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REVIEW

Dissecting the role of redox signaling in neuronal development

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Abstract

The generation of abnormally high levels of reactive oxygen species (ROS) is linked to cellular dysfunction, including neuronal toxicity and neurodegeneration. However, physiological ROS production modulates redox-sensitive roles of several molecules such as transcription factors, signaling proteins, and cytoskeletal components. Changes in the functions of redox-sensitive proteins may be important for defining key aspects of stem cell proliferation and differentiation, neuronal maturation, and neuronal plasticity. In neurons, most of the studies have been focused on the pathological implications of such modifications and only very recently their essential roles in neuronal development and plasticity has been recognized. In this review, we discuss the participation of NADPH oxidases (NOXs) and a family of protein-methionine sulfoxide oxidases, named molecule interacting with CasLs, as regulated enzymatic sources of ROS production in neurons, and describes the contribution of ROS signaling to neurogenesis and differentiation, neurite outgrowth, and neuronal plasticity.

Keywords: NADPH oxidase, MICAL, reactive oxygen species, neural progenitor cells, neuronal differentiation, NMDA receptor.

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Reactive oxygen species (ROS) have been canonically described as toxic by-products of aerobic cellular energy metabolism and are associated with the onset of several diseases, particularly those related to aging such as cancer, neurodegenerative diseases, and diabetes (Andersen 2004; Trachootham *et al.* 2009; Sohal and Orr 2012). However, in the last two decades, a growing body of evidence has clearly established that ROS may also be important mediators of normal cellular functions, particularly as second messengers in multiple intracellular signal transduction pathways (Cross and Templeton 2006; Miki and Funato 2012; Weidinger and Kozlov 2015). In 1990, seminal work by Shibanuma *et al.* (1990) provided the first evidence for the involvement of

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Abbreviations used: AngII, angiotensin II; AMPA, alpha-amino-3-hydroxy-5-methylisoxazole-4-propionate; AMPAR, AMPA receptor; AT2R, angiotensin type 2 receptor; BDNF, brain-derived growth factor; BMP, bone morphogenetic protein; CGD, chronic granulomatous disease; CICR, calcium-induced calcium release; CREB, cAMP-responsive element-binding protein; CRMP, collapsin response mediator protein; Dcx, doublecortin; DFO, deferoxamine; DMT-1, divalent metal transporter 1; DMTG, dimethyloxalyl glycine; Duox, dual oxidase; ERK, extracellular signal-regulated kinase; GSK-3β, glycogen synthase kinase 3β; H2O2, hydrogen peroxide; MAPK, mitogen-activated protein kinase; MICAL, molecule interacting with CasL; MRTFA, myocardin-related transcription factor A; mTOR, mammalian target of rapamycin; NAC, N-acetyl cysteine; NGF, nerve growth factor; NMDAR, NMDA receptor; nNOS, neuronal NOS; NOS, nitric oxide synthase; NOX, NADPH oxidase; NPC, neural progenitor cell; LTP, long-term potentiation; O2•−, superoxide anion radical; •OH, hydroxyl radical; ONOO-, peroxynitrite; PDGF, platelet-derived growth factor; PHD2, prolyl hydroxylase domain 2; PI3K, phosphatidylinositol-3 kinase; PIP3, phosphatidylinositol (3,4,5)-trisphosphate; PTEN, phosphatase and tensin homolog; RA, retinoic acid; ROS, reactive oxygen species; RyR, ryanodine receptor; Sema3a, semaphorin 3a; SO, sulfoxide; SRF, serum response factor; TrkB, tyrosine receptor kinase B; Trx, thioredoxin; VCAM1, vascular cell adhesion molecule 1; vGlut1, vesicle glutamate transporter 1.

ROS in signal transduction by showing that platelet-derived growth factor induces the endogenous cellular production of ROS, which is essential for DNA replication. Multiple reports have since been published that provide evidence that the role of ROS in signal transduction is a common feature shared by most organisms, tissues, and cell types (Cooper *et al.* 2002; Forman *et al.* 2004; Chiu and Dawes 2012; Miki and Funato 2012; Bigarella *et al.* 2014). Here, we will discuss accumulating evidence supporting a role for redox-active enzymes-mediated cell signaling in several aspects related to the development and function of neurons.

NADPH oxidases and ROS generation in neurons

Superoxide anion radicals (O2•−), hydroxyl radicals (•OH), peroxynitrite (ONOO−), and hydrogen peroxide (H2O2) are the main ROS produced in cells. These molecules display different reactivity, concentration and lifetime, and most probably play different roles in signal transduction and oxidative stress. Oxidation of cysteine thiol side chains mediated by H2O2 is the most recognized and studied redox reversible post-translational modification, whereas •OH and ONOO- are the products of secondary reactions of H2O2 and O2•−, respectively. Both •OH and ONOO- are highly reactive to proteins, lipids and DNA, being mainly involved in oxidative damage (Dickinson and Chang 2011). The NADPH oxidase family (NOX) of redox-active enzymes represents a regulated source of ROS in many cell types, including neurons (Bedard and Krause 2007; Nayernia *et al.* 2014). These include the classical NOX enzymes, which constitute a family of five members (NOX1–5), and two additional proteins, named Dual oxidase (DUOX) 1 and 2. NOX1, 2, 3, and 5 catalyze the NADPH-dependent conversion of O_2 to $O_2^{\bullet-}$, whereas DUOX 1 and 2 and, more recently, NOX4 have been shown to produce H_2O_2 (Geiszt *et al.* 2003; Gough and Cotter 2011; Takac *et al.* 2011). Irrespective of the species produced primarily, most of $O_2^{\bullet-}$ turns into H_2O_2 enzymatically (by superoxide dismutases) or by spontaneous dismutation (Winterbourn 2008).

