidative stress. A large cohort of studies support the notion that release of H_2O_2 from cell surface-resident NOX can diffuse back into the cytosol to effect physiological redox signalling, subsequent to cell surface receptor activation. This autocrine function of secreted ROS is discussed in detail in the accompanying review (Haslund-Vinding *et al.*, 2016), while we here discuss the potential paracrine role of ROS and antioxidants.

While the diffusion range of H_2O_2 in the highly reducing cytosol is limited to a few micrometers (Mishina *et al.*, 2011), the diffusion range is considerably extended in the extracellular space. Although supported by only a few studies, an intriguing possibility is that oxidants released from microglia can function as paracrine first messengers of intercellular communication by altering the redox status of the cell surface or cytosol of nearby cells.

Microglia adapt their activation phenotype to existing conditions in part by engaging in close reciprocal interactions with other CNS cell types including neurons and T-lymphocytes. Under some circumstances, the presentation of self-antigen by microglia to T-lymphocytes is suggested to have immunomodulatory effects that translate into a neuroprotective environment (Shechter and Schwartz, 2013). Interestingly, the outcome of antigen presentation (T-cell activation or tolerance) is to a large extent regulated by NOX-mediated oxidant production by macrophages that alters the T-cell redox status of (unidentified) either receptors on the T-cell surface or cortical signalling machinery localized close to both T-cell and macrophage membranes in the immunological synapse (Gelderman *et al.*, 2006; Gelderman *et al.*, 2007; Holmdahl *et al.*, 2013). Granted the expression of MHC II molecules in microglia in a range of pathological conditions not overtly associated with the adaptive arm of the immune system, the communication between these two cell types and the role of NOX-derived ROS in this conversation seems interesting to pursue.

Intriguingly, some antioxidants are also secreted to fulfil important signalling roles that do not necessarily rely on the antioxidative properties of these molecules. The release of thioredoxin from different cell types by an unconventional secretory mechanism has been recognized for long (Rubartelli *et al.*, 1992). When released, thioredoxin acts as a chemoattractant for monocytes and other immune cells in a way that depends on the catalytic site cysteines in thioredoxin, but is independent of GPCRs (which typically mediate chemotaxis) (Bertini *et al.*, 1999), implying that thioredoxin may act by modifying redox targets on target cells rather than engaging a receptor. Trx treatment alone or in combination with macrophage-activating stimuli also potently enhances synthesis and release of proinflammatory cytokines from macrophages and other cell types (Schenk *et al.*, 1996; Sahaf *et al.*, 2005). Recently, it has been shown that inflammatory stimuli, such as LPS, cause the secretion of Prx 2 in its oxidized (glutathionylated) form from macrophages (Salzano *et al.*, 2014). Once secreted, glutathionylated Prx 2 activates macrophages to produce and secrete TNFα and other proinflammatory factors. Interestingly, thioredoxin, the substrate of Prx 2, was co-secreted suggesting that the two antioxidants in combination with oxidant production could cooperate to alter redox status of

cell surface receptors in an autocrine or paracrine manner (Salzano *et al.*, 2014). Other antioxidant molecules, however, can utilize receptors for signalling purposes. Thus, following ischaemic insult necrotic brain cells release Prx, which through a highly conserved central motif present in all Prx not containing active cysteines, activates blood borne macrophages through TLR2 and TLR4 to produce inflammatory cytokines (Shichita *et al.*, 2012). Also, the DAMP high mobility group box 1 can be released in an oxidatively modified form, which signals through TLR4 (Hoppe *et al.*, 2006; Agalave *et al.*, 2014).

As for communication with neurons, the culling of surplus neurons in the developing CNS by microglia-derived oxidants could in a sense be regarded as the physiological, but deadly, result of paracrine effect of NOX redox signalling (Marin-Teva *et al.*, 2004; Wakselman *et al.*, 2008).On a more subtle scale, the structural modification of neuronal connectivity in the adult CNS by the microglial and complement receptor 3-mediated engulfment of synaptic terminals represent a response to (lack of) synaptic activity (Schafer *et al.*, 2012; Kettenmann *et al.*, 2013). However, in a recent turn of events, MacVicar *et al.* demonstrated that LPS-induced activation of microglial complement receptor 3, when combined with hypoxia, caused long-term synaptic depression (LTD) in the hippocampus by internalization of the AMPA receptor (Zhang *et al.*, 2014a). The LTD was expressed rapidly (15 min), and the effect was abolished in the presence of antioxidants and replicated by xanthine/xanthine oxidase-generated superoxide, suggesting that NOX-mediated O_2^- production by microglia via unknown redox relayers activated phosphatase 2A in neurons and enhanced internalization of the AMPA receptor. Importantly, LTD occurred independently of NMDA receptors or mGluR activation, signifying that the stimulus for LTD originated from microglia, rather than presynaptic terminals, which has considerable implications for the understanding of the mechanism of NMDA receptor-independent cognitive decline that is linked to immune responses in a range of neurological disorders. The picture extends beyond the confines of local immune responses in the brain, for, in a separate study, AMPA- and NMDA-mediated currents were elevated in hippocampal slices prepared from rats with chemically induced chronic peripheral inflammation (Raizi *et al.*, 2015). Further work established that microglial activation played a key role in the response, because changes in the electrical activity of the slices were abolished by prior chronic administration of minocycline, which is known to block activation of microglia. At the molecular level, Liu *et al.* (2015) showed that microglial cell activation reduces phosphorylation of the GluR1 receptor subunit. A similar sequence of events may give rise to cognitive impairment in multiple sclerosis. Studies using the EAE rat model of multiple sclerosis show that release of IL-1β from infiltrating lymphocytes or activated microglia facilitates hippocampal long-term potentiation via inhibition of GABA-mediated synaptic activity and increased likelihood of glutamate-mediated excitotoxicity (Nisticó *et al.*, 2013a, 2013b). In contrast, Aβ inhibits induction of NMDA receptor-dependent long-term potentiation in hippocampal slices, and deficiency of iNOS or inhibitors of NADPH oxidase both relieved this inhibitory effect, implicating microglia-derived oxidants (Wang *et al.*, 2004).

Open questions and conclusions

Clearly, direct and destructive damage to proteins can occur as a consequence of increased oxidant production and release (Wu *et al.*, 2006). However, the high interspecies level of oxidative stress markers and pro-oxidant redox balance present in certain long-lived organisms (Andziak *et al.*, 2006) raises the critical question - to what extent does oxidation of macromolecules disturb the normal metabolism and function of cells and ultimately cause their demise? Recent evidence indicates that slight alterations of the level or subcellular localization of oxidant production can have large effects on individual redox modifiable proteins and circuits of redox signalling (Zhang *et al.*, 2014a) that can change, without overall disturbances to the global redox level of cells (Jones and Sies, 2015) or destruction of macromolecules. The extent to which antioxidants also relay the redox signal, and by protein structure and subcellular localization contribute yet another regulatory level to redox signalling, will be interesting to determine. In this respect, the co-secretion of oxidants and antioxidant systems from activated macrophages is alluring in terms of paracrine redox signalling and should be explored in the CNS where the extracellular space and distance to neighboring cells or cell structures is extremely limited, relative to the conditions in the periphery.

Acknowledgements

The present work was supported by the European Cooperation in Science and Research (COSTAction BM1203/EU-ROS).

Conflict of interest

The authors declare no conflicts of interest.

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