"Estudio de la relación funcional entre el complejo NADPH oxidasa y la liberación de Ca^{2+} del retículo endoplasmático en el establecimiento de la polaridad en neuronas de hipocampo"

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Se informa a la Escuela de Postgrado de la Facultad de Ciencias que la Tesis de Doctorado presentada por el candidato.

Carlos Antonio Wilson Rodríguez

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Resumen biográfico

Nací el 29 de septiembre de 1987 en Santiago, Chile. Luego de egresar del Instituto Nacional el año 2005, ingresé a estudiar Bioquímica en la Universidad de Chile durante el período 2006-2011. Realicé mi memoria de título bajo la supervisión del Dr. Manuel Estrada, profesor de la Facultad de Medicina. En marzo del 2012 comencé mis estudios en el programa de Doctorado en Ciencias, mención Biología Molecular, Celular y Neurociencias, realizando mi tesis de doctorado bajo la supervisión de los profesores Christian González Billault y Marco Tulio Núñez.

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Index

Introduction

Reactive oxygen species (ROS) mainly composed by the hydroxyl radical, anion superoxide and hydrogen peroxide, deeply impact on neuronal functions. High ROS levels lead to cell death and neurodegeneration whereas physiological concentrations of ROS are needed to support several neuronal processes ranging from neuronal development to neurotransmission (Bedard and Krause, 2007). Both neuronal development and neurotransmission depend on proper cytoskeleton remodeling to shape neuronal morphology in response to the physiological demands of the nervous system.

Neurons are highly polarized cells characterized by the development of both the somato-dendritic and axonal compartments (Caceres et al., 2012). The acquisition of this characteristic morphology is the result of a well stereotyped sequence of transformations called *The Establishment of Neuronal Polarity*. Briefly, cultured neurons are initially symmetric cells, including a peripheral actin rich structure (stage 1, 6 h *in vitro*). Then, plasma membrane projections emerge from the cell periphery developing minor neurites (stage 2, 18-24 h *in vitro*). At this stage all the neurites have the same average length. After 24-48 h *in vitro* the neuron breaks the symmetry of the cell, which means that one of the minor neurites grows 5-6 times faster than the others, specifying the putative axon of the neuron (stage 3). Later, both minor neurites and axon keep growing developing several branching points (stage 4, 3-10 days *in vitro*). Finally, the axon initial segment is assembled and minor neurites acquire the dendritic identity. At this point neurons are able to establish communication with other neuronal and non-neuronal cells through the propagation of an action potential (stage 5, 10 days *in vitro*) (Dotti et al., 1988).

From a molecular point of view, the establishment of neuronal polarity may be summarized in 5 key cellular concepts: 1) Development of a polarized cytoplasmic flow to the first neurite, a necessary condition to specify the axon (Bradke and Dotti, 1997), 2) Selective destination/retention of axonal and dendritic molecular determinants in axon and dendrites, respectively, which allows neuronal polarization (Sampo et al., 2003), 3) Appearance of a proper actin and tubulin cytoskeleton dynamics that support the morphological transformation of the cell, leading to neurite outgrowth (Stiess and Bradke, 2011), 4) Selective degradation and removal of inhibitory molecules for the

axonal growth, present in this structure (Cheng et al., 2011) and 5) Establishment of the *Axon Initial Segment,* a molecular filter between the axon and dendrites. This structure blocks the exchange between both compartments, recruits ion channels needed for action potential development and support the maintenance of neuronal polarity (Rasband, 2010). In addition, extracellular signals like the neurotrophic factors NT-3 and BDNF, secreted by neurons during the initial stages of polarization (1-2 days *in vitro*) (Nakamuta et al., 2011), as well as the nucleotides cGMP and cAMP, among others, promote the establishment of polarity through the activation of signaling pathways needed to this purpose (Namba et al., 2015). Together, both intracellular and extracellular factors contribute to the morphological transformation of the neuron during polarity acquisition.

Actin filaments and microtubules confer a structural platform to the establishment of neuronal polarity (Stiess and Bradke, 2011). The regulation of cytoskeleton dynamics is a complex molecular event in which several signaling pathways participate. Actin filaments and microtubule dynamics depend on many signaling events that include redox balance, which means a proper regulation between the concentration of oxidative and reductive molecules inside the neurons (Wilson and Gonzalez-Billault, 2015). Whereas high concentration of oxidative molecules lead to F-actin severing and microtubule catastrophe, the inhibition of ROS synthesis impairs actin polymerization affecting neurite outgrowth, neuronal development and polarization of neurons. Based on these data, it is possible to suggest that the redox balance is crucial for the normal development of neurons.

The NADPH oxidase (NOX) enzymes represent the main enzymatic and regulated source of ROS in neurons (Lambeth, 2004; Nayernia et al., 2014). The NOX enzymes are a family of proteins composed by 7 isoforms (NOX1-5 and Duox1-2) which catalyze the conversion of O_2 into the superoxide radical $O_2^{\cdot-}$. NOX1, 2 and 4 are expressed in the nervous system being the NOX2 the best characterized of them (Nayernia et al., 2014). NOX2 has 5 molecular interactors ($p22^{phox}$, $p40^{phox}$, $p47^{phox}$, $p67^{phox}$ and the Rho GTPase protein Rac1), which together establish the NOX complex to produce ROS according to the physiological demands (Bedard and Krause, 2007).

This thesis describes that the loss of function of the NOX complex, utilizing both genetic and pharmacological models, decreases the F-actin content, impairs F-actin dynamics and reduces both the polarization and axonal growth of hippocampal neurons. In contrast, specific activation of the NOX complex promotes the establishment of neuronal polarity as well as increases the axonal growth. From our data is possible to propose that ROS from the NOX complex is needed to sustain a proper F-actin dynamics through the regulation of the Rho GTPase protein Rac1, suggesting a physiological redox control of the actin cytoskeleton dynamics in neurons. This work also explores the mechanistic aspects that link ROS production with actin dynamics regulation. We study Ca^{2+} release from the endoplasmic reticulum (ER) mediated by the Ryanodine receptor (RyR) as a likely intermediate between ROS synthesis and F-actin dynamic regulation. This hypothesis was elaborated based on the following evidence. First, ROS regulate Ca^{2+} release from ER in several cell types including neurons (Espinosa et al., 2006; Hidalgo et al., 2006; Zhang and Forscher, 2009; Donoso et al., 2011; Riquelme et al., 2011; Adasme et al., 2015). Second, Ca^{2+} release from the ER, mainly mediated by the RyR, promotes Rac1 activation in cell lines and neuronal models (Price et al., 2003; Jin et al., 2005). On the other hand, it has been previously described that Rac1 is a key molecule for neuronal polarization and axonal growth (Gonzalez-Billault et al., 2012). We find that the NOX complex is functionally coupled to the RyR in neurons undergoing polarization, situation that impact positively on both Rac1 activity and axonal growth of neurons. Collectively, these data suggest that the NOX complex contributes to the establishment of neuronal polarity and axonal growth through the regulation of the actin cytoskeleton.

This manuscript is composed by 5 chapters corresponding to original reports and reviews either published or submitted to peer-review. Chapter 1, entitled *"Dissecting the role of redox signaling in neuronal development"*, published in 2016, summarizes the main evidence that supports the notion that ROS may act as signaling molecules required for the development and function of the nervous system (Borquez et al., 2016). Chapter 2, entitled *"Contribution of the NADPH oxidase to the establishment of neuronal polarity in culture"*, is an original research article published in the Journal of Cell Science in 2015. This work describes the contribution of NOX2 to the acquisition of neuronal polarity, suggesting that basal ROS levels are required for both the polarization and axonal growth of neurons involving the actin cytoskeleton (Wilson et

al., 2015). Chapter 3, *"Regulation of cytoskeletal dynamics by redox signaling and oxidative stress: implications for neuronal development and trafficking"* published in Frontiers in Cellular Neuroscience on 2015, summarizes the main evidence that supporst the notion that actin and microtubule cytoskeletons depend on redox balance, phenomena that impact on vesicle trafficking, polarity and general neuronal development (Wilson and Gonzalez-Billault, 2015). Chapter 4, *"Neuronal actin microfilaments: a target for redox species"* is a recently submitted review to Cytoskeleton. This manuscript summarizes main findings supporting the notion that the actin cytoskeleton is a target of redox species, with emphasis on NADPH oxidase (NOX) and MICAL (from the Molecule Interacting with CasL) enzymes as source of ROS. Finally, Chapter 5 is an original research article entitled *"A feed-forward mechanism involving the NOX complex and calcium release from the endoplasmic reticulum mediated by the ryanodine receptor promotes axonal growth"* also recently submitted to Cell Reports. On one hand, this article suggests that increasing levels of ROS derived from the NOX complex promote the polarization of neurons. On the other hand, this article also suggests that the NOX complex is required to promote ER Ca^{2+} release mediated by RyR in hippocampal neurons during the initial stages of neuronal polarization. Moreover, cultured neurons derived from the $p47^{phox}$ null mice ($Ncf1^{-/-}$ in this text) do not polarize properly and axonal growth is impaired compared to wild type neurons. In addition, $Ncf1^{-/-}$ neurons exhibit reduction of both ER Ca^{2+} release mediated by the RyR and Rac1 activity. Collectively, these data support the notion that the functional coupling between NOX and RyR impacts positively on the activation of Rac1 and axonal growth as well as the establishment of neuronal polarity.

In summary, this thesis proposes a novel function for ROS, supporting the hypothesis that, under physiological concentrations, ROS may act as signaling molecules required for the proper development and function of neurons.

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Chapter 1

Dissecting the role of redox signaling in neuronal development

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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; $O_2^{\bullet-}$, 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.* 2015).

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

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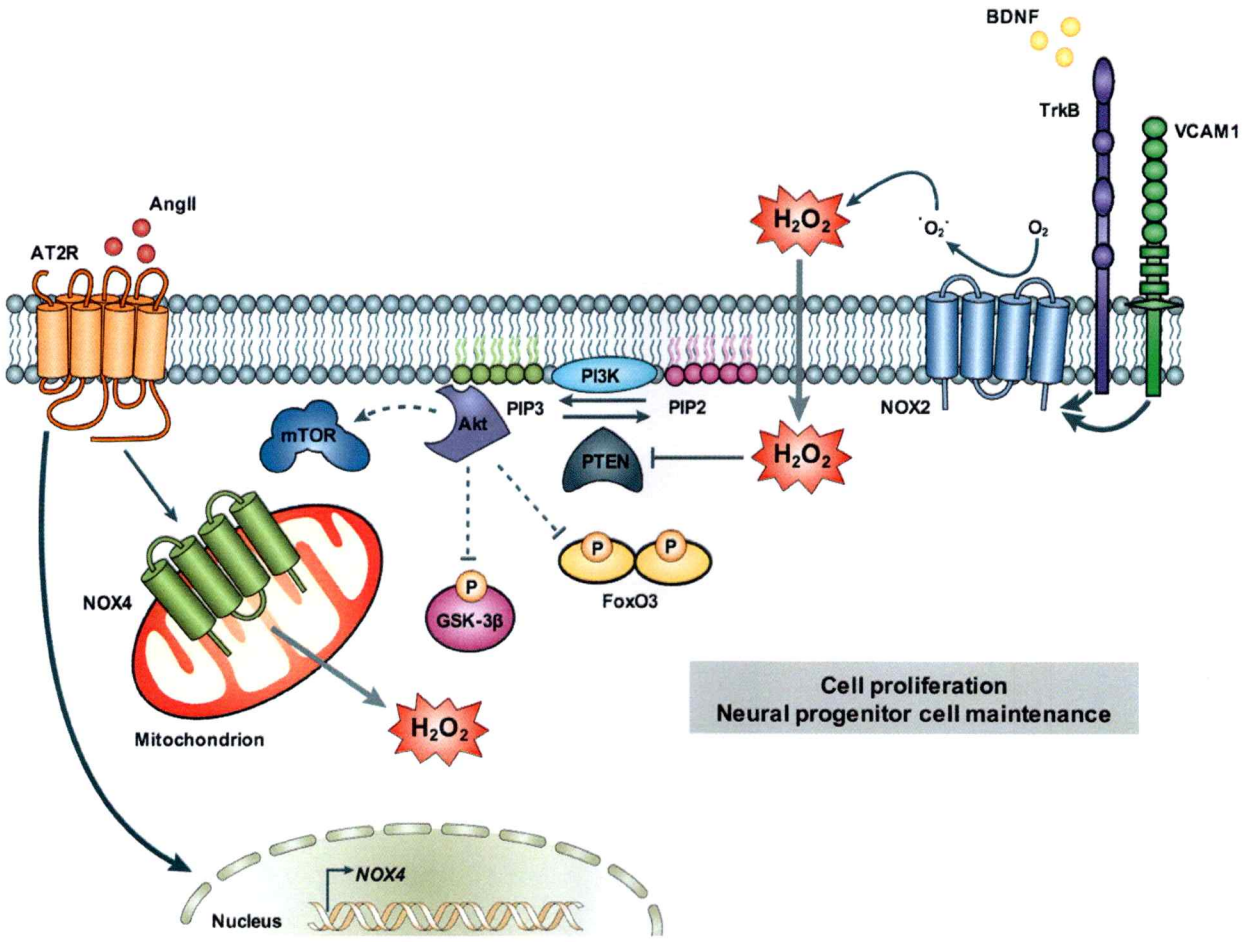