ROS production mediated by NOXs has many implications in normal physiology, including the immune response, cell signaling, hormone synthesis, and others (Bedard and Krause 2007).

The best-characterized NOX is the NOX2 complex, which is composed of six catalytic and regulatory subunits arranged in a multimeric enzymatic complex at the plasma or luminal membrane: three cytosolic subunits named p40phox, p47phox, and p67phox; two integral membrane subunits named gp91phox (renamed NOX2) and p22phox; and the small Rho GTPase Rac1 (Bedard and Krause 2007). The most well-characterized isoform is NOX2, which is considered a prototypical NOX. Sequence analyses and hydropathy profiles suggest that NOX2 is a six-pass transmembrane protein, with its N- and C-termini facing the cytoplasmic compartment. NOX2 possesses NADPH- and FADH-binding domains in its C-terminal domain. Transmembrane domains III and V contain two histidine residues that links two heme groups, which are necessary for electron transfer from NADPH to O_2 (Harper *et al.* 1985; Rotrosen *et al.* 1990; Paclet *et al.* 2004; Groemping and Rittinger 2005).

Upon assembly of these subunits in the membrane, this enzyme produces a burst of $O_2^{\bullet-}$ on the extracellular side of the membrane by transferring electrons from NADPH to oxygen (Babior 1999). The membrane protein p22phox interacts with NOX2, the catalytic subunit of the complex, stabilizing the catalytic subunit of the complex and promoting O2•− production (Dinauer *et al.* 1987; Parkos *et al.* 1988; Ambasta *et al.* 2004). Under these conditions, NOX produces $O_2^{\bullet-}$ under a basal steady state called the dormant state; under certain circumstances, however, such as an immune response or growth factor stimulation, $O_2^{\bullet-}$ production increases. In addition to p22phox-mediated stabilization, NOX2 displays molecular partners that enhance ROS synthesis. Interaction with the cytoplasmic protein p67phox increases NOX2 activity, although the p67phox/NOX2 interaction does not always take place, because these proteins reside in different subcellular locations, with p67phox in the cytoplasm and NOX2 at the plasma membrane. Therefore, an additional subunit, $p47^{phox}$, is necessary to promote translocation of $p67^{phox}$ from the cytoplasm to the plasma membrane, where it is involved in NOX2 activation (Nauseef 2004; Groemping and Rittinger 2005; Sumimoto *et al.* 2005). The $p47^{phox}$ functions are regulated by post-translational modifications in its C-terminal domain, which lead to the recruitment of $p67^{phox}$ to the plasma membrane where it binds NOX2. Therefore, the $p47^{phox}$ subunit is considered the assembly organizer subunit of the NOX complex. Finally, two additional subunits are required for proper assembly and function of the NOX complex: the $p40^{phox}$ protein and the actin cytoskeleton regulator Rac. While the precise role of p40phox on ROS production is not clearly known, Rac1/2 are required to induce ROS synthesis after physiological demands (Glogauer *et al.* 2003; Diebold *et al.* 2004; Bokoch and Zhao 2006; Bedard and Krause 2007; Roepstorff *et al.* 2008).

Several isoforms and subunits of NOXs are widely expressed in the central nervous system, especially NOX1, NOX2, and NOX4 (reviewed in Sorce and Krause 2009). The widespread expression of NOX2 and its associated subunits had been detected by inmunohistochemistry in murine brain (Serrano *et al.* 2003; Kim *et al.* 2005). Additionally, NOX isoforms expression has been detected specifically in many neuronal types, including superior cervical ganglion, dorsal root ganglion, and celiac ganglion sympathetic neurons (Hilburger *et al.* 2005; Cao *et al.* 2009; Kallenborn-Gerhardt *et al.* 2012), cerebellar granule neurons (Coyoy *et al.* 2008) and dopaminergic neurons (Choi *et al.*

2012). In cultured primary neurons, isolated from mouse and rat embryonic hippocampus, the presence of the subunits that compose NOX2 has been recently detected (Wilson *et al.* 2015), confirming previous findings (Tejada-Simon *et al.* 2005). Similarly, quantitative PCR analyses for NOX isoforms in murine cortical neurons suggest that the most abundant isoform is NOX4, although low levels of NOX2, p22phox, and DUOX are also detected (Ha *et al.* 2010). The prevalence of NOX4 is replicated in the intact mouse/rat/human midbrain and hindbrain, but not in the forebrain, where the most highly expressed isoform is NOX2 (Infanger *et al.* 2006). NOX4 is also abundant in mouse/rat/human neurons in all-cortical layers, in hippocampal neurons, and in cerebellar Purkinje cells (Vallet *et al.* 2005).

Although not as well characterized, DUOX expression is detected in the brain. Damiano *et al.* (2012) recently detected DUOX expression by immunohistochemistry in rat cerebral cortex. It is particularly enriched in brain membrane fraction and induced by platelet-derived growth factor in SK-N-BE human neuroblastoma cells. The expression of DUOX1 and its maturation factor DUOXA1 is also increased during neuronal differentiation of neuroblastoma P19 cells (Ostrakhovitch and Semenikhin 2011; Ostrakhovitch *et al.* 2012). Recently, Weaver *et al.* (2015) described the expression of nox1, nox2/cybb, nox5, and duox (should this not be NOX1, NOX2 etc.) during the development of the nervous system in zebrafish larvae. Authors found that nox1, nox5, and duox have a variable expression pattern after 2 days post-fertilization. At this time, nox2 expression presented a stable gene expression pattern across several regions of the emerging nervous system of zebrafish. These evidences support the notion that NOX proteins are early expressed and widely distributed across the nervous system in the CNS of zebrafish.

