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REVIEW ARTICLE

Regulation of neuronal development and function by ROS

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Reactive oxygen species (ROS) have long been studied as destructive agents in the context of nervous system ageing, disease and degeneration. Their roles as signalling molecules under normal physiological conditions is less well understood. Recent studies have provided ample evidence of ROS-regulating neuronal development and function, from the establishment of neuronal polarity to growth cone pathfinding; from the regulation of connectivity and synaptic transmission to the tuning of neuronal networks. Appreciation of the varied processes that are subject to regulation by ROS might help us understand how changes in ROS metabolism and buffering could progressively impact on neuronal networks with age and disease.

Keywords: axon; cytoskeleton; dendrite; NADPH oxidase; nervous system; neuronal polarity; pathfinding; plasticity; reactive oxygen species; synapse

The increase in atmospheric oxygenation is presumed to have set the pace of evolutionary change. The symbiotic acquisition of mitochondria 1.45 billion years ago generating the eukaryota further allowed diversification *via* the efficient metabolic use of diatomic oxygen. Reactive oxygen species (ROS), highly reactive molecules and free radicals derived from molecular oxygen are produced as natural by-products of normal respiratory metabolism, with the major source being the mitochondria. Mitochondria leak bursts of ROS as a function of respiration [1] pointing to a link between metabolism and ageing-related damage. Other subcellular locations of ROS production continue to be identified and include the endoplasmic reticulum (ER) [2], peroxisome [3], the cytosol [4], plasma membrane [5] and extracellular space [6]. However, there is a growing opinion and body of evidence that ROS can act as physiological signalling molecules. In this review we will focus on the role of ROS as a physiological signal in the nervous system during development and as a regulator of neuronal function.

By their highly reactive nature, ROS are damaging to the cell, oxidising proteins, lipids and DNA and are normally regarded as detrimental to cell function.

Abbreviations

BH_4, tetrahydrobiopterin; CamKII, calcium/calmodulin-dependent kinase II; CRMP2, collapsin response mediator protein 2; ER, endoplasmic reticulum; ERK, extracellular signal–regulated kinase; Grx1, glutaredoxin 1; HFS, high-frequency stimulation; IP3Rs, inositol-3-phosphate receptors; JNK, Jun-N-terminal Kinase; LTD, long-term depression; LTP, long-term potentiation; MICAL, molecule interacting with CasL; MsrB, methionine sulfoxide reductase; NGF, nerve growth factor; PKC, protein kinase C; PP, protein phosphatase; PTEN, phosphatase and tensin homolog; PVIs, parvalbumin-expressing inhibitory interneurons; redox, reduction–oxidation; ROS, reactive oxygen species; RyRs, ryanodine receptors.

Indeed, an overwhelming of the defences against ROS is termed oxidative stress and is commonly associated with cellular damage seen in neurodegenerative disorders, including Parkinson's [7] and Alzheimer's disease [8]. ROS and the accumulation of ROS-related damage are also associated with ageing; oxidised lipids, DNA damage and the accumulation of lipofuscin (the 'aging pigment', autofluorescent material found in endosomes consisting of oxidised lipids, proteins, transition metals and senescent mitochondria) [9]. Increasing evolutionary complexity and concomitant demand for oxygen-dependent energy production *via* reduction–oxidation (redox) reactions also produced a diversification in defence mechanisms against ROS. This is particularly notable in energy demanding tissues such as heart and liver. The nervous system is anomalous in this framework as nerve cells are very energy demanding yet at the same time inadequately equipped with antioxidant defence [10,11]. Interestingly, much of the ROS defence within the nervous system occurs in glia [12]. It is becoming increasingly apparent, however, that evolution may have made a virtue out of a necessity and co-opted ROS for cellular signalling mechanisms. For such a framework lowered antioxidant defence or ROS buffering in neurons would be permissive and necessary.