Fig. 1 Redox signaling in neurogenesis. Neural progenitor cell (NPC)s proliferation and stemness are maintained by enzymatic reactive oxygen species (ROS) production. Adhesion-mediated VCAM1 signaling or the stimulation of AT2R or the brain-derived growth factor (BDNF) receptor, TrkB, induces the activation 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 signaling not only is involved in 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 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.* 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

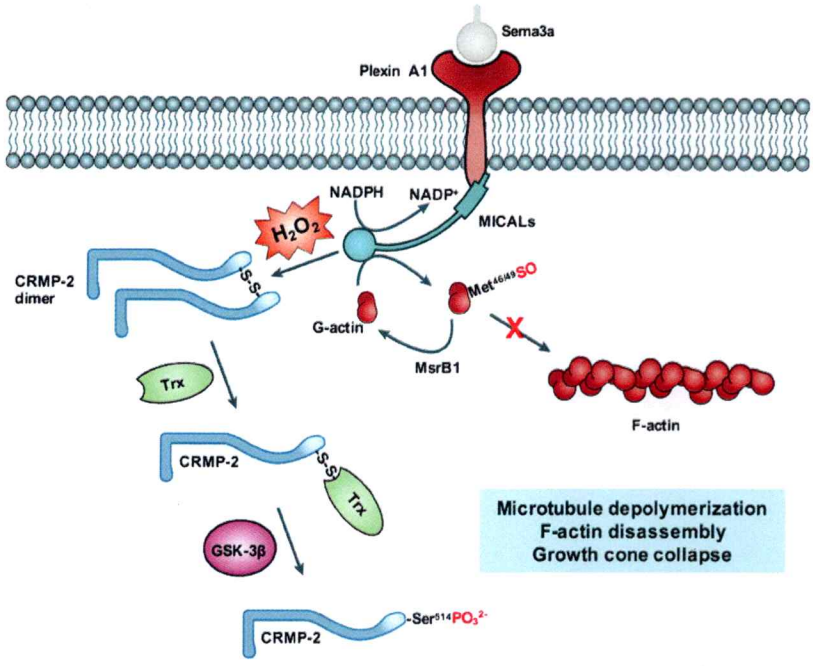

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Fig. 2 Redox signaling in axonal growth cone cytoskeletal dynamics. The binding of the axon-repulsive cue Semaphorin 3a (Sema3a) to its membrane receptor, Plexin A1, releases the autoinhibitory conformation of MICALs, a group of FAD-dependent monooxygenases, 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 NMDAR-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

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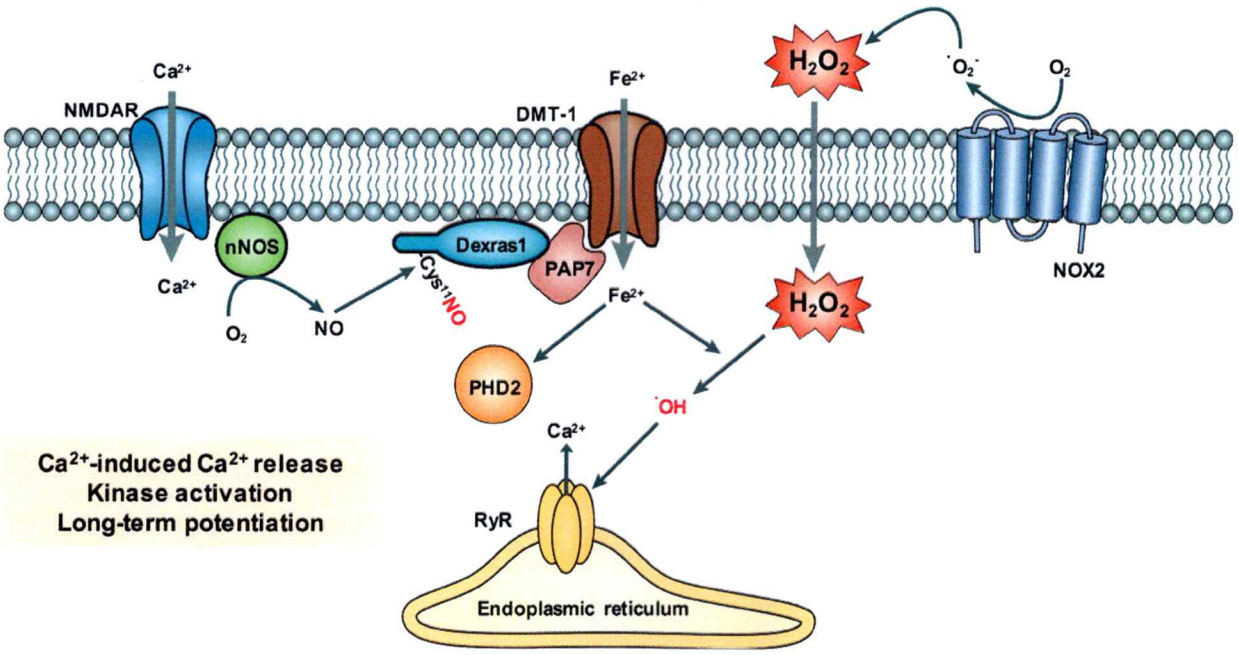

Fig. 3 Redox-dependent NMDA signaling. NMDA receptor (NMDAR) activation at the postsynaptic membrane induces calcium entry and activation of neuronal NOS (nNOS). NO modifies a specific cysteine residue in the small GTPase Dexras1, thus enhancing iron uptake, partially through the formation of a ternary 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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All experiments were conducted in compliance with the ARRIVE guidelines. The authors have no conflict of interest to declare.

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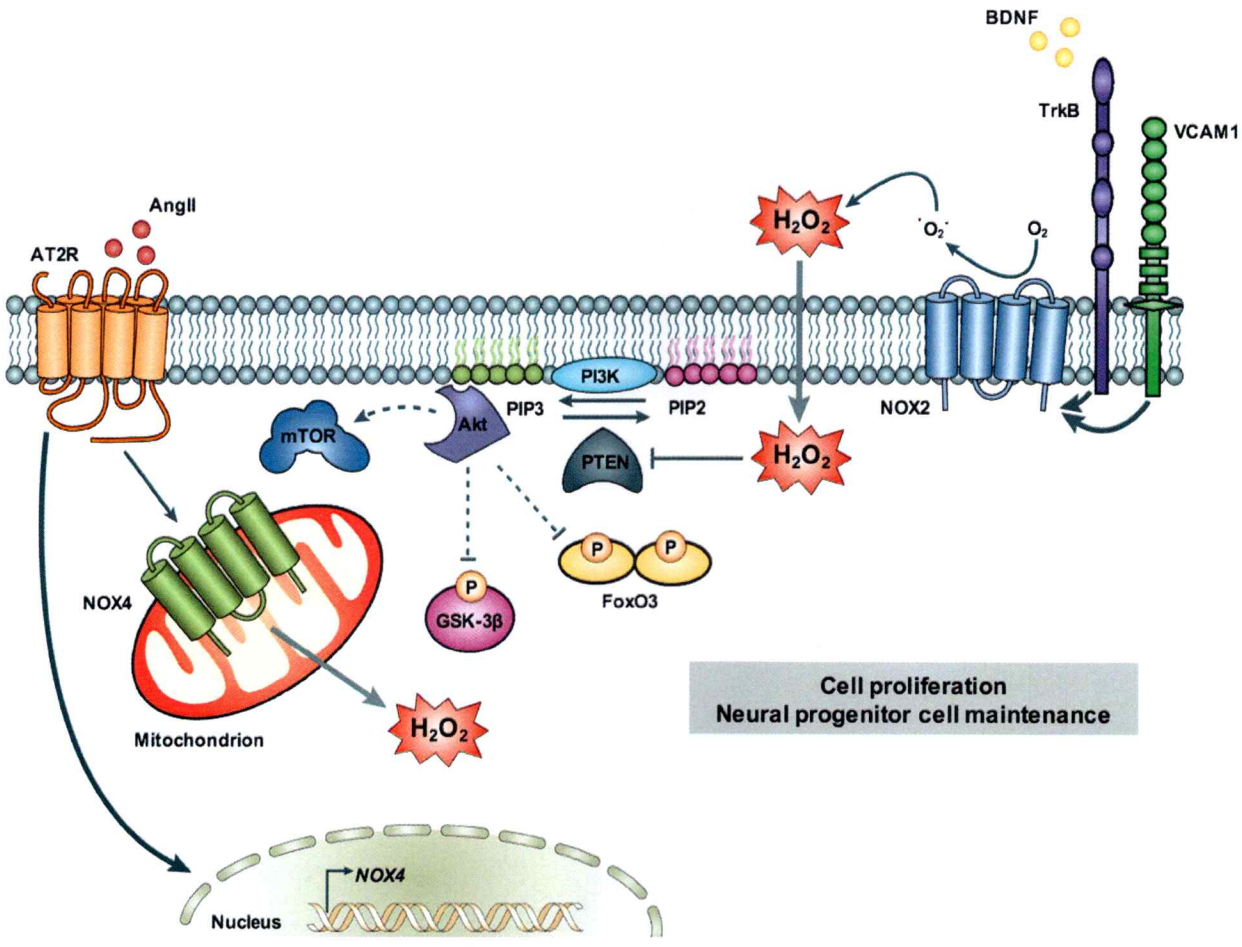

We review the role of reactive oxygen species (ROS) in neurogenesis, axon growth, and guidance and NMDA-receptor-mediated plasticity, LTP, and memory. ROS participation is presented in the context of NADPH oxidase and MICAL functions and their importance for brain functions.

Chapter 2

Contribution of the NADPH oxidase to the establishment of hippocampal neuronal polarity in culture

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SHORT REPORT

Contribution of NADPH oxidase to the establishment of hippocampal neuronal polarity in culture

Carlos Wilson, M. Tulio Núñez and Christian González-Billault*

ABSTRACT

Reactive oxygen species (ROS) produced by the NADPH oxidase (NOX) complex play important physiological and pathological roles in neurotransmission and neurodegeneration, respectively. However, the contribution of ROS to the molecular mechanisms involved in neuronal polarity and axon elongation is not well understood. In this work, we found that loss of NOX complex function altered neuronal polarization and decreased axonal length by a mechanism that involves actin cytoskeleton dynamics. These results indicate that physiological levels of ROS produced by the NOX complex modulate hippocampal neuronal polarity and axonal growth *in vitro*.

KEY WORDS: NOX, ROS, Neuronal polarity, Actin cytoskeleton

INTRODUCTION

Reactive oxygen species (ROS) participate in pathological and physiological aspects of neuronal functions. A basal level of ROS produced by the NADPH oxidase (NOX) complex is necessary for neurotransmission, learning and memory (Kishida et al., 2006; Knapp and Klann, 2002; Massaad and Klann, 2011; Nayernia et al., 2014; Serrano and Klann, 2004). The NOX family consists of NOX1–NOX5, and DUOX1 and DUOX2 (Bedard and Krause, 2007), with NOX1, NOX2 and NOX4 being the main enzymes expressed in the central nervous system (CNS) (Sorce et al., 2012). NOX2 interacts with five regulatory proteins – $p22^{phox}$, $p40^{phox}$, $p47^{phox}$, $p67^{phox}$ (also known as CYBA, NCF4, NCF1 and NCF2, respectively) and Rac1 (Bedard and Krause, 2007; Lambeth, 2004; Nayernia et al., 2014). NOX proteins have been detected in several regions of the adult mouse brain (Serrano et al., 2003). Mutations in $gp91^{phox}$ (also known as CYBB), $p47^{phox}$, $p67^{phox}$ and $p22^{phox}$ are linked to chronic granulomatous disease (CGD), which is associated with cognitive impairment (Pao et al., 2004).

Neurons are highly polarized cells that have two functionally independent compartments, the somato-dendritic region and the axon, that emerge during the establishment of neuronal polarity (Caceres et al., 2012; Dotti et al., 1988; Szu-Yu Ho and Rasband, 2011).

The actin cytoskeleton is essential for neuronal polarization (Bradke and Dotti, 1999; Stiess and Bradke, 2011). Thus, Rac1 and Cdc42, members of the small GTPase Rho family, promote neuronal polarization and axonal growth (Gonzalez-Billault et al., 2012). Oxidation of actin decreases its ability to polymerize (Hung et al., 2011, 2010; Sakai et al., 2012; Terman and Kashina, 2013). However, inhibition of NOX reduces both the F-actin content at the growth cone and the retrograde actin flow in neurons, suggesting a crosslink between NOX and actin dynamics (Munnamalai and Suter, 2009; Munnamalai et al., 2014).

In this work, we studied the contribution of the NOX complex to the development of neuronal polarity. Inhibition of the NOX complex affected polarity acquisition and reduced the axonal length of cultured neurons. NOX inhibition also affected actin organization, and decreased both the filopodial dynamics and the activity of Rac1 and Cdc42. These findings suggest that physiological levels of ROS, which are maintained by NOX, are needed to support neuronal polarization *in vitro*.