Contribution of ROS to neurogenesis

Redox signaling plays an important role in the differentiation of various cell lineages from their respective precursors (Chaudhari *et al.* 2014), as well as in the clonal expansion of stem cells in their proliferative niches (Wang *et al.* 2013). Therefore, tight regulation of ROS production is likely needed to maintain stemness properties of neuronal precursors in the brain (Dickinson *et al.* 2011; Forsberg *et al.* 2013; Forsberg and Di Giovanni 2014). Both pharmacological inhibition of the NOX complex and the use of antioxidants significantly inhibits proliferation of embryonic hippocampal-derived neural progenitor cells (NPCs) (Yoneyama *et al.* 2010). Moreover, NOX2 knock-out mice ($NOX2^{-/-}$) exhibit a decrease in the number of proliferating progenitors in the adult hippocampus, suggesting that basal ROS production sustained by NOX2 is required for NPCs maintenance (Dickinson *et al.* 2011). In addition, a cross-talk between redox balance, metabolism, and p53 protein regulates the differentiation of neuronal progenitors to neurons (Forsberg and Di Giovanni 2014). These authors also showed that $p53^{-/-}$ mice exhibited an increase in the content of ROS at telencephalic neuronal progenitors, associated with an enhancement of doublecortin, vesicle glutamate transporter, and glutamate decarboxylase GAD65/GAD67 positive cells. These findings suggest that p53 loss-of-function increases ROS levels in neuronal progenitors promoting its differentiation toward neuron lineage. Moreover, both p53 ectopic expression and the treatment with the general antioxidant N-Acetyl cysteine decreased both neurogenesis and neurite outgrowth (Forsberg *et al.* 2013; Forsberg and Di Giovanni 2014), suggesting that ROS levels regulate *in vitro* and *in vivo* NPCs commitment. Although the down-regulation of ROS decreases neurogenesis, the up-regulation stops the differentiation of neural precursors into neurons, suggesting that ROS fine-tunes the maintenance of NPCs population (Tsatmali *et al.* 2006; Dickinson *et al.* 2011; Forsberg *et al.* 2013; Forsberg and Di Giovanni 2014).

Our knowledge on the mechanisms that regulates NOX activity by extracellular/intracellular ligands is still fragmentary. It is relevant, therefore, to identify and define extracellular stimuli involved in NOX activation in the brain. Recent evidences suggest that angiotensin II (AngII) triggers NPCs proliferation by binding to type II receptors (Chao *et al.* 2013). AngII-induced NPCs proliferation is dependent on the production of ROS by NOX4, the major NOX isoform present in these cells (Topchiy *et al.* 2013). Pharmacological or genetic loss-of-function of NOX4 abrogates AngII-induced ROS production and NPCs proliferation (Topchiy *et al.* 2013). AngII induces both mitochondrial and extra-mitochondrial production of ROS in neuronal cells, as observed by partial localization of NOX4 in this organelle (Case *et al.* 2013; Topchiy *et al.* 2013). The precise mechanism by AngII regulates NOX4-mediated ROS production is still unknown. AngII increases NOX4 protein levels in NPCs, suggesting a transcriptional or translational regulation (Topchiy *et al.* 2013). However, treatment with AngII at short intervals (5-60 min) increases ROS production in NPCs and other cell types, an effect inhibited by NOX4 genetic loss-of-function, suggesting that in addition to transcriptional regulation, a direct regulation of NOX activity is likely (Gorin *et al.* 2003; Massey *et al.* 2012; Case *et al.* 2013; Topchiy *et al.* 2013; Somanna *et al.* 2016).

Another extracellular ligand associated with ROS-dependent control of neurogenesis is the vascular cell adhesion molecule-1. This adhesion receptor regulates NOX2 expression and activation, in order to maintain adequate ROS levels required to preserve quiescence of NPCs in the subventricular zone (Fig. 1) (Kokovay *et al.* 2012).

In addition, brain-derived growth factor (BDNF) also regulates NOX2-dependent ROS production in order to maintain the self-renewal of NPCs. Accordingly, BDNF