Regulation of ROS in the nervous system, the case for the defence

Studies of ROS in the nervous system have primarily focused on ROS as damaging agents and the defence against ROS. Protection against ROS is mediated by multilayered constitutive and adaptive forms of defence. In the brain, static defences against ROS generally are seen to be (a) constitutive and enzymatic as seen in the standing high concentrations of enzymes such as superoxide dismutases, catalases, thioredoxin reductases and glutathione peroxidases or (b) constitutive and nonenzymatic as mediated by defence molecules (termed ROS scavengers) present in the cell, *via* synthesis or diet, such as alpha-tocopherol (vitamin E), ascorbic acid, ß-carotene and tetrahydrobiopterin (BH_4). BH_4 is a molecule of particular interest. For example, BH_4 is a highly sensitive scavenger of H_2O_2 and Hydroxyl ions, while also important for the synthesis of the neurotransmitters dopamine, serotonin and noradrenaline. This suggests a functional link between ROS abundance, buffered by BH_4 levels, and neurotransmitter function (for review see [13]). A second set of defence mechanisms is adaptive and mediated by transcription factors [14]. Two pathways are prominent: (a) the NRF2/Keap system, predominant in glial cells [12] and (b) the Jun-N-terminal Kinase (JNK)/AP-1 system, seen to be a major protective mechanism in neurons. Both promote the transcription of genes encoding antioxidant response proteins. For example, NRF2 promotes the expression of glutathione-*S*-transferases in glia [15] while in neurons AP-1 activation upregulates sulfiredoxin [16]. NRF2 activity in glia mediates neuronal protection in a nonautonomous manner, partly through the ensheathing nature of symbiotic glial–neuron interactions. Indeed, keeping NRF2 function low in neurons allows dendritic and synaptic development and their regulation *via* redox-sensitive signalling pathways, such as JNK/AP-1 and WNT [17].

Neuronal polarity

Most neuronal cell types are explicitly polarised, endowed with a major axonal neurite that mediates long-range connectivity and is primarily presynaptic, dedicated to passing on information, while the somato-dendritic compartment of the cell is composed of branched smaller diameter neurites that are largely postsynaptic. The establishment of neuronal polarity has until recently mostly been studied *in vitro*, using low-density cultures of cortical and hippocampal neurons. Under such conditions neuronal polarity first manifests with the emergence of a primary neurite, which extends more rapidly than others and develops into the axon, while the other minor neurites adopt postsynaptic dendritic characteristics. Several signalling pathways, including TGF-ß, growth factors (e.g. BDNF), LKB and PI3 kinases, have been implicated in bringing about and maintaining asymmetries of the cytoskeleton. Characteristically, axons contain microtubules whose plus ends face away from the cell body, while dendrites have microtubules of mixed (mammals) or opposite polarity (e.g. *Drosophila* and *Caenorhabditis elegans*; for reviews see [18,19]). Perhaps inspired by work from other systems that have associated NADPH oxidase-generated ROS with the regulation of cell polarisation and growth, for example, *Arabidopsis* hair cell outgrowth [20] and the enforcement of apical dominance in *Aspergillus* hyphae [21], ROS have been investigated as signals regulating the polarisation of neurons. Indeed, *in vitro* studies suggest that ROS produced by NADPH oxidases were required alongside growth factors for the differentiation of neuronal characteristics by PC12 and SH-SY5Y cells, such as axonal outgrowth [22–25]. *In vivo*, gene expression profiles show that subunits of the NOX2 NADPH oxidase complex are present at the right place and time in mouse and rat embryonic hippocampal neurons

[26,27]. In the context of neuronal differentiation mediated by nerve growth factor (NGF), Neuregulin or Retinoic Acid ROS appear to be permissive, acting in parallel to these growth factors. As documented for several growth factors, ROS can enhance downstream kinase signalling by inhibition of phosphatases, whose active sites contain cysteines that are susceptible to oxidation [28]. The activity of protein kinases is critical during cellular polarisation, meditating positive feedback loops and signal amplification. Multiple pathways converge onto PI3 kinase, which is enriched at the tip of the future axon as neurons adopt a polarised morphology [29,30] (reviewed in [18]). Interestingly, PI3 kinase signalling can be regulated by ROS, by redox-mediated inhibition of the phosphatase and tensin homolog (PTEN), which antagonises the PI3 kinase product phosphoinositide-3-phosphate [31,32].