RESULTS AND DISCUSSION

Loss of function of the NOX complex modifies both neuronal polarity acquisition and axonal growth

To evaluate the contribution of the NOX complex in the establishment of neuronal polarity, we used genetic and pharmacological strategies. First, embryonic hippocampal neurons were transiently co-transfected, after plating, with GFP and the P156Q mutant of the regulatory subunit $p22^{phox}$ (here denoted $DNp22^{phox}$), which has a dominant-negative effect on ROS production, affecting the NOX1–NOX3 enzymes (Kawahara et al., 2005). Transfected neurons were fixed after 24 h in culture (Fig. 1). $DNp22^{phox}$ expression delayed neuronal polarity acquisition (Fig. 1A,B). To evaluate the contribution of NOX to axonal growth, neurons transfected with $DNp22^{phox}$ were cultured for 3 days *in vitro* (DIV) and were then fixed and immunostained for MAP2 and Tau (also known as MAPT) (somato-dendritic and axonal markers, respectively) (Fig. 1C). $DNp22^{phox}$ expression decreased the length of axonal, but not minor neurites (Fig. 1D,E). In addition, MAP2 was detected in axons after $DNp22^{phox}$ expression (Fig. 1C), suggesting that NOX inhibition disrupted neuronal polarization. To confirm that $DNp22^{phox}$ indeed reduced ROS content, neurons were co-transfected with the genetically encoded biosensor Hyper, which detects intracellular H_2O_2 (Lukyanov and Belousov, 2014), an indicator of NOX activity. $DNp22^{phox}$ expression (48 h) significantly reduced H_2O_2 content compared with control neurons (Fig. 1F,G). The Hyper-H_2O_2 map revealed that the highest H_2O_2 production was at the periphery of the soma as well as at the axonal tip (Fig. 1F, arrows), whereas $DNp22^{phox}$ expression abolished this pattern. Expression of $DNp22^{phox}$ was confirmed in N1E115 cells and cultured neurons (supplementary material Fig. S2). Taken together, these results suggest that NOX inhibition alters neuronal polarity acquisition and axonal growth.

As a second strategy to reduce NOX activity, neurons were treated with NOX inhibitors at 6 h after plating. Those chosen were gp91 ds-tat (5 μM), a peptide that inhibits $p47^{phox}$ association with $gp91^{phox}$ (Rey et al., 2001), VAS2870 (5 μM), a molecule that blocks the assembly of the NOX complex (Altenhofer et al., 2012) and apocynin (100 μM), which blocks $p47^{phox}$ translocation to the plasma membrane (Ximenes et al., 2007). Neurons were fixed at

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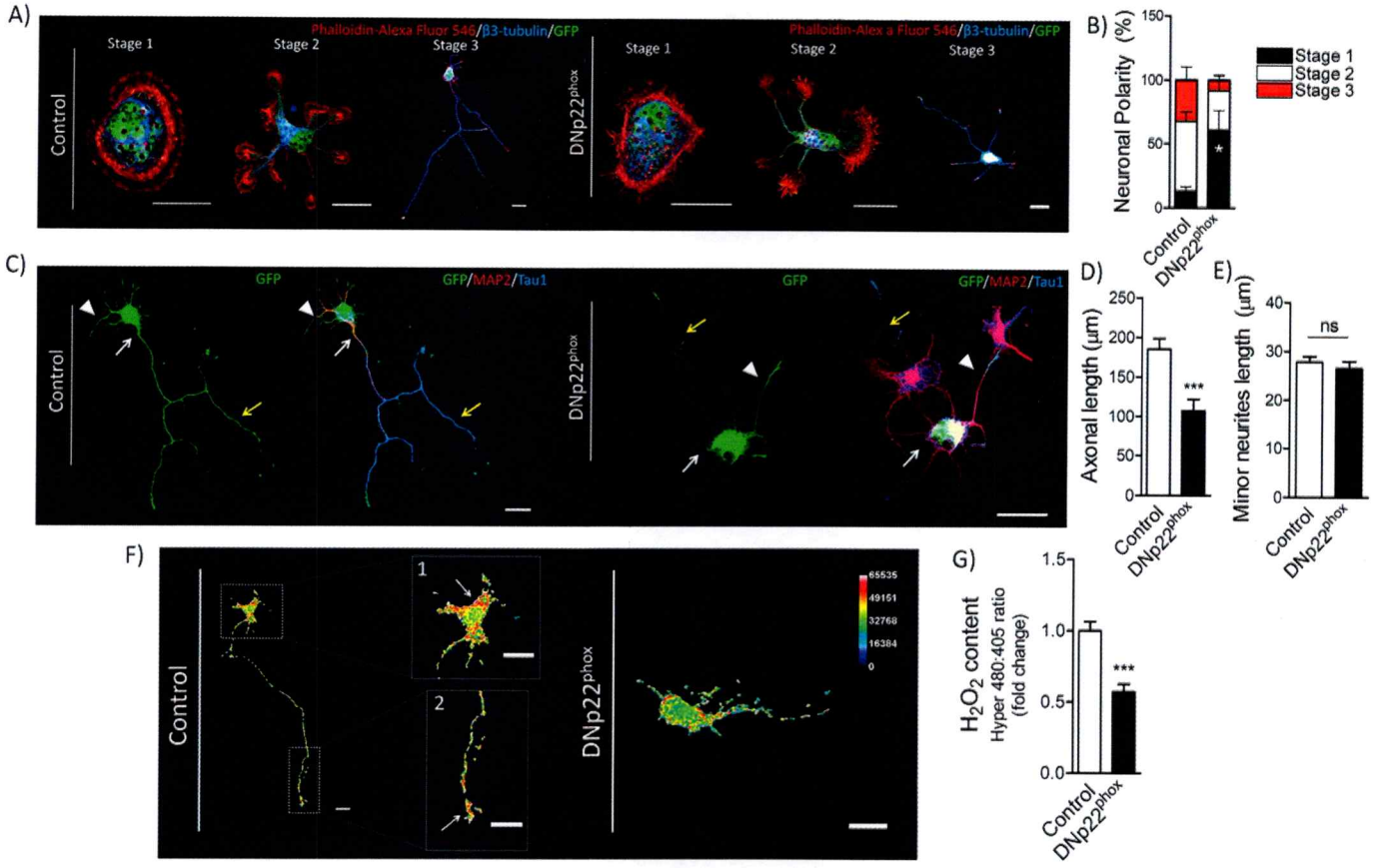

Fig. 1. DNp22^{phox} expression altered neuronal polarity acquisition and axonal growth. Neurons were transfected with GFP (control) or co-transfected with GFP and DNp22^{phox}. (A) Representative images of control and DNp22^{phox} neurons at stage 1, 2 and 3. F-actin was labeled with phalloidin–Alexa-Fluor-546 (red) and for β3-tubulin (blue). (B) Percentage of neurons that displayed polarity after DNp22^{phox} expression. *P<0.05 versus control stage 1 (Student's t-test). (C) Representative 3 DIV control and DNp22^{phox} neurons stained for MAP2 and Tau. White arrows indicate transfected neurons. (D) Axonal length (yellow arrows in C) of control and DNp22^{phox}-transfected neurons at 3 DIV; ***P<0.001 versus control (Student's t-test). (E) Length of minor neurites (arrowheads in C) in control and DNp22^{phox}-transfected neurons at 3 DIV. ns, not significant (Student's t-test). (F) H_2O_2 content evaluated with Hyper in control and DNp22^{phox} neurons. Magnifications 1 and 2 corresponds to soma and axonal tips, respectively. (G) Quantification of H_2O_2 in control and DNp22^{phox} neurons from images in F. ***P<0.001 versus control (Student's t-test). Quantitative results are mean±s.e.m.; 40 transfected neurons were analyzed per condition. Scale bars: 20 μm.

18 h of culture to evaluate the development of neuronal polarity (Fig. 2A). Under these treatments, most of the neurons remained at stage 1 (Fig. 2B–D), which supports the idea that NOX inhibition modifies neuronal polarity acquisition. We used DCFH-DA (a probe to measure oxidative species; LeBel and Bondy, 1990) to check ROS content after NOX inhibition (Fig. 2E). To rule out any non-specific effects of gp91 ds-tat, we used a scrambled gp91 (scr) peptide, which neither affected ROS content nor inhibited neuronal polarity (Fig. 2B,E).

Next, we sought to study the contribution of NOX to axonal growth. Neurons were treated with gp91 ds-tat, gp91 scr, VAS2870 and apocynin at 18 h of culture, when neurons are already at stage 2 and only display minor neurites. Neurons were fixed at 2 and 3 DIV to quantify the length of the axon and minor neurites (supplementary material Fig. S1; Fig. 2F–H). After 3 DIV, most neurons were fully polarized (stage 3) (92%±1), but this percentage decreased after NOX inhibition (gp91 ds-tat, 62%±13; VAS2870, 35%±15, P<0.01 and apocynin: 30%±3.5, mean±s.e.m., P<0.01). The remaining neurons did not develop an axon, resembling stage 2 of polarity. Control neurons exhibited somatic MAP2 and axonal Tau segregation at 3 DIV (87%±3) (Fig. 2F). In contrast, Tau and MAP2 distribution was reduced to 38%±5 of neurons treated with gp91 ds-tat (P<0.01), 3%±3 with VAS2870 (P<0.001) and 8%±1 with apocynin (P<0.001) (Fig. 2F), indicating loss of polarity. Moreover, NOX inhibition reduced the axon length but not the minor neurite length (Fig. 2G,H). These results are consistent with DNp22^{phox}-dependent NOX inhibition.

NOX complex expression and cellular localization in embryonic brain and hippocampal neurons

The NOX complex has been detected in mouse adult brain and in mature cultured hippocampal neurons (Serrano et al., 2003; Tejada-Simon et al., 2005), but not in embryonic brain or developing neurons. We detected gp91^{phox}, p22^{phox}, p47^{phox} and p67^{phox} subunits by immunoblotting in embryonic (E18.5) hippocampus and cerebral cortex (Fig. 3A) and also in stage 2 and 3 cultured neurons (18 h and 48 h, respectively) (Fig. 3B). Rac1, another component of the NOX complex, is expressed in hippocampal neurons at these stages (Santos Da Silva et al., 2004). gp91^{phox}, p22^{phox} and p47^{phox} were detected by immunofluorescence both in the soma and in minor neurites of stage 2 neurons (Fig. 3C). Interestingly, NOX subunits were also detected at the axon and axonal tip at stage 3 (Fig. 3D), which suggests that local production of ROS might be involved in axonal growth. Thus, NOX subunits are expressed in a timely manner to support neuronal polarity acquisition.

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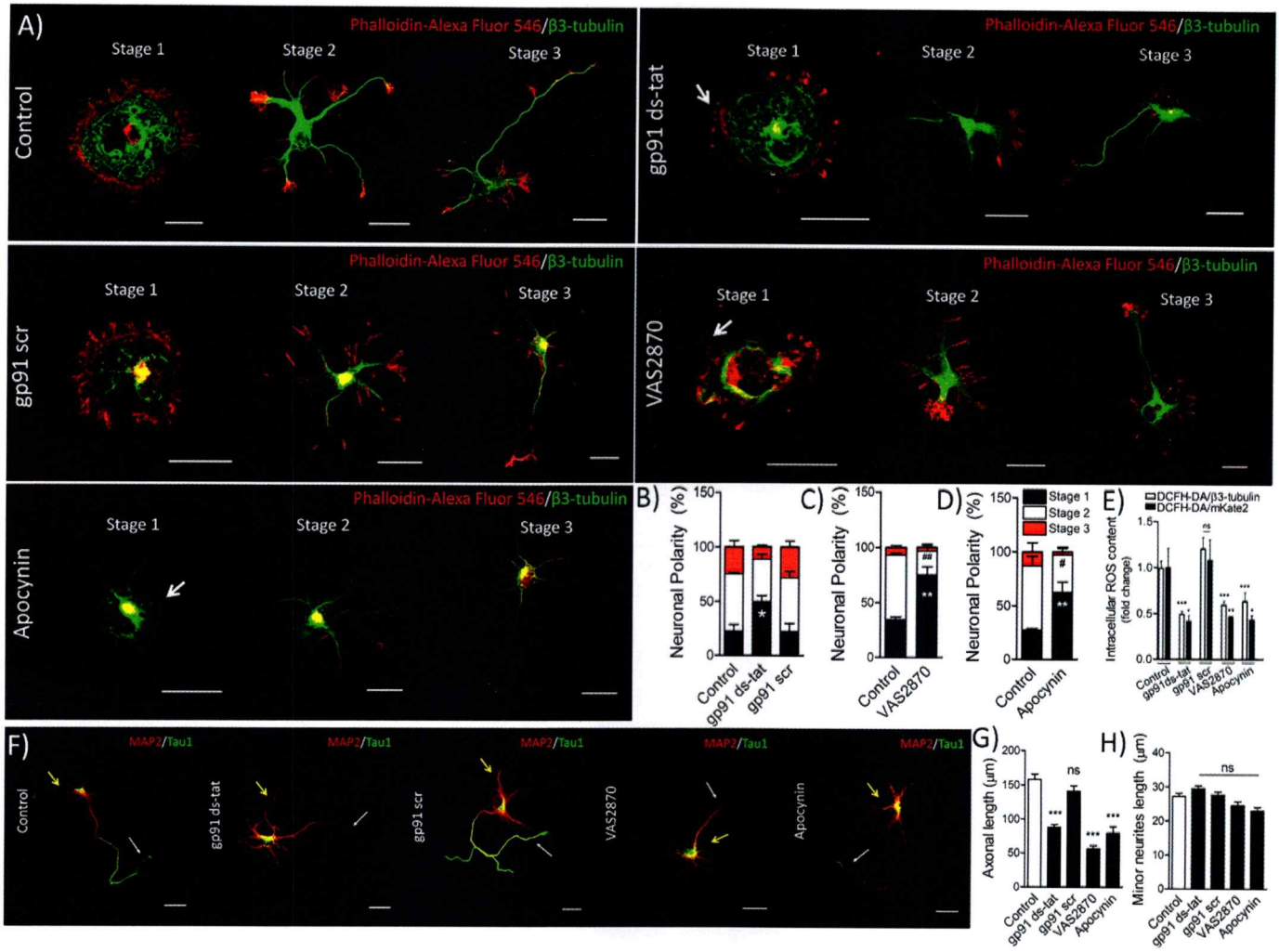

Fig. 2. NOX pharmacological inhibition altered neuronal polarity acquisition and axonal growth. (A) Neurons were treated with gp91 ds-tat peptide, gp91 scr, VAS2870 and apocynin at 6 h in culture and fixed after 12 h of treatment to evaluate neuronal polarity. (B–D) Neuronal polarity stages after treatments in as described in A. Results are from four independent experiments. (B) $^{*}P<0.05$ versus stage 1 control (ANOVA with Dunnett's post-test). (C) $^{**}P<0.01$ versus stage 1 control; $^{\#\#}P<0.01$ versus stage 2 control (Student's *t*-test). (D) $^{**}P<0.01$ versus stage 1 control, $^{\#}P<0.05$ versus stage 3 control (Student's *t*-test). (E) Intracellular ROS content measured with DCFH-DA. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ versus control (ANOVA, Dunnett's post-test). (F) 3 DIV neurons stained with MAP2 and Tau after NOX inhibition. Neurons were treated at 18 h of culture and fixed after 3 DIV to evaluate polarity and the length of both the axon and minor neurites. Axonal length (G) and length of minor neurites (H). $^{***}P<0.001$ versus control; ns, not significant (ANOVA, Dunnett's post-test). Results are from three independent experiments. Quantitative results are mean±s.e.m.; 80–120 neurons were analyzed for each treatment. Scale bars: 20 μm.