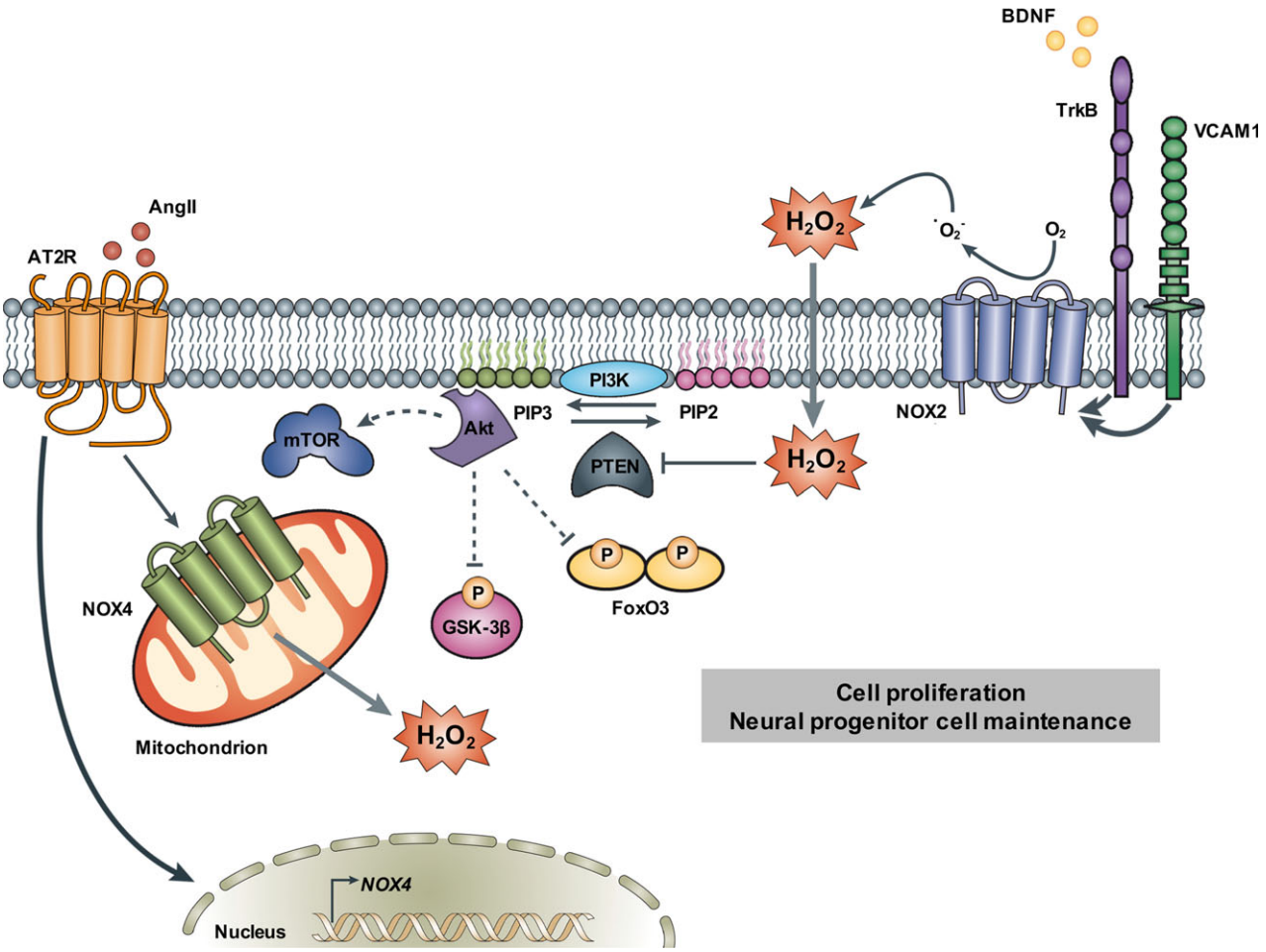

Fig. 1 Redox signaling in neurogenesis. Neural progenitor cell (NPC)s proliferation and stemness are maintained by enzymatic reactive oxygen species (ROS) production. Adhesion-mediated VCAM1 signaling or the stimulation of AT2R or the brain-derived growth factor (BDNF) receptor, TrkB, induces the activation of NADPH oxidase family members such as NOX2 and NOX4 to increase H_2O_2 levels. A local increase in H_2O_2 could inactivate the Phosphatase and tensin homolog (PTEN), altering the phosphoinositide-phosphate balance toward phosphatidylinositol (3,4,5)-trisphosphate (PIP3). The increase in PIP3 activates Akt, which in turn would activates mammalian target of rapamycin (mTOR) and inhibits glycogen synthase kinase 3β (GSK-3β) or FoxO3, controlling NPCs proliferation.

induces endogenous production of superoxide, but is unable to stimulate the self-renewal of NPCs derived from NOX2 knockout mice (Le Belle *et al.* 2011). BDNF binding to its tyrosine kinase receptor tyrosine receptor kinase B, is coupled to activation of phosphatidylinositol-3 kinase (PI3K), that rises phosphatidylinositol (3,4,5)-trisphosphate (PIP3) levels. Subsequently, PIP3 stimulates Akt activity in order to regulate several process including neuroprotection (Chen *et al.* 2013), synapsis formation (Luikart *et al.* 2008), and neural crest-derived cell proliferation (Dewitt *et al.* 2014). The oxidative inactivation of phosphatase and the tensin homolog (PTEN), the phosphatase involved in PIP3 dephosphorylation, could be a key event triggered by ROS for regulating this process (Lee *et al.* 2002). The administration of exogenous H2O2 to NPCs oxidizes and reversibly inactivates PTEN, favoring the activation of PI3K-Akt. Consistently, PTEN-deficient cells do not respond to BDNF stimulation or to treatment with exogenous H2O2. Likewise, pharmacological inhibition of PI3K eliminates the positive effects of ROS in neurogenesis (Le Belle *et al.* 2011). Activation of the PI3K-Akt pathway has also been associated with the phenotype of premature neurogenesis observed in the p53 knockout mice, but a role for NOXs has not been established in this model (Forsberg *et al.* 2013). Downstream targets of Akt in this process are unknown, although some interesting candidates arise. Activated Akt phosphorylates and inactivates glycogen synthase kinase 3β (GSK-3β). Indeed, decreased GSK-3β activity promotes NPCs proliferation (Sato *et al.* 2004; Ying *et al.* 2008; Kim *et al.* 2009). Akt also inhibits the transcription factor FoxO3. A constitutive active FoxO3 expressing transgenic mice shows disminished NPCs numbers and reduced brain size (Sch-