Studies on embryonic hippocampal neurons and cerebellar granule cells in culture have underpinned the idea that ROS contribute to the establishment of neuronal polarity. As cerebellar granule cells differentiate during the first 3 days *in vitro* overall ROS levels increase and become specifically enriched at growth cones and other cytoskeletally dynamic sites. This is paralleled by a ROS-dependent rise in Tau and MAP2 expression levels, which are representative of axonal and dendritic cytoskeletal specialisation [33]. Underlining a requirement for ROS in these processes, neurons derived from NOX2 knockout mice showed reduced neurite length compared to control cells [33]. Similarly, in embryonic hippocampal neurons pharmacological or genetic reduction in NADPH oxidase activity, e.g. by expression of the dominant negative $DNp22^{phox}$ regulatory subunit, led to delayed and reduced axon outgrowth [26]. Conversely, overactivation of NADPH oxidase by overexpression of the accessory protein $p47^{phox}$ promoted axonal growth [34]. These studies suggest that NOX2 activity could indeed contribute to neuronal polarisation and promote neurite outgrowth.

The mechanisms by which physiological levels of ROS support neurite outgrowth and axon specification appear to involve release of calcium from intracellular stores, a potent second messenger regulating cytoskeletal organisation and dynamics (reviewed in [35,36]). Short respiratory bursts of NADPH oxidase-generated ROS promote calcium release by redox modification of ryanodine (RyRs) and inositol-3-phosphate receptors (IP3Rs). These in turn lead to increased expression of Rac1, an activator of the NOX2 complex, thus generating a positive feedback loop that can convert initially transient ROS bursts into sustained ROS activation and high levels of intracellular calcium [34]; (Fig. 1). However, the extent to which ROS signalling promotes the establishment of neuronal polarity *in vivo* remains to be determined. Phenotypes of NADPH oxidase knockout and RNAi knockdown experiments in several experimental animal models suggest that removal of any one NADPH oxidase still allows the nervous systems to form and function remarkably well, at least to a level sufficient for survival under laboratory conditions [37,38]. This could indicate a degree of functional redundancy. It also suggests that ROS might have a modulatory role rather than being critically required for the establishment of neuronal polarity. This would not be entirely surprising, as during nervous system development neural progenitor cells and their progeny are invariably located within asymmetric environments where local enrichment of numerous cues, including cell adhesion and extracellular matrix proteins, can contribute to cellular polarisation [39–41] (for review see [18]).

Cytoskeletal modifications by ROS and growth cone pathfinding

Reactive oxygen species can regulate cytoskeletal change at multiple levels; directly *via* redox modification of structural cytoskeletal proteins and indirectly by modification of proteins or signalling pathways that regulate cytoskeletal dynamics. For example, actin and tubulin monomers contain multiple cysteine and methionine residues exposed to the cytoplasm that are subject to redox modifications, notably glutathionylation, nitrosylation and carbonylation [42–49]. Indeed, all major cytoskeletal elements and many cytoskeleton-associated proteins are subject to direct redox modifications of some kind [50–52], with substantial fractions of actin, tubulin and neurofilaments found glutathionylated under normal physiological conditions [53]. The question of which residues are modified, under what conditions and how this impacts on protein function and dynamics remains live. For alpha-actin purified from rabbit muscles, *in vitro* and cell culture studies have shown Cys 374 sensitive to glutathionylation, leading to a reduced rate of actin polymerisation and altered actin dynamics [44,54]. Actin Cys 374 glutathionylation has been proposed to occur in response to growth factor and integrin-stimulated signalling following interactions with the extracellular matrix [54]. This actin modification is thought to regulate the disassembly of the actinomyosin complex during cell spreading [52]. Along the same lines, disruption of protein deglutathionylation by mutation of glutaredoxin 1 (Grx1), the gene coding for an enzyme that catalyses actin deglutathionylation, led to reduced actin polymerisation and impaired

polarisation, chemotaxis, adhesion, and phagocytosis by neutrophils. Conversely, blocking NOX activity led to increased formation of filamentous actin [55]. Thus, ROS have increasingly been recognised as important regulators of actin dynamics.