Contribution of the NOX complex to actin cytoskeleton dynamics

Based on our findings, we hypothesized that the NOX complex affects actin dynamics during neuronal polarization. First, we measured the neuronal lamellar area at stage 1. A well-structured lamella is important because minor neurites and the axon will emerge from this region (Caceres et al., 2012). Neurons were transfected with GFP or co-transfected with DNp22^{phox} and GFP immediately after plating, and fixed after a short time in culture to measure the area of the lamella. The F-actin and tubulin cytoskeleton were detected with phalloidin–Alexa-Fluor-546 and β3-tubulin immunolabeling, respectively (Fig. 4A). DNp22^{phox} expression significantly reduced the lamellar area compared with control neurons (Fig. 4B). Moreover, gp91 ds-tat, VAS2870 and apocynin also reduced phalloidin labeling in neurons (Fig. 2A, arrows). These results are consistent with the finding that actin at the growth cone of *Aplysia* bag cells is disorganized after NOX inhibition (Munnamalai and Suter, 2009).

Second, based on the possible influence of NOX on the integrity of the actin cytoskeleton, we evaluated filopodial dynamics at the tip of the axon as a parameter for actin polymerization. Neurons were transfected with either the genetically encoded probe Lifeact, which allows visualization of actin polymerization in real time (Riedl et al., 2008) or co-transfected with Lifeact and DNp22^{phox} (Fig. 4C). The number, length and lifetime of filopodia at the axonal tip were reduced after DNp22^{phox} expression (Fig. 4D–F). These results suggest that adequate amounts of ROS are needed to maintain the dynamics of the actin cytoskeleton.

Third, and considering that both lamellar and filopodial dynamics were altered after NOX inhibition, we sought to measure Rac1 and Cdc42 activities after DNp22^{phox} expression. To this end, 1 DIV neurons were transfected with the Raichu FRET biosensors for Rac1 or Cdc42 in control and DNp22^{phox} expression conditions (Nakamura et al., 2006). Representative FRET maps for Rac1 and Cdc42 are shown to indicate their local activity (Fig. 4G–J).

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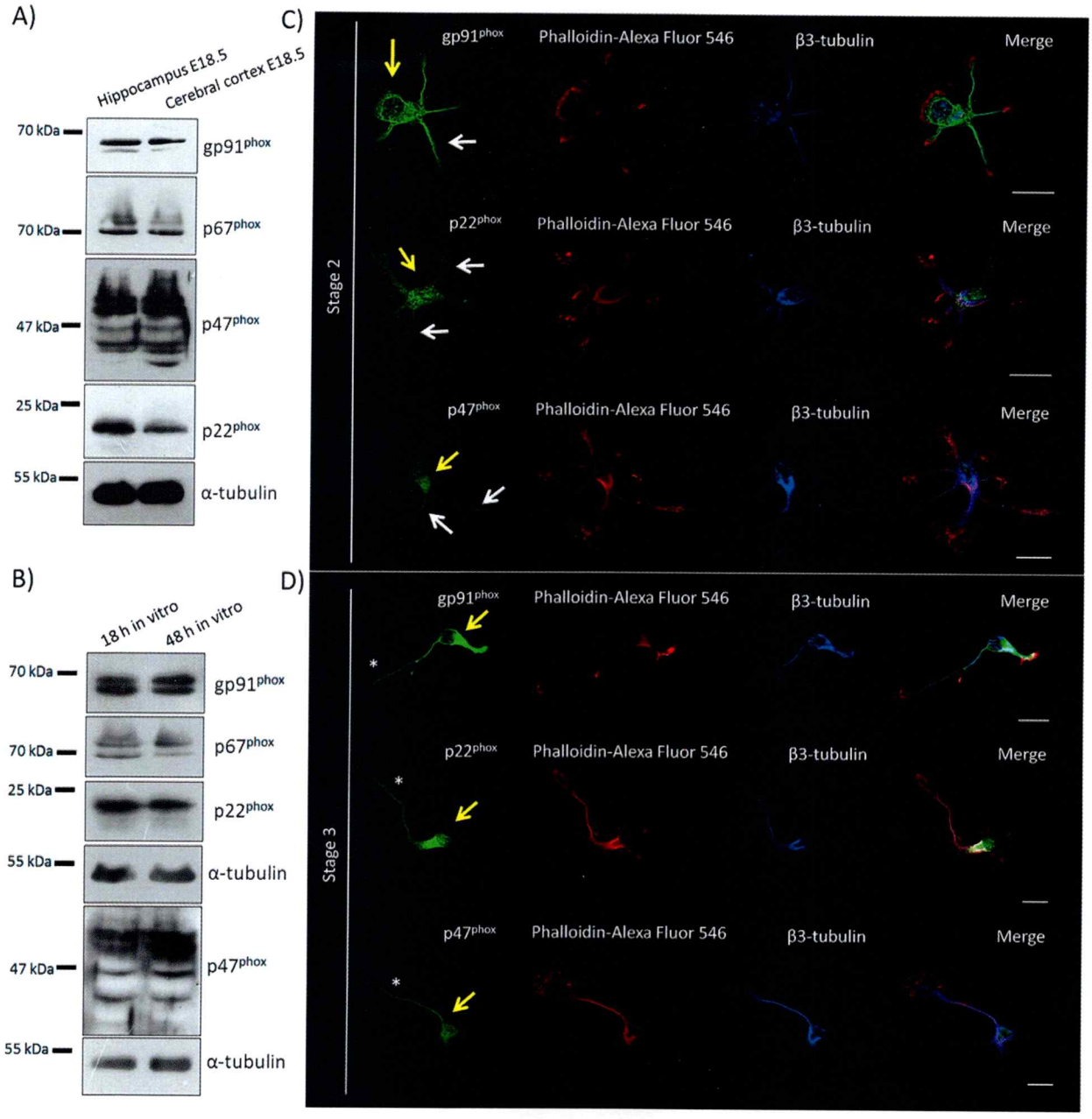

Fig. 3. Expression and cellular localization of the NOX complex in hippocampal neurons. (A) Immunoblotting of gp91^{phox}, p67^{phox}, p47^{phox}, p22^{phox} and α-tubulin in E18.5 hippocampus and cerebral cortex. (B) Stage 2 and 3 cultured neurons were assessed for the presence of NOX complex proteins gp91^{phox}, p67^{phox}, p47^{phox}, p22^{phox} and α-tubulin by immunoblotting. (C) Localization of NOX proteins in stage 2 and (D) stage 3 neurons by immunofluorescence (white arrows, minor neurites at stage 2; yellow arrow, soma; asterisk, axon at stage 3). Scale bars: 20 µm.

Quantification of FRET efficiency was performed at the soma and for the whole axon, and its proximal and distal segments. The expression of DNp22^{phox} decreased Rac1 FRET globally (Fig. 4H), which is consistent with the decrease in axonal length and lamellar area (Fig. 1D; Fig. 4A). Cdc42 FRET efficiency, in turn, was decreased only within the distal axon (Fig. 4J), the same region where we observed a decrease in filopodial dynamics (Fig. 4C–F). Filopodial dynamics can also be regulated by Arp2/3 (Spillane et al., 2011), supporting the idea that Rac1 is also involved in this process, which is consistent with the decrease in Rac1 FRET efficiency shown in Fig. 4G. These results suggest that NOX inhibition modifies actin dynamics by decreasing the activity of Rho GTPase proteins. However, F-actin is also regulated by post-translational modifications of actin monomers that depend on redox balance (Hung et al., 2011; Terman and Kashina, 2013). Further experiments are thus required to explore the possibility that these modifications are required as well as regulation of the Rho GTPase protein activity.

gp91^{phox}- and p47^{phox}-knockout mice have normal brains, cortex and hippocampus (Kishida et al., 2006). However, axonal elongation, dendritic arborization and synaptic development have not been explored in these mice, even though the long-term potentiation (LTP) response is impaired and CGD patients present cognitive impairments (Kishida et al., 2006; Pao et al., 2004). Polarity acquisition studies *in vivo* could provide clues about the loss in neuronal functions observed in these models.

In conclusion, we propose that ROS production by the NOX complex contributes to the establishment of hippocampal neuronal

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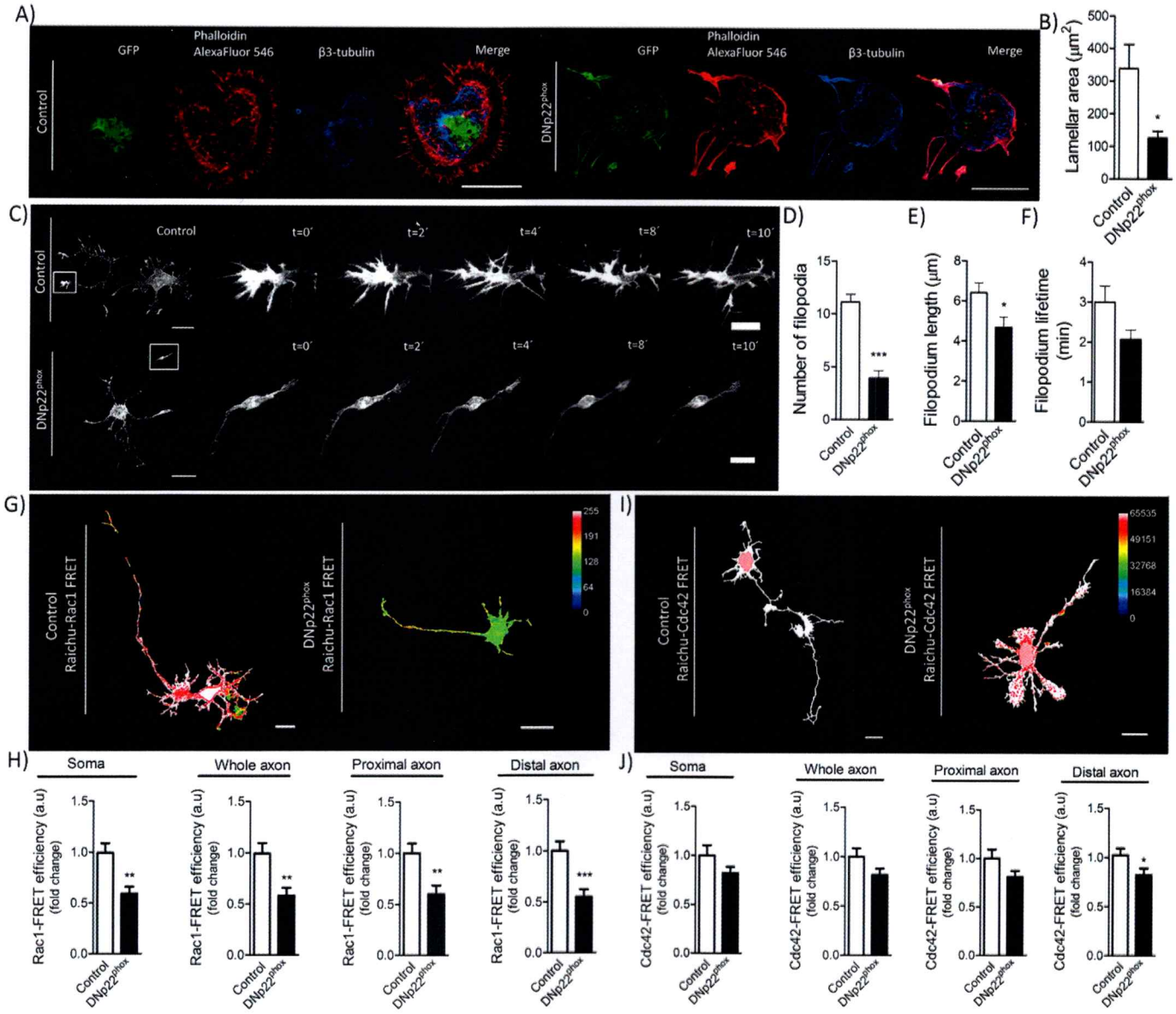

Fig. 4. Contribution of the NOX complex to actin cytoskeleton dynamics. (A) Stage 1 neurons transfected with GFP alone or with GFP and DNp22^{phox}. (B) Quantification of the lamellar area of control and DNp22^{phox} neurons at stage 1. *P<0.01 versus control (Student's t-test). (C) Time-lapse with Lifeact in control and DNp22^{phox} neurons to visualize F-actin dynamics. (D–F) Quantification of the number (D), length (E) and lifetime (F) of filopodia from inset in C (15 neurons for each condition). *P<0.05, ***P<0.001 versus control (Student's t-test). (G,I) Rac1- and Cdc42-FRET map in control and DNp22^{phox} neurons. (H,J) Local quantification of Rac1 and Cdc42 activity using the FRET biosensors in control and DNp22^{phox} neurons. (H) **P<0.01 versus control, ***P<0.001 versus control (Student's t-test); (J) *P<0.05 versus control (Student's t-test). Results are from three independent experiments. Quantitative results are mean±s.e.m.; 20 transfected neurons were analyzed per condition. Scale bars: 20 μm, 5 μm (magnified images in C).

polarity and axonal growth *in vitro* through the regulation of Rac1, Cdc42 and actin cytoskeleton dynamics. These findings support the idea that physiological levels of ROS are indeed necessary for normal neuronal development and function.