midt-Strassburger *et al.* 2012). Finally, Akt activates mammalian target of rapamycin, and a conditional deletion of mammalian target of rapamycin in NPCs impairs self-renewal (Fig. 1) (Ka *et al.* 2014). How variations in the production of ROS mediate successive steps of proliferation and differentiation is still a matter of debate. On the one hand, some studies suggest that NOX2-mediated ROS production is associated with self-renewal and multipotency of NPCs (Le Belle *et al.* 2011; Forsberg *et al.* 2013). On the other hand, other studies have indicated that high levels of ROS induce acquisition of a differentiated neuron phenotype through the increased expression of mitochondrial respiratory chain proteins (Tsatmali *et al.* 2005, 2006). This apparent contradiction may reflect specific requirements of ROS signaling when NPCs are either proliferating or quiescent (Le Belle *et al.* 2011).

Redox signaling in axonal outgrowth and guidance

ROS functions are not only related with the regulation of NPCs proliferation and commitment. There are redox-dependent mechanisms that promote neuronal differentiation. This process depends mainly on dynamic changes that affect microtubules and actin filaments in response to extracellular signals (Neukirchen and Bradke 2011). The regulation of cytoskeleton dynamics by ROS had been recently reviewed, underlining the relevance of cytoskeleton as effector of redox signaling (Stanley *et al.* 2014; Valdivia *et al.* 2015; Wilson and González-Billault 2015). Studies in *Aplysia* neurons revealed that pharmacological inhibition of NOX using apocynin or VAS2870 reduces actin polymerization in growth cones, decreases retrograde actin flow, reduces neurite outgrowth and modifies the structure of actin in the growth cone transition zone, impairing growth cone formation (Munnamalai and Suter 2009; Munnamalai *et al.* 2014; Altenhofer *et al.* 2015). Moreover, NOX inhibition using several strategies, including the expression of dominant negative variant of $p22^{phox}$, delayed axon specification and outgrowth, possibly through decreased activity of Rho GTPases, Rac1 and Cdc42 (Wilson *et al.* 2015). Reduced neurite outgrowth is also been observed in cerebellar granule neurons derived form $NOX2^{-/-}$ mice (Olguin-Albuerne and Moran 2015). Likewise, either pharmacological inhibition or siRNA-mediated knock down of NOX2 decreased bone morphogenetic protein-7-induced dendritic growth in cultured rat sympathetic neurons (Chandrasekaran *et al.* 2015). Neuronal cell lines differentiation is also dependent on the role of ROS. Nerve growth factor-induced neurite outgrowth in PC12 cells is inhibited by antioxidants, pharmacological inhibition of NOX or dominant-negative Rac1 expression (Suzukawa *et al.* 2000). Similar results are observed in several models of neurite outgrowth, including retinoic acid (RA)-differentiated SH-SY5Y human neuroblastoma cells (Nitti *et al.* 2010), neuregulin- or staurosporin-treated PC12 cells (Goldsmit *et al.* 2001; du Kim *et al.* 2013) and staurosporin-treated HN33 hippocampal cells (Min *et al.* 2006).

Once generated, axons navigate to its final destination guided by positive and negative extracellular cues (Tessier-Lavigne and Goodman 1996). Semaphorins belong to a prototypical family of secreted and membrane-associated proteins that inhibit axonal growth to specific regions in the nervous system. Semaphorins exert their effects by binding to cell surface receptors of the Plexin and neuropilin families (Pasterkamp 2012). Molecule interacting with CasL (MICAL), is a Plexin effector, originally identified in a genetic screen for Plexin A-binding partners in *Drosophila melanogaster* (Terman *et al.* 2002). Its structure is characterized by the presence of a flavin monooxygenase domain (FAD) as well as several protein–protein interaction motifs, including calpostin homology domain, a **LIM**(Lin11, Isl-1 & Mec-3) domain, and a coiled-coil domain. In vertebrates, three genes encode for MICAL-1, MICAL-2, and MICAL-3, while two additional genes encode shorter versions of the protein known as MICAL-like1 and MICAL-like2 (Giridharan & Caplan, 2014). These last two short isoforms lack FAD domain, but include the rest of protein–protein interaction motifs. Semaphorin-induced MICAL activation target neuronal cytoskeleton proteins, involved in actin and microtubule dynamics. On one hand, MICAL oxidize G-actin monomers leading to growth cone collapse (Hung *et al.* 2010). MICAL-mediated actin oxidation can be reversed by the methionine sulfoxide reductase MsrB1, providing a regulated redox modification on G-actin (Fig. 2) (Hung *et al.* 2011, Hung *et al.* 2013; Lee *et al.* 2013). In addition, MICAL-2 specifically promotes depolymerization of nuclear actin, which stimulate transcriptional mechanisms dependent on the Serum response factor/myocardin-related transcription factor-A (MRTF-A), to enhance neurite outgrowth (Lundquist *et al.* 2014). It is therefore conceivable that redox regulation of actin microfilament pools in nuclei and cytoplasm differentially contribute to neuronal differentiation.