One of the principal ROS sources in neurons is NAPDH oxidases. Their activity is highly regulated, making them prime candidates as regulators of growth cone cytoskeletal dynamics (for reviews see [56] and [57]). How NADPH oxidase-generated ROS regulate cytoskeletal dynamics in neuronal growth cones remains poorly understood. Pharmacological inhibition of NADPH oxidase activity or lowering cytosolic ROS levels led to reduced F-actin content, retrograde flow and neurite outgrowth. Few studies have documented the localisation of NOX complex components in neuronal growth cones *in vitro*, none as yet *in vivo* [58,59]. In cultured *Aplysia* bag cell neurons the main enzymatic subunit of a NOX2-type NADPH oxidase, NOX2/gp91^{phox}, was seen localised to the plasma membrane, largely distinct from the regulatory subunit p40^{phox} found associated with filopodial actin bundles (Fig. 1). Interestingly, a local stimulus of growth cone interaction with apCAM-coated beads triggered colocalisation of both subunits to the site of growth cone–substrate interaction. Thus, in growth cones NADPH oxidase subunits are localised to the periphery at sites of actin assembly [58]. Their activity is regulated by multiple pathways, including activation following translocation of regulatory subunits, such as p40^{phox} or Rac1, but also by other signalling pathways, such as protein kinase C (PKC) [59]. The extent to which local NAPDH oxidase activation regulates growth cone dynamics directly, *via* oxidation of actin and tubulin, or indirectly, through modulation of other signalling pathways, remains to be seen. Both are likely. A study using cultured marsupial kidney cells demonstrated that hydrogen peroxide-induced chemotaxis and filopodial dynamics were in large part indirectly regulated by ROS *via* local extracellular signal–regulated kinase (ERK) pathway activation, which promoted actin retrograde flow by differential activation and recruitment of cofilin and the Arp2/3 nucleator at the leading edge [60]. It is conceivable that ROS signalling in the nervous system might similarly be utilised for noncell autonomous communication, either during the development of synaptic connections or their subsequent adjustment. For example, in a mouse model for multiple sclerosis, persistent activation of NOX2/gp91^{phox} in microglia leads to the impairment of synaptic plasticity in adjacent hippocampal neurons [61].

Thus far, the clearest evidence for post-translational redox modification of cytoskeletal proteins directing growth cone pathfinding derives from studies of Semaphorin-Plexin signalling. The cytoplasmic tail of Plexin interacts with the NADPH-dependent monooxygenase, molecule interacting with CasL (MICAL), which is activated upon binding of Semaphorin guidance cues [62–67] (for review see [68]; Fig. 1). Elegant experiments by the Terman laboratory and collaborators demonstrated that the amino-terminal NADPH-dependent redox domain of MICAL-1 binds F-actin and directly oxidises the methionine residues Met44 and Met47 [64,65]. These redox modifications are regulated and can be reversed by a methionine sulfoxide reductase, (MsrB/SelR), which catalyses the reduction of methionine sulfoxide to methionine [66]. MICAL-1-mediated oxidation of actin weakens inter-actin contacts while simultaneously increasing the binding affinity of the F-actin severing protein cofilin by more than an order of magnitude. This synergistic effect of actin destabilisation by oxidation and concomitantly increased binding of cofilin promotes F-actin disassembly. In addition, MICAL-mediated oxidation decreases the capacity of actin for repolymerisation, thus further impacting on the dynamics of the actin cytoskeleton [67]. Thus, Semaphorin-Plexin guidance cue–receptor interactions are directly transduced to redox modification of the actin cytoskeleton, leading to F-actin disassembly and altered reassembly dynamics.

In addition, MICAL activation also impacts on the microtubule cytoskeleton *via* another binding partner, collapsin response mediator protein 2 (CRMP2). CRMP2 is responsible for Semaphorin-induced growth cone collapse [69] and interacts with tubulin heterodimers regulating microtubule dynamics 1 [62,70]. Current data suggest that MICAL-1 binds CRMP2, and that upon MICAL monooxygenase activation (e.g. by Semaphorin binding to Plexin), hydrogen peroxide is produced that oxidises CRMP2. Oxidation of CRMP2 promotes formation of disulfide-linked CRMP2 homodimers, which interact with thioredoxin, which in turn promotes their phosphorylation by GSK-3ß, microtubule disassembly and growth cone collapse [71]; (Fig. 1). In summary, direct redox modifications of cytoskeletal elements, notably actin and tubulin, as well as of regulators of cytoskeletal dynamics, such as CRMP2, lie at the heart of Semaphorin-Plexin growth cone guidance. It is conceivable that redox modification of cytoskeletal proteins extends to other guidance cue signalling pathways, either directly or in a modulatory capacity where the cellular redox state determines growth cone responses to extracellular cues.