MATERIALS AND METHODS

Primary culture of hippocampal neurons from rat brain embryos

Pregnant Sprague-Dawley rats were killed, embryos (E18.5) were removed and neurons were cultured according to Kaech and Banker (2006). All animal experiments were performed according to approved guidelines.

N1E115 neuroblastoma cell culture

N1E115 cells (ATCC, VA) were cultured in Dulbecco's modified Eagle's medium (DMEM) with 5% fetal bovine serum (FBS) to check DNp22^{phox} expression.

Transient transfection of cDNA coding vectors

Neurons were transiently transfected with Lipofectamine 2000 (Life Technologies, CA) in Neurobasal medium. After 2 h, neurons were supplemented with B27, Glutamax, sodium pyruvate and antibiotics. Experiments were performed 18–72 h after cDNA transfection.

Primary antibodies

Antibodies against gp91^{phox} (mouse, ab109366, lot YH081212C; 1:1000 for immunoblotting and 1:100 for immunofluorescence), p67^{phox} (rabbit, ab80897, lot GR23630-9; 1:500 for immunoblotting) and p22^{phox} (rabbit, ab75941, lot GR83982-1; 1:1000 for immunoblotting and 1:100 for immunofluorescence) were purchased from Abcam (MA). The antibody against α-tubulin (1:10,000, mouse) was from Sigma (MO), that against p47^{phox} (rabbit, sc-14015, lot A2113; 1:500 for immunoblotting and 1:100 for immunofluorescence) was from Santa Cruz Biotechnology (TX), and those against MAP2 (rabbit, 1:500) and Tau (mouse, 1:500) were from

Merck Millipore (Darmstadt, Germany). Anti-β3-tubulin antibody (mouse, 1:1000) was from Promega (WI). For solutions and general considerations for immunoblotting and immunofluorescence experiments, please see Henriquez et al. (Henriquez et al., 2012).

Hyper H_2O_2 measurement

Neurons (4×10^4 cells/well) were cultured on glass coverslips. Immediately after plating, neurons were transfected with Hyper (Evrogen, Moscow, Russia), an intracellular and ratiometric sensor to detect local H_2O_2 production (Lukyanov and Belousov, 2014). Transfected neurons were excited at 488 and 405 nm and emission was collected at 505–530 nm. Fluorescence emission from excitation at 488 nm was divided by fluorescence emission at 405 nm excitation (488:405) as a measure of the H_2O_2 content (Belousov et al., 2006).

DCFH-DA ROS measurement

Neurons were incubated with 1 µM DCFH-DA (Sigma) for 20 min at 37°C to evaluate intracellular ROS levels. DCFH-DA detects intracellular oxidative species by increasing fluorescence emission after oxidation (LeBel and Bondy, 1990). Neurons were fixed and permeabilized as described previously (Henriquez et al., 2012). β3-tubulin immunofluorescence and the transient expression of the far-red fluorescent protein mKate2 (Evrogen, Moscow, Russia) were used to normalize DCFH-DA emission, similar to as described previously (Munnamalai and Suter, 2009).

Measurement of lamellar area

Neurons (24 h in culture) were fixed and immunostained against β3-tubulin. Phalloidin–Alexa-Fluor-546 was incubated for 1 h at room temperature during secondary antibody incubation for F-actin detection. Binary masks of F-actin- and β3-tubulin-positive areas were generated to measure the lamellar area. The lamellar area of stage 1 neurons was defined as the area of F-actin minus the area of β3-tubulin (Laishram et al., 2009).

Real-time filopodial dynamics

Neurons were transfected with the Lifeact–GFP biosensor, and imaging was carried out 18 h after transfection. Time-lapse images were taken every 30 s for 10 min to visualize filopodial dynamics. Later, the number, length and lifetime of filopodia were measured using Fiji-ImageJ (NIH, Bethesda). Protrusions shorter than 2 µm and longer than 15 µm were not considered for the analysis. The lifetime was defined as the time during which a filopodium emerges and disappears.

Measurement of Rac1 and Cdc42 activity

Neurons (4×10^4 cells/well) were transfected with the Raichu-Rac1 and Raichu-Cdc42 FRET biosensors (provided by Alfredo Cáceres, IMMF, Córdoba, Argentina) to measure Rho GTPase activity. Raichu probe expression and FRET efficiency measurements were performed as described previously (Nakamura et al., 2006). Briefly, transfected neurons were excited at 450 nm, and emissions were collected at 460–490 and 505–530 nm (donor and acceptor emission wavelengths, respectively). The ratio of the acceptor to donor emission was established as the FRET efficiency. The FRET map was achieved by dividing the acceptor to donor ratio image by the binary mask of the same image. Measurement of FRET efficiency was carried out by selecting a region of interest at the soma, the whole axon or the proximal and distal axon.

Statistics

Results are the mean±s.e.m. of at least three independent cultures. The number of neurons per experiment (n) is indicated in the figure legends. ANOVA, Dunnett's post-test and Student's t-test tests were carried out with the GraphPad Prism 5 software.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

C.W., M.T.N. and C.G.-B. conceived and designed the experiments. C.W. performed the experiments. C.W. and C.G.-B. analyzed the data. C.W., M.T.N. and C.G.-B. wrote the paper.

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Supplementary material

Supplementary material available online at
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Chapter 3

Regulation of cytoskeletal dynamics by redox signaling and oxidative stress: implications for neuronal development and trafficking

frontiers in Cellular Neuroscience

REVIEW
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Regulation of cytoskeletal dynamics by redox signaling and oxidative stress: implications for neuronal development and trafficking

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A proper balance between chemical reduction and oxidation (known as redox balance) is essential for normal cellular physiology. Deregulation in the production of oxidative species leads to DNA damage, lipid peroxidation and aberrant post-translational modification of proteins, which in most cases induces injury, cell death and disease. However, physiological concentrations of oxidative species are necessary to support important cell functions, such as chemotaxis, hormone synthesis, immune response, cytoskeletal remodeling, Ca^{2+} homeostasis and others. Recent evidence suggests that redox balance regulates actin and microtubule dynamics in both physiological and pathological contexts. Microtubules and actin microfilaments contain certain amino acid residues that are susceptible to oxidation, which reduces the ability of microtubules to polymerize and causes severing of actin microfilaments in neuronal and non-neuronal cells. In contrast, inhibited production of reactive oxygen species (ROS; e.g., due to NOXs) leads to aberrant actin polymerization, decreases neurite outgrowth and affects the normal development and polarization of neurons. In this review, we summarize emerging evidence suggesting that both general and specific enzymatic sources of redox species exert diverse effects on cytoskeletal dynamics. Considering the intimate relationship between cytoskeletal dynamics and trafficking, we also discuss the potential effects of redox balance on intracellular transport via regulation of the components of the microtubule and actin cytoskeleton as well as cytoskeleton-associated proteins, which may directly impact localization of proteins and vesicles across the soma, dendrites and axon of neurons.

Keywords: redox, cytoskeleton, neurons, development, trafficking

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The Nervous System as a Target for Oxidative Species

The chemical reduction-oxidation (redox) balance commands physiological and pathological responses at different levels ranging from cells to tissues to biological systems. Among organs, the brain is especially vulnerable to oxidation for three main reasons. First, the brain consumes high levels of O_2—up to 20% of the amount used by the entire body (Sparaco et al., 2006). Given that the brain represents only 2% of the total body mass, metabolites derived from O_2 in the brain are highly concentrated in a restricted space, increasing the risk of oxidation. Second, Fe^{2+} is abundant in many specific areas of the brain (Gerlach et al., 1994), contributing

to non-reversible oxidation. Fe^{2+}, the redox-active form of iron, catalyzes the conversion of hydrogen peroxide (H_2O_2) into the hydroxyl radical (HO⊕) through the Fenton reaction. The hydroxyl radical represents the most chemically reactive of all reactive oxygen species (ROS; Núñez et al., 2012). Finally, the brain lacks effective mechanisms to remove accumulated pro-oxidative molecules (Halliwell, 1992). Together, these factors necessitate that the brain must utilize effective biochemical measures to counter oxidative stress.

Increased oxidation of molecules in both central and peripheral neurons is often associated with aging, oxidative stress and disease (Andersen, 2004). The term oxidative stress was coined to reflect an imbalance between oxidative and reductive molecules that leads to increased accumulation of pro-oxidant species, with deleterious consequences in most cases (Sies, 1997). This is the case for several neuronal pathologies, including Parkinson's disease, Alzheimer's disease, Huntington's disease and amyotrophic lateral sclerosis (Andersen, 2004). Most sporadic versions of these pathologies are linked to aging and have a documented positive correlation between oxidative stress and development of pathology.

Although oxidation of intracellular components owing to increased oxidative stress is a natural consequence of long-term exposure to pro-oxidant conditions, it is important to remember that physiological synthesis of oxidative species is required for normal cellular function (Rhee, 2006; Dáux and Toledano, 2007). The innate immune response depends on proper synthesis of ROS produced by NADPH oxidases (NOXs). Particularly, phagocytosis, chemotaxis and cellular locomotion of immune cells require basal ROS synthesis (Lambeth, 2004). Other processes requiring physiological concentrations of ROS include thyroid hormone synthesis, Ca^{2+} homeostasis, ion channel dynamics and cytoskeletal remodeling (Bedard and Krause, 2007; Hidalgo and Nunez, 2007; Espinosa et al., 2009; Sakai et al., 2012; Contreras-Ferrat et al., 2014).

In the central nervous system (CNS), enzymatic production of physiological levels of ROS contributes to synaptic plasticity and memory consolidation (Knapp and Klann, 2002; Massaad and Klann, 2011). Genetic models in which $gp91^{phox}$ and $p47^{phox}$ proteins are inactivated (the catalytic and one of the regulatory subunits of the NOX complex, respectively) exhibit abnormal long-term potentiation responses after electrical stimulation, which is an *ex vivo* paradigm to evaluate synaptic plasticity in hippocampal neurons (Kishida et al., 2006). $gp91^{phox}$ and $p47^{phox}$ knockout mice also have decreased consolidation of spatial memory, suggesting a neuronal disorder owing to impaired NOX activity and ROS signaling (Kishida et al., 2006). Humans with chronic granulomatous disease (CGD), an inherited syndrome caused by point mutations in the NOXs proteins $gp91^{phox}$, $p47^{phox}$, $p67^{phox}$ and $p22^{phox}$, affecting the immune response to pathogens, develop cognitive impairments and reduced intellectual coefficients compared with healthy control individuals (Pao et al., 2004). CGD develops during childhood and often requires repeated long-term hospitalization throughout life. It has been proposed that the intellectual deficits in CGD children could be linked to irregular school attendance and that this could even be the main cause for their low intellectual coefficients. However, other infectious diseases with similar periods of hospitalization do not result in cognitive deficits, suggesting that CGD patients develop specific neuronal alterations mainly attributable to reduced NOX activity and decreased ROS synthesis (Pao et al., 2004).

Taken together, these observations suggest that basal physiological ROS synthesis is required for normal cellular function, including the regulation of neurotransmission, but that high and unregulated ROS concentrations lead to oxidative stress and disease.

General Overview of Redox Balance

Intracellular Sources of ROS

The main oxidative species derived from O_2 are H_2O_2, HO⊕ and the superoxide anion ($O_2⊕^-$) and are collectively called ROS (Bedard and Krause, 2007). Nitrogen (N_2), the principal gas in the atmosphere we breathe, also induces intracellular oxidation via the production of physiologically reactive nitrogen species (Weidinger and Kozlov, 2015).