On the other hand, MICALs forms a complex with Plexin A and collapsin response mediator proteins (CRMPs), providing a molecular link connecting repulsive extracellular cues with microtubules (Schmidt *et al.* 2008). MICAL-dependent oxidation of CRMP-2 Cys504 promotes the formation of disulfide-linked homodimers (Morinaka *et al.* 2011). Oxidization of CRMP-2 can then be reduced by thioredoxin, which in turn generates a disulfide-bridged intermediate. Such cysteine-linked intermediate complex promotes CRMP-2 phosphorylation by GSK-3β, favoring microtubule depolymerization (Morinaka *et al.* 2011) (Fig. 2). Consistently, MICAL-1 knockout mice present developmental defects in the nervous system that result from abnormal actin cytoskeleton dynamics and cell adhesion (Van Battum *et al.* 2014). Given the limited number of described substrates for MICAL, we anticipate that such

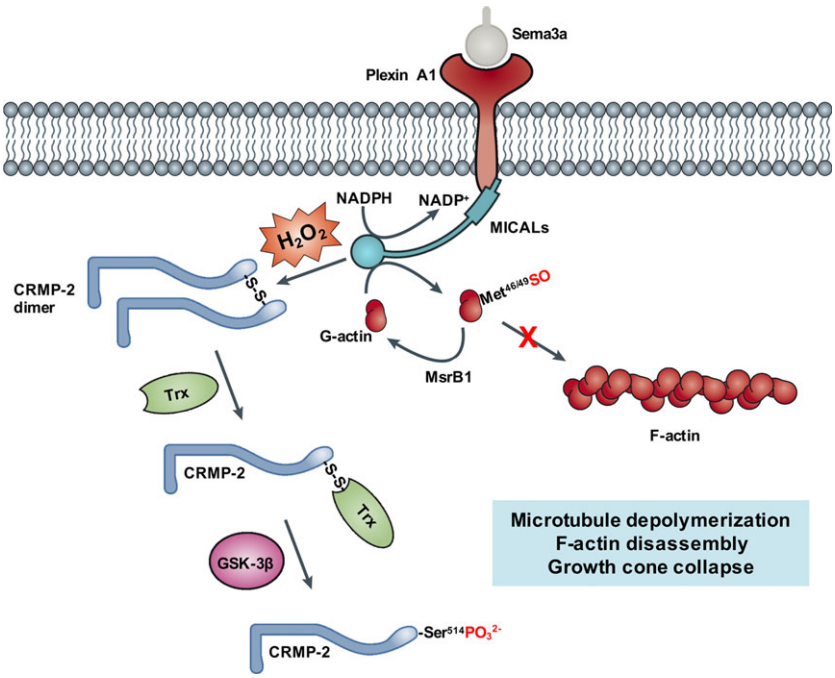

Fig. 2 Redox signaling in axonal growth cone cytoskeletal dynamics. The binding of the axon-repulsive cue Semaphorin 3a (Sema3a) to its membrane receptor, Plexin A1, releases the autoinhibitory conformation of MICALs, a group of FAD-dependent monooxygenases, which mediate the oxidative modification of actin and CRMP-2. Two methionine residues (Met46 and Met49) in G-actin are reversibly oxidized to methionine sulfoxide (SO), inhibiting its incorporation into actin filaments. In contrast, oxidation of Cys504 in CRMP-2 forms a disulfide-linked dimer, favoring thioredoxin (Trx)-mediated GSK-3β phosphorylation of collapsin response mediator protein (CRMP)-2 in Ser 514, which leads to microtubule depolymerization and growth cone collapse.

redox regulation will likely be involved in other aspects of neuronal differentiation. For example, MICAL3 regulates Rab6/Rab8 exocytic vesicles docking and fusion, through the oxidative modification of hitherto unknown proteins (Grigoriev *et al.* 2011), possibly regulating neuronal functions associated with vesicular traffic, such as neurite outgrowth (Villarroel-Campos *et al.* 2014).

The role of ROS in NMDA receptor-mediated plasticity, LTP and memory

One of the most studied forms of synaptic plasticity is hippocampal long-term potentiation (LTP), in which activation of synaptic N-methyl-D-aspartate receptors (NMDARs) leads to insertion of AMPA receptors into the postsynaptic membrane, a process driven by calcium entry, CaMKII and/or the Ras-extracellular-regulated kinase (ERK) pathways (Malenka and Nicoll 1999; Lisman *et al.* 2012). Calcium entry through synaptic NMDARs also activates the ERK-mitogen-activated protein kinase signaling cascade that phosphorylates cAMP-responsive element binding protein, a transcription factor that can translocate to the nucleus to mediate gene transcription of multiple "synapse-associated genes" required for memory consolidation (Greer and Greenberg 2008). The fact that NOX proteins are also expressed at synaptic sites of mature hippocampal neurons suggest that NOX may have a role in neurotransmission (Tejada-Simon *et al.* 2005; Vallet *et al.* 2005; Sorce and Krause 2009; Massaad and Klann 2011). NMDAR activation promotes $O_2^{\bullet-}$ production by NOX in hippocampal mature neurons, suggesting that glutamatergic and excitatory synapses are intimately related to ROS production and NOX activity (Brennan *et al.* 2009; Reyes *et al.* 2012). In agreement, patients affected with the inherited syndrome called chronic granulomatous disease (CGD), in which NOX proteins exhibit missense mutations that are unable to produce physiological concentrations of $O_2^{\bullet-}$, show cognitive dysfunction and lower intellectual coefficient compared to control population (Pao *et al.* 2004). The consequence of NOX2 deficiency in the intellectual disability has been questioned in patients with CGD (Cole *et al.* 2013). A criticism to these data is that children with CGD required long-term hospitalization, affecting normal school attendance and normal intellectual development during childhood. However, children that present other infectious diseases, that also required long-term hospitalization, did not develop cognitive deficits, suggesting a NOX-specific phenotype. Further analysis will be required to understand the impact of NOX loss of function in cognitive development and neuronal function in humans. Consistent with the phenotype observed in CGD patients by Pao *et al.*, mice lacking gp91phox or p47phox (mouse models for CGD) show impaired LTP and hippocampus-dependent memory (Kishida *et al.* 2006). The absence of a severe phenotype can be explained by a putative compensatory effect, since over-expression of NOX4 has been observed in the gp91phox knockout mouse (Pendyala *et al.* 2009). However, NOX isoforms are not completely equivalent, showing differential response to agonists and selective activation of signaling cascades (Anilkumar *et al.* 2008).