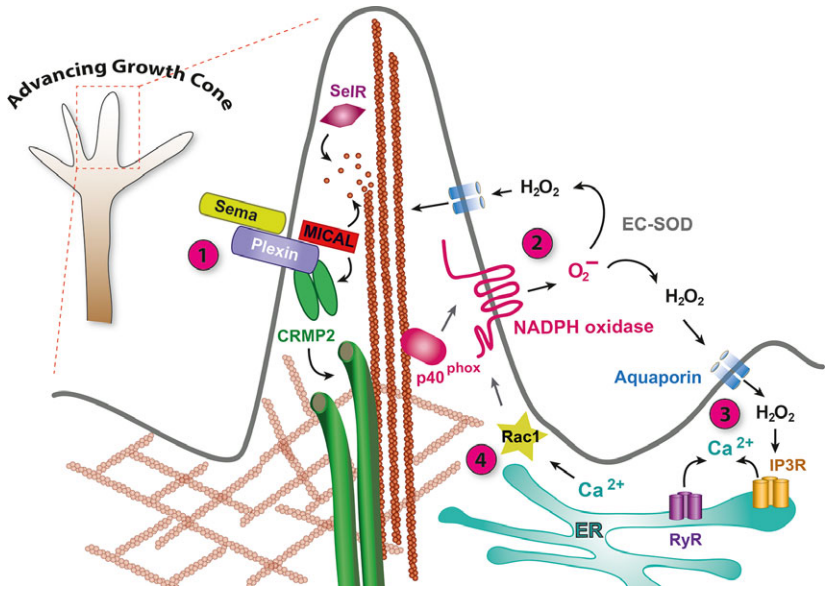

Fig. 1. Regulation of the growth cone cytoskeleton by ROS. (1) Semaphorin binding to Plexin activates MICAL monooxygenase. MICAL interactions with F-actin lead to oxidation of the conserved amino acid Met44 [64]. Oxidation of actin destabilises F-actin filaments and promotes binding of the actin severing protein Profilin, thus promoting F-actin disassembly [67]. Oxidised actin monomers have a reduced propensity for polymerisation. MICAL redox activity is opposed by the MsrB enzyme SelR, which specifically reduces Met-44-R-sulfoxide [66]. MICAL activation *via* Semaphorin-Plexin binding also generates H_2O_2 that can oxidise CRMP2, enabling it to form a disulfide-linked homodimer and to transiently interact with Thioredoxin, which stimulates phosphorylation of CRMP2 by glycogen synthase kinase-3, promoting CRMP2 modification of microtubules and growth cone collapse [71]. (2) Superoxide produced by the NOX2 NADPH oxidase catalytic subunit ($gp91^{phox}$) is regulated by translocation of the $p40^{phox}$ regulatory subunit from its association with F-actin to the plasma membrane upon growth cone engagement with a substrate or guidance cue [58]. Converted into hydrogen peroxide by extracellular superoxide dismutase (EC-SOD), H_2O_2 enters the cytoplasm *via* aquaporins and can oxidise cytoskeletal proteins (e.g. F-actin) [132,133]. (3) H_2O_2 also modifies RyRs and IP3Rs triggering release of calcium from internal stores in the ER. (4) Changes in intracellular calcium modify activities of cytoskeletal regulatory proteins, directly or indirectly, e.g. *via* the regulation of Calcium/calmodulin-dependent kinase II (CamKII) or the phosphatase calcineurin or activation of the protease calpain. Elevated calcium levels also lead to expression of the cytoskeletal and NADPH oxidase regulator Rac1, thus generating a positive feedback loop that can amplify and sustain transient respiratory bursts [34].

Connectivity and structural plasticity

For decades ROS have been implicated in neurodegenerative conditions, largely thought of as destructive agents [72,73]. Increasingly ROS have also been viewed as regulators and modulators of signalling pathways and gene expression, of which many are known to regulate neuronal growth and plasticity (reviewed in [56,57,74]). Several years ago we provided the first direct *in vivo* evidence of ROS as regulators of synaptic terminal growth, under pathological conditions in an experimental animal model (*Drosophila*) for lysosomal storage disease [75]. The study demonstrated that oxidative stress resulting from lysosomal storage dysfunction led to activation of the JNK cascade and activation of the immediate early genes c-Jun and c-Fos (AP-1) which in turn led to altered growth of neuromuscular junction terminals [75]. Along with a prior study by Sanyal and colleagues [76], this work identified AP-1 as the major adaptive response to ROS in neurons. The JNK/AP-1 signalling pathway has long been known to be critical in many neuronal functions and is a well-known mediator of synaptic and oxidative stress responses (reviewed in [74]). AP-1 is a heterodimer composed of the leucine-zipper transcription factors Fos and Jun. Fos is one of the major immediate early transcription factors mediating long-term synaptic changes during long-term potentiation (LTP) [77,78], although the actual mechanism inducing JNK phosphorylation and subsequent AP-1 activation in this process remains obscure. Activation of JNK/AP-1 by oxidative stress is thought to reinforce autophagy, and many genes encoding autophagy proteins are direct transcriptional targets of AP-1 [79]. Some evidence suggests that activation of autophagy *via* the oxidative stress-induced JNK/AP-1 pathway can regulate synaptic terminal size and strength at the *Drosophila* larval neuromuscular synapse [75,76,80]. The data point to the importance of the JNK/AP-1