The main sources of intracellular ROS are mitochondria and NOXs. In mitochondria, complexes I and III of the electron transfer chain produce the short-lived $O_2⊕^-$, a radical derived from O_2 (Murphy, 2009; Bigarella et al., 2014). No intracellular signaling pathway that regulates mitochondrial superoxide synthesis has yet been described, suggesting that mitochondria could be a source of constitutive ROS production. Synthesis and release of ROS from mitochondria depend on the tissue and its intrinsic metabolism. Mitochondrial dysfunction quickly leads to oxidative stress that targets DNA, membrane lipids and proteins, directly affecting cell physiology (Tahara et al., 2009).

NOXs represent the other major cellular source of ROS (Bedard and Krause, 2007). The NOX family includes seven members that catalyze the production of $O_2⊕^-$ in an NADPH-dependent reaction. The family is composed of five canonical NOXs (NOX1 to NOX5) and two dual oxidases (Duox1 and Duox2; Lambeth et al., 2007). NOXs represent the main enzymatic source of ROS, and several signal transduction pathways are involved in their regulation (Dang et al., 2001; Park et al., 2001; Chen et al., 2003; Hoyal et al., 2003). NOX1, NOX2 and NOX4 are expressed in the CNS (Sorce and Krause, 2009), with NOX2 being the principal enzyme expressed in neurons. NOX2 can produce superoxide by itself but requires interaction with regulatory proteins for stabilization and to increase ROS levels under physiological circumstances. Together with its partners $p22^{phox}$, $p47^{phox}$, $p67^{phox}$ and $p40^{phox}$, NOX2 synthesizes superoxide to meet the physiological requirements of neurons (Bokoch and Diebold, 2002; Glogauer et al., 2003; Nauseef, 2004; Decoursey and Ligeti, 2005).

ROS as Signaling Molecules

Superoxide reactivity is fairly low, mainly owing to its short life-time and restricted diffusion area (Weidinger and Kozlov, 2015). However, superoxide can be converted to H_2O_2 either spontaneously or enzymatically via superoxide dismutase

(Núñez et al., 2012). H_2O_2, the most stable ROS, is converted to H_2O by several antioxidant enzymes, e.g., glutathione peroxidase and catalase, and this is likely the reason why oxidative modifications induced by H_2O_2 are transient and reversible (Weidinger and Kozlov, 2015). Thus, under normal conditions, the synthesis of superoxide and H_2O_2 are enzymatically regulated and their levels remain under a physiological threshold. In the presence of Fe^{2+}, however, H_2O_2 is rapidly converted to HO⊕ through the Fenton reaction (Núñez et al., 2012). Hydroxyl radicals modify molecules in a non-reversible way, leading to permanent modifications of proteins and other targets. To consider ROS as signaling molecules, they should meet certain spatial and regulatory criteria, namely they should be produced locally and their levels regulated by intracellular molecular systems. According to these criteria, superoxide and H_2O_2, but not HO⊕, are considered signaling molecules (Dáux and Toledano, 2007; Janssen-Heininger et al., 2008; Gerich et al., 2009).

Regulation of Protein Function by Oxidation via Post-Translational Modification

Cysteine thiol groups (SH) of proteins are the main targets for oxidation (Stadtman and Berlett, 1997). In general terms, oxidation of SH groups leads to glutathionylation, nitrosylation and disulfide bond formation. These modifications are enzymatically reversed through the glutaredoxin (Grx), thioredoxin and peroxiredoxin systems, among others (Ghezzi, 2005; Shelton et al., 2005; Janssen-Heininger et al., 2008). Oxidation also leads to sulfenic acid formation and protein carbonylation, two non-reversible modifications that permanently affect protein structure and function (Bigarella et al., 2014).

The functions of several proteins, including cytoskeletal proteins, depend on ROS signaling and oxidation (Sparaco et al., 2006). Several studies have shown that redox balance affects both *in vitro* and *in vivo* cytoskeletal dynamics, which directly impacts cell morphology and morphometrics. In neurons, cytoskeletal rearrangement commands cell development, polarization and neurotransmission (Jaworski et al., 2009; Hoogenraad and Akhmanova, 2010; Stiess and Bradke, 2011; Caceres et al., 2012; Gonzalez-Billault et al., 2012). Neurons are highly polarized cells, having a cell body from which emerge several dendrites and an axon to establish functional communication networks with other neurons and glial cells (Caceres et al., 2012). Acquisition of this morphology depends directly on the dynamics of actin microfilaments and microtubules (Neukirchen and Bradke, 2011). Given the influence of the redox state on neuronal function and its potential role in modifying the cytoskeleton, it is interesting to review the contribution of the redox balance to cytoskeletal organization in neurons.

Contribution of Redox Balance to Organization of the Neuronal Cytoskeleton

Redox State of Neuronal Cytoskeleton Proteins

The main components of the neuronal cytoskeleton network, namely actin microfilaments, microtubules and neurofilaments, are susceptible to oxidation (Sparaco et al., 2006). Post-mortem histological studies from non-pathological human samples have revealed a basal pool of glutathionylated proteins in the prefrontal cortex, cerebellum and spinal cord. Cellular analysis of the prefrontal cortex revealed that neurons are more highly glutathionylated than oligodendrocytes and astrocytes. Biochemical analysis revealed that actin, tubulin and neurofilaments are glutathionylated, suggesting that the redox state of neurons and cytoskeletal proteins under basal conditions is slanted toward oxidation (Sparaco et al., 2006). The relevance of cytoskeleton oxidation depends on the spatiotemporal context in which a defined modification occurs as well as the source of the ROS. Whereas physiological ROS production is needed for proper cytoskeleton polymerization, oxidation tends to disrupt polymerization and impair cytoskeletal dynamics under oxidative stress conditions (Munnamalai and Suter, 2009; Hung et al., 2010; Morinaka et al., 2011; Wilson et al., 2015).

Actin Modification and Regulation of F-Actin Dynamics by Oxidative Species

Intracellular ROS production is needed for proper cell migration and chemotaxis, which are actin-dependent processes (Roberts et al., 1999; Ambruso et al., 2000; Kim and Dinauer, 2001). Actin monomers contain 5 Cys and 16 Met residues (Dalle-Donne et al., 2002, 2003). Of these, only Cys 374 is fully exposed to the cytoplasm (Dalle-Donne et al., 2003). By contrast, Met 44, 47 and 355 are prominently exposed to the cytoplasm and Met 176, 190, 227 and 260 are also susceptible to the action of oxidative molecules (Dalle-Donne et al., 2002). *In vitro* assays suggested that an oxidative environment inhibits actin polymerization (Dalle-Donne et al., 2003). In addition, both Cys and Met residues can be carbonylated after *in vitro* treatment with hypochlorous acid (HOCl), a common derivative product released by leukocytes during the initial phase of the immune response. This polymerization assay was designed to recapitulate *in vitro* the exact oxidative environment of the immune cell response; importantly, this environment inhibits actin polymerization (Dalle-Donne et al., 2001). This analysis also revealed that specific glutathionylation at Cys 374 decreases actin polymerization (Dalle-Donne et al., 2003). In neutrophils, glutaredoxin 1 (Grx1), a deglutathionylating enzyme that reduces oxidized Cys residues, is needed to maintain actin dynamics (Sakai et al., 2012). ROS depletion using the NOX inhibitor diphenyleneiodonium or Grx1 overexpression decreases actin glutathionylation, increases the amount of filamentous actin (F-actin) and impairs proper cellular migration. In contrast, loss of Grx1 function via knockdown and knockout strategies increases the amount of glutathionylated actin and decreases the level of F-actin. Sakai et al. (2012) proposed that physiological levels of ROS and redox balance regulate actin dynamics, which are required for chemotaxis and migration of immune cells. On the other hand, physiological ROS levels induce local membrane protrusions in marsupial kidney epithelium Ptk1 cells in a mechanism that involves cofilin, the actin-related protein 2/3 complex (Arp2/3) and the extracellular signal–regulated kinase (ERK), thereby enhancing the retrograde flow of actin at the leading edge of these migrating cells (Taulet et al., 2012).

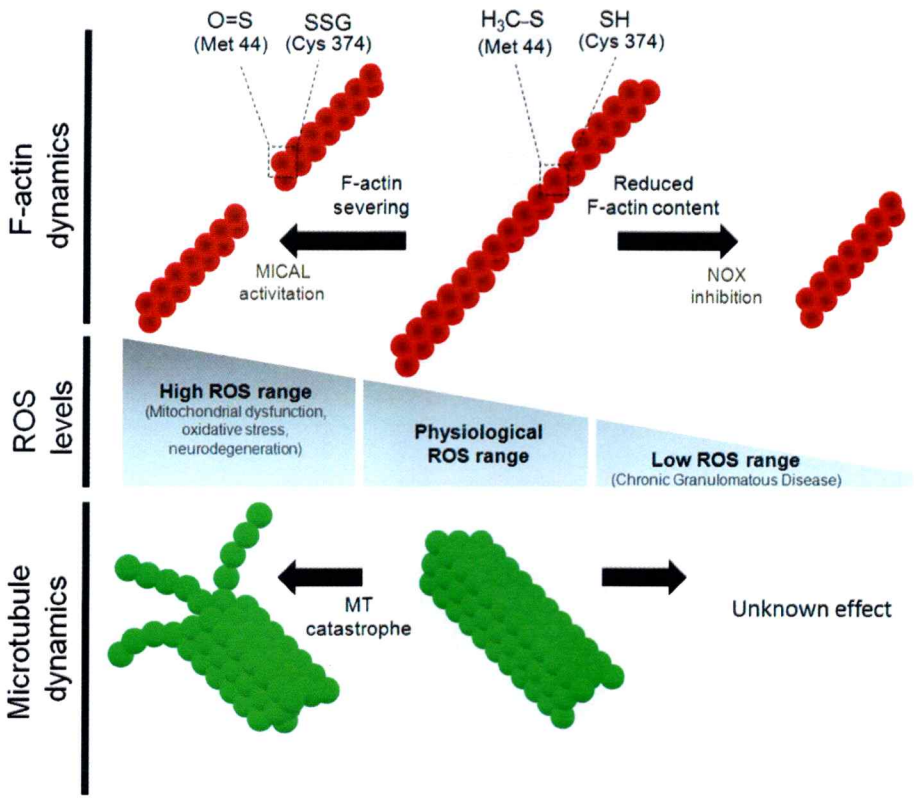

FIGURE 1 | Regulation of the cytoskeleton dynamics by differential reactive oxygen species (ROS) levels. Physiological concentrations of ROS are needed for proper F-actin and microtubule dynamics both *in vitro* and *in vivo*. Oxidative stress induces the severing of F-actin and impairs microtubule polymerization. In the particular case of actin monomers, both Cys 374 and Met 42 residues are targets for oxidative molecules during oxidative stress conditions. Oxidation of Cys 374 leads to actin glutathionylation. On the other hand, oxidation of Met 44, which occurs specifically by MICAL1, leads to Met oxidation and severing of the actin filaments (Hung et al., 2011). Despite both α and β tubulin have several Cys residues that are susceptible to oxidation, it has not been described which Cys residues of tubulin monomers are modified by oxidation. In contrast, down-regulation of ROS synthesis, mainly through NOX inhibition, decreases the F-actin content and alters the dynamic properties of the actin cytoskeleton. None of the post-translational modification of actin monomers have been described after NOX inhibition and ROS down-regulation. Finally, the effect of the inhibition of ROS synthesis (below physiological levels) on microtubule dynamics has not been explored.

Figure 1 summarizes the effect of the oxidative power on actin polymerization and F-actin dynamics.

ROS also target the actin cytoskeleton in neuronal cells. The Semaphorin/Plexin signaling pathway represents a major repulsive cue for axonal guidance (Hung and Terman, 2011). Semaphorin 3A (Sema3A) signaling induces a local increase in H_2O_2 at the growth cones of dorsal root ganglion neurons by activating MICAL1 and MICAL3 (Morinaka et al., 2011). MICAL1 is a binding partner for the cytosolic domain of Plexin A, a Sema3A receptor (Terman et al., 2002). The principal target of MICAL1 is actin (Hung et al., 2010, 2011; Giridharan and Caplan, 2014). In bristle cells of Drosophila melanogaster, oxidation of actin at Met 44 by MICAL1 severs actin filaments (Hung et al., 2011). MICAL1-dependent actin oxidation is reversed by Selenoprotein R (SelR) activation, restoring F-actin dynamics and polymerization (Hung et al., 2013). After neuronal injury, Sema3A levels rise above normal levels to inhibit the ability of axons to regenerate and regrow. These findings represent a redox mechanism by which Sema3A induces the collapse of axonal growth cones and impedes axon guidance, supporting the hypothesis that redox imbalance—particularly oxidative stress—leads to neuronal damage. Therefore, MICAL1 contributes to axonal pathfinding by regulating ROS signaling at dorsal root ganglion growth cones. In addition to directly regulating the actin redox balance, MICAL1 may modify actin dynamics by promoting interaction between proteins. MICAL1 interacts with Cas and CasL proteins, which may be involved in cross-talk between actin and intermediate filaments (Suzuki et al., 2002). Moreover, MICAL1 negatively regulates the nuclear dbf2-related kinase NDR2 in non-neuronal cells (Zhou et al., 2011). NDR1 and NDR2 contribute to targeting the Par3/Par6/aPKC complex to growing axons, a mechanism that promotes neuronal polarity (Yang et al., 2014). Together, these findings suggest that MICAL1 both directly and indirectly regulates the neuronal actin cytoskeleton, contributing to neuronal function and development under both normal and stress conditions.