Additional studies using ROS scavengers and pharmacological manipulations to alter NOX activity also demonstrate that NOX-induced $O_2^{\bullet-}$ production is required for NMD AR-mediated ERK pathway activation, the full expression of NMDAR-mediated LTP, and hippocampal-dependent memory tasks (Klann 1998; Thiels *et al.* 2000; Massaad and

Klann 2011). Studies that monitor intracellular accumulation of fluorescent oxidized dihydroethidium (dHEh) have shown that $O_2^{\bullet-}$ in hippocampal neurons is produced as a result of NMDAR activation (Bindokas *et al.* 1996; Brennan *et al.* 2009). NOX activity has been identified as a source of NMDA-induced $O_2^{\bullet-}$ production (Brennan *et al.* 2009; Girouard *et al.* 2009; Guemez-Gamboa *et al.* 2011), although other sources, including mitochondria and nitric oxide synthase (NOS), have also been implicated in ROS generation in neurons (Dugan *et al.* 1995; Bindokas *et al.* 1996; Massaad and Klann 2011). While the above-mentioned studies indicate that NMDA-induced $O_2^{\bullet-}$ production can function as an intracellular messenger in LTP, at the same time production of this anion by activation of NMDARs can also promote neurotoxicity (Lafon-Cazal *et al.* 1993; Patel *et al.* 1996; Suh *et al.* 2008; Brennan *et al.* 2009), including in neighboring neurons and astrocytes (Reyes *et al.* 2012). Whether ROS function as beneficial intracellular messengers or as neurotoxic molecules likely depends on which NMDAR subtype is activated, its specific localization, and duration of activity. NMDARs are composed of two obligatory NR1 subunits plus two NR2A-D and/or NR3A-B subunits; the precise subunit combination determines the physiological and pharmacological properties of the receptor, their binding partners and downstream signaling effects (van Zundert *et al.* 2004). Functional NMDARs are located both at the synaptic and extrasynaptic membrane, however, they are linked to different underlying signaling cascades and can have opposite functions in physiological (van Zundert *et al.* 2004) and pathological (Hardingham and Bading 2003) neuronal processes.

As discussed above, it is well established that activation of synaptic NMDARs induces calcium entry into postsynaptic terminals that can activate ERK signaling pathways to induce local synaptic plasticity and/or gene transcription required for memory consolidation (Greer and Greenberg 2008). Interestingly, more recently it has also been shown that a rise in calcium levels in dendritic spines can trigger the opening of ryanodine receptors (RyR), stimulating additional calcium release from the endoplasmic reticulum; a phenomenon termed calcium-induced calcium release (Emptage *et al.* 1999). Interestingly, RyRs are extremely sensitive to redox modifications, with oxidizing reagents activating the channel, whereas reducing compounds inhibiting this receptor (Murayama *et al.* 1999; Hidalgo *et al.* 2005). Studies in hippocampal slices have indicated that increased ROS (H_2O_2 or $O_2^{\bullet-}$) levels stimulate ERK and cAMP-responsive element binding protein phosphorylation through oxidative