pathway as regulating synaptic function and to ROS as critical upstream signals during normal physiological conditions as well as under oxidative stress.

In a follow-up study we since asked whether ROS also act as regulators of synaptic terminal growth and plasticity under normal physiological conditions, which until now has remained largely unexplored. Indeed, we found that ROS, in particular hydrogen peroxide, are necessary for activity-induced synaptic terminal growth and are sufficient to drive synaptic terminal growth [81]. Specifically, overactivation of motoneurons leads to increased mitochondrial ROS levels at the presynaptic neuromuscular junction of *Drosophila* larvae, previously also reported for cultured hippocampal neurons [82,83]. In *Drosophila* larvae, activity-generated ROS promote altered synaptic terminal growth, generating more, albeit smaller synaptic varicosities (boutons) along with a reduction in the number of synaptic release sites. Postsynaptic dendrites similarly undergo homeostatic structural adjustments in response to activity-generated ROS, leading to smaller dendritic arbours, which we previously showed equates to reduced synaptic input sites and reduced synaptic drive [84]. The signalling and downstream effector pathways of neuronal activity-generated ROS are only just being sketched out. We found that neuronal ROS signal *via* the conserved redox-sensitive protein DJ-1ß, a homologue of vertebrate DJ-1 (PARK7) [85,86], which appears to act as a neuronal redox sensor. Oxidation of DJ-1ß increases inhibitory interactions with the phosphatase PTEN thus leading to disinhibition of PI3kinase signalling, a known regulator of synaptic terminal growth [87–90]. Whether changes in dendritic growth are also regulated by PTEN-PI3kinase as a ROS effector pathway, and how synaptic terminal growth might be coupled to synaptic connectivity remain to be determined. Importantly, ROS are obligate signals for activity-regulated structural plasticity of synaptic terminals, either instructive or permissive. Targeted abrogation of neuronal ROS signalling in *Drosophila* larval motoneurons, by expression of a modified form of the DJ-1β redox sensor (the conserved Cysteine 106 mutated to a nonoxidisable Alanine [86,91]) prevented the locomotor network from adjusting homeostatically in response to increased levels of network activity, resulting in abnormal motor output [81].

Precisely how neuronal activation leads to the generation of ROS signals is not clear and is likely context specific. In general, ROS are formed as obligate by-products of mitochondrial respiratory ATP synthesis, by 'leakage' of the electron transport chain [72]. Thus, mitochondrial ROS could potentially provide neurons with a readout of their energetic demand. In addition, NMDA receptor stimulation has been shown to trigger ROS generation by either mitochondria [92,93] or NADPH oxidases [94]. NADPH oxidase activity is subject to complex regulatory pathways, of which many are associated with neuronal activation, such as elevated intracellular calcium levels, Protein kinases C and A, as well as calmodulin and calcium/calmodulin-dependent kinase II (CamKII) [34,56,95–98].

It has become increasingly evident that during normal nervous system development and function ROS act as second messengers that regulate multiple aspects, from neuronal polarity and axon growth cone behaviour to structural plasticity. As such ROS have more recently been investigated as potentially involved in the aetiology of psychiatric disorders with neurodevelopmental origins. For example, in mammalian brains cortical parvalbumin-expressing inhibitory interneurons (PVIs) are critical to the excitation–inhibition balance and therefore function of many cortical networks. These fast-spiking neurons are arguably highly susceptible to ROS generated by mitochondrial ATP metabolism and it is thought that their surrounding perineuronal nets confer protection of the PVIs to oxidative challenges [99]. Imbalances in ROS metabolism and buffering are thought of as potentially impacting on the development of cortical networks during so-called critical periods, leading to sub-optimal network performance and neuropsychiatric disorders [100]. Supporting this hypothesis are reports of *post mortem* brain tissue from patients with schizophrenia, bipolar or autism spectrum disorder exhibiting reductions in cortical PVIs [101–103]. Moreover, a recent study did indeed find that conditions of elevated oxidative stress preceded and correlated with reduced integrity of PVIs in mouse models for these disorders [104].