In the marine mollusk Aplysia, NOX-derived ROS are required to maintain proper F-actin dynamics in the growth cones of bag cells (Munnamalai and Suter, 2009; Munnamalai et al., 2014). Inhibition of NOX activity using pharmacological inhibitors like apocynin, diphenyleneiodonium and VAS2870 reduce F-actin content in these growth cones and reduce both retrograde actin flow and neurite outgrowth, supporting the idea that actin dynamics and neurite elongation require basal NOX activity (Munnamalai and Suter, 2009). Cultured embryonic hippocampal neurons that express the mutant P156Q $p22^{phox}$, which down-regulates ROS synthesis by the NOX complex, show a decrease in the number, length and lifetime of filopodia at axonal growth cones (Wilson et al., 2015). Moreover, lamellar actin organization of stage 1 neurons, the initial morphology from which neurons develop, is disrupted after loss of NOX function (Wilson et al., 2015). Together, this evidence supports the hypothesis that local ROS signaling is needed to maintain normal F-actin dynamics in neurons and is consistent with other reports proposing that ROS are needed to support membrane protrusions, lamellar structures and filopodia at the leading edge of migrating cells (Taulet et al., 2012). Interestingly, NOX2 and $p40^{phox}$co-distribute with F-actin at the growth cone of neuronal bag cells in Aplysia, suggesting that local ROS production may be involved in neurite outgrowth (Munnamalai et al., 2014). NOXs are expressed in axons and dendrites of embryonic and adult neurons, suggesting that local ROS synthesis that may be involved in filopodial dynamics and neurite growth (Tejada-Simon et al., 2005; Wilson et al., 2015). **Figure 2** summarizes the differential effect of ROS on the organization of the growth cones of axons.

Tubulin Modification and Regulation of Microtubule Dynamics by Oxidative Species

α-Tubulin and β-tubulin contain 12 and 8 Cys residues, respectively, and each of these residues can be oxidized by endogenous and exogenous oxidizing agents (Luduena and Roach, 1991; Löwe et al., 2001; Landino et al., 2002, 2004a). The functions of these Cys residues are linked to GTP binding, microtubule polymerization and drug response (Mellon and Rebhun, 1976; Luduena et al., 1985; Luduena and Roach, 1991). In *in vitro* polymerization assays using purified tubulin from adult bovine brain, oxidative species added to the reaction medium dramatically reduced tubulin polymerization. Peroxynitrite ($ONOO^-$), a ROS produced from the reaction between superoxide and nitric oxide ($NO\oplus$), progressively oxidizes the thiol groups of tubulin monomers, thereby decreasing the ability of microtubules to polymerize *in vitro* (Landino et al., 2007). The same results were obtained with $NO\oplus$ and nitroxyl donors. Moreover, $ONOO^-$ promotes disulfide bond formation between α- and β-tubulin (Landino et al., 2004a). In addition, *in vitro* assays revealed that tubulin is glutathionylated after treatment with $ONOO^-$, and that this modification is reversed by the glutathione/glutathione reductase system, composed of glutathione, glutathione reductase, Grx and NADPH (Landino et al., 2004a). The reversal of tubulin glutathionylation by this system is interesting because intracellular signaling pathways may modulate microtubule polymerization in a reversible manner. **Figure 1** summarizes the effect of high oxidative power on microtubule dynamics. However, the inhibition of ROS synthesis below a physiological range has not been explored in terms of tubulin modifications nor microtubule dynamics.

Another layer of regulation is provided by proteins that stabilize or destabilize microtubules. Microtubule-associated protein 2 (MAP2) and tau are MAPS that specifically regulate MT polymerization in dendrites and axon. MAP2 and tau contain one and seven Cys residues, respectively (Lewis et al., 1988). Oxidation of MAP2 and tau Cys residues decreases microtubule polymerization *in vitro*, suggesting that redox balance regulates tubulin not only through direct interaction but also by regulating their stabilization by MAPs (Landino et al., 2004b). It is plausible that oxidized/reduced MAPs present differential microtubule stabilization. Moreover, binding of MAPs to microtubules may promote differential regulation of molecular motors in axons and dendrites (Dixit et al., 2008), affecting trafficking and cargo destination. Therefore, redox-dependent MAP modifications may be an additional mechanism for regulating cytoskeletal dynamics in neurons. Indeed, increased nitrosylation of MAP1B at Cys 2457 is involved in neurite retraction through a mechanism that couples microtubule stability and dynein function (Stroissnigg et al., 2007; Villarroel-Campos and Gonzalez-Billault, 2014).

Microtubule function depends on its intrinsic polymerization properties (Mitchison and Kirschner, 1984, 1988) as well as the specific tubulin isotype (Kavallaris, 2010) and post-translational modifications (Janke, 2014). Microtubule proteins can be modified by redox state, but understanding the functional consequences of such modifications can be challenging. For example, tubulin modifications induced by $ONOO^-$ treatment *in vitro* can be difficult to interpret because $ONOO^-$is unstable at physiological pH, and thus *in vitro* microtubule polymerization assays are performed at basic pH (typically pH 10). In addition, tubulin is glutathionylated in both cell-specific and tissue-specific ways (Sparaco et al., 2006, 2009). Prefrontal cortex, cerebellum and spinal cord tissue samples from non-pathological humans exhibit tubulin glutathionylation under basal conditions. In addition, after treatment with oxidized glutathione, neurons are preferentially glutathionylated compared with astrocytes and oligodendrocytes (Sparaco et al., 2006). Thus, it seems that tubulin Cys residues can be modified by redox balance in both *in vitro* and *in vivo* contexts.

Neurite outgrowth is affected by tubulin oxidation in cellular models. In the motor neuron-derived neuroblastoma cell line NSC34, oxidation induced by oxidized glutathione promote the formation of retraction bulbs and thin axon-like processes (Carletti et al., 2011). Under these conditions, glutathionylated tubulin levels are increased and interestingly, tyrosination of α-tubulin is simultaneously decreased. These findings suggest that an oxidative cytoplasmic environment induces tubulin glutathionylation, leading to neurite retraction and degeneration.

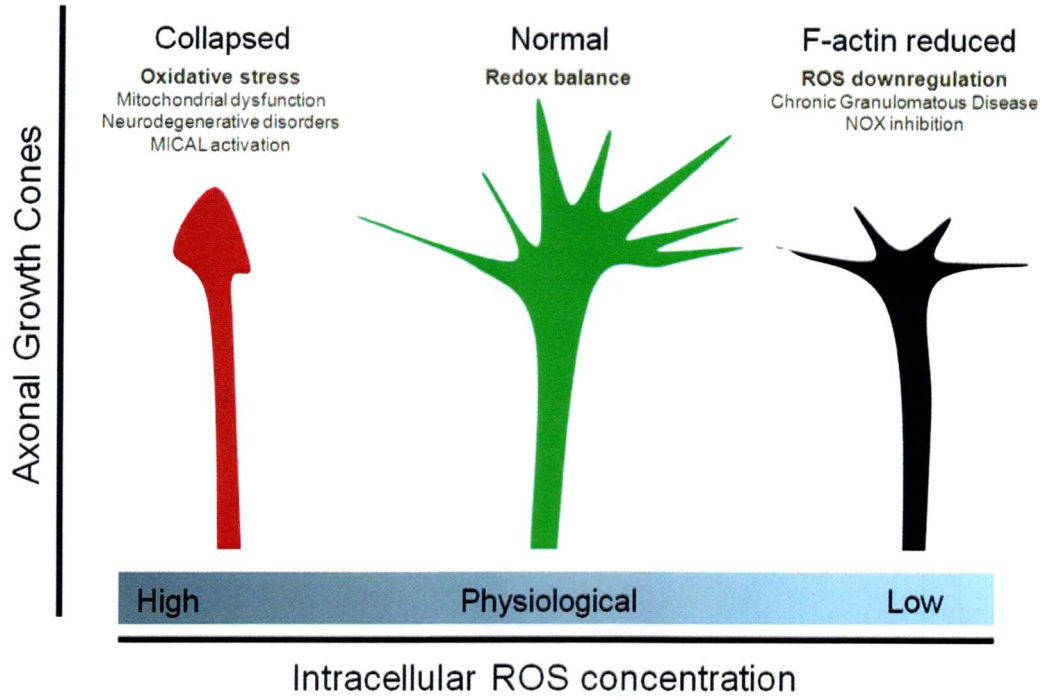

FIGURE 2 | Effect of physiological and non-physiological ROS on axonal growth cone organization. Oxidative stress (due to mitochondrial dysfunction, Sema3A/Plexin signaling and MICAL1 activation, neurodegenerative disorders, among others) induces the collapse of the axonal growth cones of neurons. In contrast, down-regulation of ROS synthesis, particularly due to NOX inhibition as well as Chronic Granulomatous Disease (CGD), decreases the F-actin content at the growth cone and affect filopodial dynamics by decreasing the number and length of filopodia at the axonal growth cone. Physiological concentrations of ROS, principally maintained by NOX enzymes and basal mitochondrial activity, are needed for a proper organization of the growth cones. In this context, the implicances for vesicular trafficking due to altered cytoskeleton dynamics has not been explored.

Moreover, in Friedrich's ataxia, a neuropathological condition characterized by degeneration of spinal cord pathways, immunohistochemical studies in motor neurons revealed co-distribution of tyrosinated tubulin and glutathionylated proteins (Sparaco et al., 2009). In the future, it will be necessary to establish whether there is a causal relationship between tubulin glutathionylation and changes in microtubule dynamics.

Studies in cultured primary neurons exploring phenotypes and mechanisms underlying the regulation of microtubule dynamics by redox state are still preliminary. However, there is some indirect evidence suggesting a putative link between microtubule polymerization and ROS balance. CRMP-2, a molecular regulator of microtubule polymerization, is oxidized at Cys 504, inducing homodimerization. These dimers can form a transient complex with thioredoxin, which creates a docking site for the protein kinase GSK3-β. GSK3-β-dependent CRMP-2 phosphorylation is linked to growth cone collapse in cultured dorsal root ganglion cells, recapitulating some molecular pathways involved in the initial steps of neurodegeneration (Morinaka et al., 2011). Therefore, new research may help establish a direct link between regulation of microtubule dynamics and redox balance in neuronal systems.

Participation of Oxidative Species in the Central Nervous System and Neuronal Development

Genetic models that reduce ROS production in the nervous system, such as $gp91^{phox}$ and $p47^{phox}$ knockout mice, are characterized by a macroscopically normal brain overall, including the cerebral cortex and hippocampus (Kishida et al., 2006). However, they display cognitive impairments as well as impaired synaptic plasticity, a phenomenon that involves, among other molecular events, cytoskeletal remodeling (Kishida et al., 2006). Therefore, a complete understanding of the contribution of ROS to normal nervous system physiology is important. Some insights have emerged in the last few years. For example, ROS levels have been shown to contribute to the commitment of neuronal progenitors to differentiate into mature neurons (Forsberg et al., 2013; Quadrato and Di Giovanni, 2013; Forsberg and Di Giovanni, 2014). Along the same line, loss of NOX function (using either neurons from NOX2 knockout mice or NOX2 knockdown in cultured neurons) has been shown to decrease the proliferation rate and number of neural stem cells *in vivo* and *in vitro*, suggesting that physiological ROS levels derived from NOX2 are needed to maintain a basal population

of adult hippocampal neuronal progenitors (Dickinson et al., 2011). It was recently observed that inhibition of NOX functions alter normal neuronal polarization and reduces axonal growth (Wilson et al., 2015). Similarly, differentiation of cerebellar granule neurons involves glutathione homeostasis and NOX activity (Olguín-Albuerne and Morán, 2015). Of note, MICAL1 knockout mice exhibit abnormal mossy fiber lamination, aberrant F-actin content and decreased Rab6 trafficking to the growth cones of hippocampal neurons, suggesting a role in the development of mossy fiber axons and of specific sub-areas of the hippocampus and supporting the notion that redox balance is needed for development of brain tissues (Van Battum et al., 2014). Together, these lines of evidence support a new hypothesis in the field of ROS, that the physiological and controlled production of ROS is needed for signaling and development in neurons. In the future, it will be important to address *in vivo* functions for ROS from various sources and their involvement in neuronal differentiation, migration and axonal guidance.

A major challenge in this field is to understand the specificity that redox imbalance have on cytoskeleton proteins compared to DNA or lipids. This is especially important considering that modifications on these molecules could also affect neuronal functions and morphology. In fact, ROS contributes to the transcription of several genes associated with metabolism, cell cycle and development (Bigarella et al., 2014), supporting the notion that ROS have pleiotropic effects on sub-cellular components. Moreover, there are technical concerns about the quantification of ROS in cells. Both ROS and cytoskeleton proteins are dynamic cellular elements with short life-times. Thus, it is hard to establish a correlation between local synthesis of ROS and the oxidation of actin filaments and microtubules in a living cell. New genetic tools based on fluorescence microscopy have emerged in the last years to measure ROS levels (Mishin et al., 2015). Genetically encoded probes to measure ROS combined with cytoskeleton biosensors will be necessary to define the spatial and temporal association between ROS synthesis and cytoskeleton remodeling.