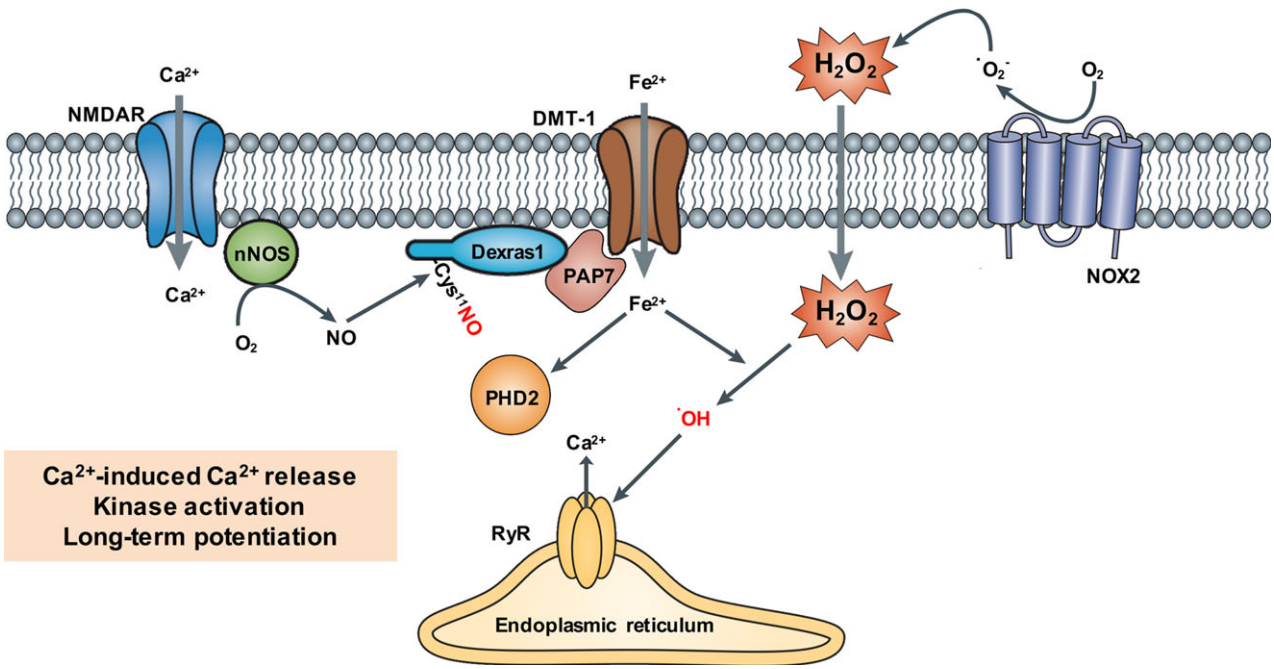

Fig. 3 Redox-dependent NMDA signaling. NMDA receptor (NMDAR) activation at the postsynaptic membrane induces calcium entry and activation of neuronal NOS (nNOS). NO modifies a specific cysteine residue in the small GTPase Dexras1, thus enhancing iron uptake, partially through the formation of a ternary complex between Dexras1, peripheral benzodiazepine receptor-associated protein (PAP7) and divalent metal transporter 1 (DMT-1). In addition, NMDAR activation is linked to NOX2 complex assembly, leading to an increase in local H_2O_2 levels. Iron and H_2O_2, through Fenton chemistry, produce hydroxyl radical-mediated oxidative sensitization of the ryanodine receptor (RyR) that is involved in calcium-induced calcium release, enhancing long-term potentiation (LTP). In addition, iron activates prolyl hydroxylases, such as PHD2, to mediate its effects on LTP.

modifications of RyRs (Kemmerling *et al.* 2007; Huddleston *et al.* 2008). In addition, recent studies show that iron stimulates NMDAR-mediated calcium-induced calcium release hereby increasing ERK activation and synaptic plasticity (Fig. 3) (Munoz *et al.* 2011). The precise mechanisms by which iron regulates the physiology of RyRs and other compounds of the postsynaptic compartment are still unknown. The capacity of iron to transition between two oxidation states under physiological conditions makes this metal a preferred co-factor in several redox enzymes, particularly hydroxylases; alternatively, free iron could also mediate the non-enzymatic transformation of H_2O_2 into the highly reactive hydroxyl radical through the Fenton reaction (Núñez *et al.*, 2012; Dixon and Stockwell 2014). How redox-active transition metals, such as iron and copper, modulate NMDAR-mediated synaptic plasticity has recently been hypothesized (Hidalgo *et al.* 2007; Gaier *et al.* 2013).

In glutamatergic neurons, nitric oxide production by NOS leads to covalent modification of Cys11 in a small GTPase named Dexras1, stimulating hereby iron entry through the two classical routes of cellular uptake: transferrin-mediated entry, which specifically incorporates iron bound to the plasma protein transferrin, and divalent metal transporter 1 (DMT-1)-mediated entry, which allows the entry of iron that is not bound to transferrin directly from the extracellular milieu (Cheah *et al.* 2006). Iron entry mediated by activation of the NMDAR apparently increases hydroxyl radical production as measured by an increase in fluorescence of hydroxyphenyl fluorescein, a hydroxyl radical-sensitive probe (Cheah *et al.* 2006). Hydroxyl radical production in the postsynaptic terminal is favored, because the activation of the NMDAR is coupled to activation of NOX2, which increases the levels of $O_2^{\bullet-}$, which then dismutates to H_2O_2 via activity of the superoxide dismutase 1 enzyme or spontaneously. These actions thus generate the conditions for the Fenton reaction in a microdomain in close proximity to the NMDAR, and can explain how iron-mediated hydroxyl radical generation induces the oxidative activation of the RyR, inducing increases in intracellular calcium and activation of ERK1/2, hereby stimulating synaptic plasticity (Fig. 3) (Munoz *et al.* 2011). Alternatively, iron may be involved in the activation of prolyl hydroxylases in the postsynaptic compartment as suggested by the observation that incubation of hippocampal slices or isolated hippocampal neurons with 10 μM deferoxamine, an iron chelator, impairs LTP, similar to the prolyl hydroxylase chemical inhibitor dimethyloxalyl glycine. Moreover, genetic models specific for inactivation of the prolyl hydroxylase domain 2 enzyme exhibit similar deficits in LTP, which cannot be exacerbated by the use of deferoxamine or dimethyloxalyl glycine (Corcoran *et al.* 2013). These findings suggest that activation of prolyl hydroxylase domain 2 by iron underlies its observed effects in promoting LTP (Fig. 3).

Future directions

Although the involvement of ROS as second messengers in cell signaling is a well-accepted concept in the physiology of multiple cell types, our understanding of ROS-mediated cell signaling in neurons is not yet complete. During the process of neuronal differentiation from NPC to their integration into neural circuits, the fragmentary evidence suggests that ROS are essential regulators in the formation and function of the central nervous system. In the next few years, studies related to this issue will likely focus on two essential mechanistic questions: the nature of the signals that regulates ROS concentrations inside the neurons and the putative targets susceptible to oxidation related to neuronal function in health and disease.

Acknowledgments and conflict of interest disclosure

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