Synaptic transmission and plasticity

Synaptic plasticity describes the ability of synapses to adjust their strength, connectivity and structure in response to previously experienced activity. The inherent plasticity of neurons is key to neuronal network development and in networks allows for adaptation, memory and learning. Synaptic strength may be enhanced or reduced depending upon the neuronal context and the nature of stimulation, the best-studied examples being LTP and long-term depression (LTD). LTP was originally described following repetitive stimulation of the perforant path fibres to the dentate area of the hippocampus [105]. The high-frequency stimulation (HFS) used for induction of LTP results in opening of NMDA receptors and thus elevated intracellular

Ca^{2+}. This leads to the adjustment of synaptic strength *via* direct and transcriptionally regulated modification of synaptic proteins, and changes in the composition of synaptic protein complexes (for review see [57,106]).

A role for ROS in synaptic plasticity has been demonstrated in various model systems and areas of the nervous system (Fig. 2). ROS production is elevated in hippocampal slice preparations following increased neuronal activity, NMDA receptor activation and subsequent LTP [92]. In mouse hippocampus NMDA receptor activation triggers ROS generation through the NOX2 NADPH oxidase, regulated by PKC [94]. Importantly, acute application of cell permeable superoxide scavengers can block HFS-induced LTP in hippocampal slices. Dysregulation of ROS *via* transgenic mis-expression of SOD1 or Catalase similarly blocked LTP, suggesting that LTP requires ROS and at the same time is sensitive to the cellular redox state [107–110]. Conversely, bath applied elevation of ROS in hippocampal slices can be sufficient to induce LTP in the CA1 region [111]. ROS are also required and sufficient for the induction and maintenance of spinal cord LTP, contributing to central sensitisation and chronic neuropathic pain [112]. Interestingly, in cerebellar Purkinje neurons superoxide is required for LTD, although in these cells synaptic depression (as opposed to potentiation) requires elevated intracellular calcium concentration [113].

The specific protein targets of ROS for regulating synaptic plasticity and the interplay between Ca^{2+} and ROS-regulated pathways are exciting and thriving research fields [57]. In particular, the link between ROS and Ca^{2+}-regulated plasticity signalling pathways raises the possibility that synaptic redox state provides a permissive environment that, depending upon context, positively or negatively tunes neuronal plasticity in response to Ca^{2+} signalling. One of the recurrent challenges is to identify the mechanisms through which ROS act, whether these are direct redox modification of effector proteins, or whether ROS impact on cellular function indirectly by modulating other signalling pathways. For example, several protein phosphatases (PP), including PP1 and PP2, PTEN and calcineurin (PP 2B), are regulated by ROS, perhaps suggesting a general role for ROS as negative regulators of kinase cascade pathways [114–119]. In the capacity of phosphatase inhibitors ROS have modulatory access to pathways known to regulate synaptic plasticity. Indeed, in response to H_2O_2 application the phosphorylation state of ERK is upregulated in hippocampal slices, cortical neurons and PC12 cells [120,121]. Similarly, activation of PKC, which is required for hippocampal LTP, is triggered by ROS [111] (Fig. 2).

Direct regulation of synaptic function by redox modification of synaptic proteins also appears likely. For example, exposure of frog NMJs to H_2O_2 revealed their capability to directly regulate synaptic release probabilities. While synaptic strength remained unchanged, the authors observed altered quantal release synchronicity when comparing proximal vs. distal parts of the NMJ, suggesting a modulatory role for

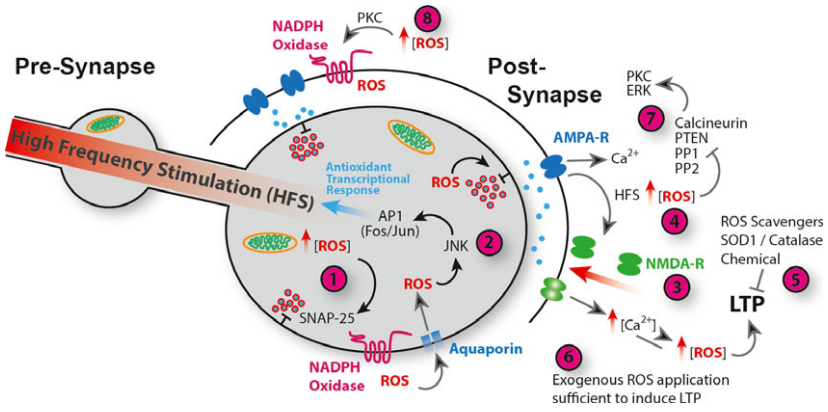

Fig. 2. ROS regulation of Synaptic Plasticity. (1) Presynaptic ROS, derived from mitochondria or NADPH oxidase activity regulates vesicle release *via* oxidation of SNAP-25 [124]. ROS regulate release probabilities with subsynaptic terminal resolution [122,123]. (2) Increases in ROS lead to activation of JNK and AP-1, which promote expression of antioxidant encoding genes [14] and others required for autophagy [79]. AP-1 mediates neuronal adaptive responses to ROS [75,76]. (3) Postsynaptic LTP, in response to HFS, drives recruitment and opening of NMDA receptors and consequent elevation of intracellular Ca^{2+} concentration. HFS causes elevated ROS production and a shift towards an oxidative environment in the synaptic terminal (4) [83,92]. HFS-induced LTP requires ROS (5) [108] and exogenous application of ROS (6) is sufficient to induce LTP in the absence of HFS [111]. ROS regulate canonical synaptic plasticity pathways *via* direct oxidative modification, and inhibition of phosphatases PP1, PP2, PTEN and Calcineurin resulting in increased kinase signalling including ERK and PKC (7) [114–121]. Also, ROS-activated PKC stimulates NADPH-oxidase activation and exacerbated ROS production (8) [107,111].

ROS or redox state with subsynaptic resolution [122,123]. In a follow-up study the same group identified SNAP-25, a component of the SNARE complex and regulator of synaptic vesicle fusion, as a direct ROS target, whose oxidation by H_2O_2 mediated down-regulation of release synchronicity [124]. Excitingly, a recent study demonstrated a role for targeted redox modifications in regulating presynaptic homeostatic adjustments of quantal content. Postsynaptic Semaphorin to presynaptic Plexin signalling activates the MICAL monooxygenase. This is known to specifically modify the actin cytoskeleton, which might impact on the presynaptic cytoskeleton in general and actin-mediated vesicle tethering in particular [125] (Fig. 2).

In summary, current evidence suggests that synaptic plasticity is regulated by both direct and indirect modes of ROS action. It will almost certainly be determined by context, such as local redox state of pre- or postsynaptic sites, the nature (e.g. hydrogen peroxide vs. superoxide vs. nitric oxide), source and subcellular localisation of ROS (e.g. mitochondrial vs. plasma membrane localised NADPH oxidase vs. cytoskeleton associated monooygenase). In addition, different protein modifications might be subject to distinct ROS concentration thresholds, which could endow ROS signalling with a further degree of flexibility and complexity.

Conclusions

A large body of work has demonstrated the importance of ROS as signals that regulate numerous processes during neuronal development and nervous system function. With the majority of work to date having been carried out in cultured cells, it will be important to verify these by studying ROS signalling *in vivo*. While whole animal knockout mutants, for example, of NADPH oxidases, are informative, future work would ideally use cell-specific mosaic manipulations with which to unambiguously determine cell type specificity of ROS requirements. One central aspect will be to determine how ROS generation is regulated, for example, the subcellular localisation of NADPH oxidases and other ROS generators. Previous studies have reported cue-dependent changes in regulatory subunits as mechanisms for controlling ROS production [58,126]. Equally important for our understanding will be genetically encoded tools for visualising ROS and cellular redox potential, ideally with specificity for different ROS species [127–130]. Under normal physiological conditions ROS signals are expected to be confined in space and time. In the nervous system, in particular, there is a need for new reagents that will allow genetically targeted *in vivo* manipulation of ROS generators and scavengers with spatial and temporal control appropriate for studying neuronal cell behaviour and synaptic transmission. Following the success of optogenetic modulators of neuronal excitability, this might take the route for engineering optogenetic solutions for ROS generation and sequestration [131].

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