Emerging Concepts in the Contribution of Redox Balance to Vesicle Trafficking

Intracellular trafficking is highly dependent on actin microfilaments, microtubules and molecular motors such as myosin, kinesin and dynein. In addition, members of the Rab family of small GTPases are essential for targeting components to discrete domains within cells. Several Rab proteins derived from the trans-Golgi network, the early/late endosome and recycling endosomes regulate neurite outgrowth and development (Villarroel-Campos et al., 2014). In preceding sections, we discussed how changes in ROS content may target tubulin and actin dynamics to regulate the tracks for intracellular trafficking. However, the link between redox balance and the vesicle components involved in neuronal trafficking remains poorly understood.

Several studies correlate MICAL activity with trafficking. It has been recovered as an interacting partner for several members of the Rab family in yeast two-hybrid experiments (Fukuda et al., 2008). MICAL1 deletion leads to aberrant destination of the IgCAM cell adhesion molecules to the growth cones of cultured hippocampal neurons in a Rab6- and actin-dependent mechanism, establishing a link between redox state and vesicle trafficking in neurons (Van Battum et al., 2014). Interestingly, MICAL1 interacts with Rab1, which is involved in vesicle trafficking from the endoplasmic reticulum to the Golgi (Fischer et al., 2005). Additionally, MICAL3 interacts with Rab8, which in turns interacts with Rab6 to promote exocytosis of secretory vesicles (Van Battum et al., 2014). Moreover, expression of a mutant isoform of MICAL3 in HeLa cells induces accumulation of vesicles at the cell cortex by inhibiting vesicle docking with the plasma membrane, ultimately decreasing release of vesicle contents (Grigoriev et al., 2011). This suggests a link between Rab-dependent vesicle trafficking and ROS. Zinc deficiency has been shown to decrease tubulin polymerization via oxidation of tubulin thiol groups (Mackenzie et al., 2011). Interestingly, tubulin oxidation also impairs translocation of the transcription factor NFκB to the nucleus, suggesting a link between redox state and microtubule-dependent trafficking in a cellular model (Mackenzie et al., 2011).

In addition to ROS, NO also plays a role in terms of neuronal function and vesicle trafficking. A recent report suggest that NO reduces the expression of the molecular motors KIF5 and KIF21B and it decreases the length of the axons of cultured cortical neurons (Redondo et al., 2015). Authors hypothesize that NO exposure could affect KIF-dependent vesicle trafficking required for normal axonal growth. In fact, axonal retraction and NO release are key issues in some neurodegenerative disorders, like Parkinson's disease (More et al., 2013; Tripathy et al., 2015). However, authors did not explored vesicle movement after NO exposure and this issue does not allow to conclude a direct effect of NO on axonal trafficking through cytoskeleton regulation. Moreover, G-protein coupled receptors that respond to NO (NO/CG) contributes positively to the physiology of neurons and neurotransmission (Hardingham et al., 2013; Russwurm et al., 2013). In summary, oxidative molecules signaling is an emerging concept in the field of cytoskeleton regulation and further studies will be required to understand the contribution to the vesicle trafficking and its impact on the physiology of neurons.

Concluding Remarks and Future Perspectives

ROS influence many different cellular functions under both physiological and pathological conditions. The targets of ROS include DNA, lipids and proteins. Among these, cytoskeletal proteins can be modified *in vitro* and *in vivo* by redox molecules. An imbalance between oxidative and reductive species leads to oxidative stress, which affects the polymerization of both F-actin and microtubules. In contrast, down-regulation of ROS also affects normal cytoskeletal organization, impacting the morphology, development and physiology of cells and neurons. Neuronal development and specification of neuronal compartments depend on redox homeostasis, in a mechanism that involves regulation of the actin cytoskeleton by the NOX complex. Further exploration of the role of redox balance

in regulating microtubule dynamics in cellular models is required. Abnormal polymerization of actin microfilaments and microtubules directly affects vesicle trafficking and specific cargo delivery throughout the soma, dendrites and axon. However, the regulation of vesicle trafficking and protein sorting by redox balance represents an unexplored field despite strong evidence in several cellular contexts that cytoskeletal proteins are targets of oxidative species. Moreover, the contribution of redox balance to the interaction between the cytoskeleton and cytoskeleton-associated proteins such as myosins, dyneins and kinesin molecular motors has not been studied, and such analysis may reveal direct effects on vesicle trafficking and cargo destination. New evidence has emerged concerning the dissection of the cellular sources of ROS that can modulate cytoskeletal dynamics. The development of new ratiometric microscopy tools to characterize the spatiotemporal production of ROS may give other important clues about how redox balance controls neuronal physiology.

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Chapter 4

Neuronal actin microfilaments: a target for redox species

Neuronal actin microfilaments: a target for redox species

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Running title: F-actin as a target of redox signaling

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Abstract

Actin cytoskeleton commands global functions in neurons. Redox signaling impacts directly on F-actin dynamics through actin post-translational modifications and also by the modulation of signaling pathways involved on the regulation of actin polymerization. In this review we summarized evidence that support the notion that actin is a target for redox molecules with a main focus on the enzymatic sources of oxidative molecules NOX and MICAL. Finally, we will discuss the impact of redox signaling on actin dynamics for neuronal function in health and disease.

Introduction

Actin microfilaments (F-actin) contribute to cellular and neuronal physiology in a global context. F-actin dynamics determine the shape and morphology of virtually all cells, promote both chemotaxis and cell motility and regulate vesicle trafficking as well as neurite outgrowth in neurons, among other actin-related functions (Ambruso et al. 2000; Roberts et al. 1999; Stiess and Bradke 2011). The regulation of F-actin cytoskeleton depends on molecular mechanisms that involve actin-associated proteins, like the Arp 2/3 complex and cofilin protein and also by endogenous signaling regulators like the Rho GTPase family proteins (Gonzalez-Billault et al. 2012; Hall 2012). Together, these partners are able to regulate F-actin dynamics at both physiological and pathological dimensions.

F-actin dynamics may be also regulated by post-translational mechanisms at both monomer and polymeric levels. Previous reports suggest that actin monomers posses several cysteine and methionine residues that are susceptible to oxidation, which impact both positively and negatively on the neuronal F-actin properties (Wilson and Gonzalez-Billault 2015). The outcome depends on the nature of the oxidative molecules and the time at which F-actin cytoskeleton is exposed to oxidation. Long-term exposure to oxidants, as well as abnormally high concentrations of oxidative molecules, tends to disrupt actin polymerization (Hung and Terman 2011; Terman and Kashina 2013; Wilson and Gonzalez-Billault 2015). In contrast, physiological and time-regulated synthesis of oxidative species is required for normal F-actin dynamics (Valdivia et al. 2015; Wilson and Gonzalez-Billault 2015). In this review, we will discuss specific issues about the influence of redox balance on neuronal actin cytoskeleton, suggesting the main effects on neuronal health and disease.

Structural basis for the redox control of F-actin polymerization

Both cysteine and methionine aminoacids are susceptible to oxidation. Actin monomer posses 5 cysteines and 16 methionines (Dalle-Donne et al. 2003; Dalle-Donne et al. 2002; Dalle-Donne et al. 2001). The actin Cys 374 residue is fully exposed to the cytoplasm, which makes it a good target for pro-oxidant molecules, like superoxide, hydroxyl radical and hydrogen peroxide (collectively called reactive oxygen species or ROS) (Dalle-Donne et al. 2003). On the other hand, 7 methionines of the actin monomer are exposed to the cytoplasm (Met 44, 47, 176, 190, 227, 260 and 375) (Dalle-Donne et al. 2002). The meaning of the actin and methionine oxidation depends on the spatio-temporal context. In vitro assays suggest that a strong oxidative environment tends to disrupt actin polymerization (Wilson and Gonzalez-Billault 2015). On the other hand, oxidative stress, an abnormal homeostasis condition in which the concentration of oxidative molecules is higher than reductive ones, usually trigger F-actin severing (Wilson and Gonzalez-Billault 2015). In contrast, the content of polymerized actin, organization and dynamics is dramatically impaired after downregulation of ROS basal levels in neurons of several models, suggesting that physiological levels of ROS are needed to maintain proper F-actin levels (Munnamalai and Suter 2009; Munnamalai et al. 2014; Wilson et al. 2015).

F-actin dynamics regulation by redox signaling: NOX and MICAL enzymes contribution

Oxidative molecules like superoxide, hydrogen peroxide and nitric oxide are considered signaling molecules due to their concentrations are spatio-temporally regulated by enzymes (Bigarella et al. 2014). In this context, the NADPH oxidase (NOX) enzymes have important functions. The NOX enzymatic family comprises 7 members called NOX 1 -5 and both Duox 1-2 (Bedard and Krause 2007). NOXs catalyze the conversion of molecular oxygen (O_2) into the superoxide radical ($O_2^{\cdot-}$), a short-lived molecule that is rapidly converted both spontaneously or enzimatically into hydrogen peroxide (H_2O_2) (Nunez et al. 2012). The main NOXs expressed at the central nervous system are NOX 1, 2 and 4 (Sorce et al. 2012). NOX2 is the best characterized isoform and it seems to be the main NOX expressed in neurons (Bedard and Krause 2007; Lambeth 2004; Nayernia et al. 2014). NOX2 (also called $gp91^{phox}$) is a plasma membrane protein of approximately 50 kDa spanning 6 trans-membrane domains, with both NADPH and

FAD binding sites towards the intracellular space (Groemping and Rittinger 2005; Harper et al. 1985; Paclet et al. 2004; Rotrosen et al. 1990). NOX2 establish a complex with $p22^{phox}$, a 22 kDa plasma membrane protein that confers further stabilization to NOX2 (Ambasta et al. 2004; Dinauer et al. 1987; Parkos et al. 1988). The complex NOX2/$p22^{phox}$ interacts with 4 additional molecular partners: $p40^{phox}$, $p47^{phox}$, $p67^{phox}$ and the Rho GTPase Rac (Lambeth 2004). The assembly of these interactors depends on the cellular demands of ROS. To increase ROS synthesis, protein kinase PKC phosphorylates $p47^{phox}$, which allows the translocation to the plasma membrane to meet NOX2/$p22^{phox}$ complex (Groemping and Rittinger 2005). After phosphorylation, $p47^{phox}$ suffer a conformational change that releases its own autoinhibitory domain, promoting the interaction with the NOX activator $p67^{phox}$ (Groemping and Rittinger 2005; Nauseef 2004; Sumimoto et al. 2005). Considering these, $p47^{phox}$ is usually referred as the organizer of NOX complex assembly, which allows increasing ROS synthesis over basal levels in response to cellular demands. Finally, Rac and $p40^{phox}$ meet the complex through $p67^{phox}$ interaction (Bokoch and Zhao 2006; Glogauer et al. 2003). Rac, a molecular regulator of the F-actin dynamics, has 3 isoforms called Rac1, Rac2 and Rac3. In neutrophils, the knock down of Rac2 but not Rac1 decreased ROS production via NOX, suggesting an isoform dependent mechanism (Ambruso et al. 2000; Kim and Dinauer 2001; Roberts et al. 1999). In neurons, both Rac1 and Rac2 are expressed and usually Rac1 is positively associated with F-actin polymerization, axonal growth and neuronal polarity (Gonzalez-Billault et al. 2012). Additional studies will be required to dissect which Rac isoform is required for ROS production in neurons, which could clarify whether the pool of Rac required for ROS production is the same that regulates at the same time F-actin dynamics and neuronal polarization. Considering this, both Rac and NOX might be involved on a kind of positive feedback loop, which could regulate the synthesis of ROS required for neuronal polarization through the regulation of actin cytoskeleton.

F-actin colocalizes with $p40^{phox}$ at the growth cone of the mollusc Aplysia, which propose a structural link between actin cytoskeleton and NOX proteins (Munnamalai et al. 2014). In addition, NOX2, $p47^{phox}$ and $p22^{phox}$ have been detected in the growing axon of both mature and immature hippocampal cultured neurons (Serrano et al. 2003; Tejada-Simon et al. 2005; Wilson et al. 2015). Moreover, these 3 proteins were also detected at the axonal tip and growth cone of young polarizing neurons (2 days in vitro)

(Wilson et al. 2015). Ectopic expression of $p22^{phox}$ P156Q in neurons, a mutant isoform of $p22^{phox}$ that inhibits ROS production via NOX1, NOX2 and NOX3 (Kawahara et al. 2005), decreased both lamellar organization of actin at stage 1 neurons (the initial neuronal morphology before axonal specification) and filopodial dynamics at axonal growth cones of cultured hippocampal neurons (Wilson et al. 2015), suggesting that physiological ROS regulates F-actin dynamics in neuronal cells. The influence of redox balance on the activity of actin-related proteins has just begun to be explored. By instance, we know that both Rac1 and Cdc-42 activities are decreased after NOX2 inhibition in cultured hippocampal neurons (Wilson et al. 2015), which propose that molecular regulators of F-actin dynamics also depend on redox balance. However, detailed mechanism about redox regulation of the Rho GTPase proteins remains unexplored.

Together, these evidences support the notion that NOX complex is spatially correlated with those actin-rich sites of a neuron, like the periphery of axonal growth cone and emerging both axon and neurites, suggesting a structural link between actin and NOX proteins.

Implicances of F-actin regulation by redox signaling in neuronal function

Previous findings suggest that humans with deleterious point mutations in $gp91^{phox}$, $p47^{phox}$, $p67^{phox}$ and $p22^{phox}$ proteins develop cognitive impairments and lower intellectual coefficients compared to controls (Cole et al. 2013; Pao et al. 2004). These mutations triggers an infectious inherited disease called Chronic Granulomatous Disease (CGD), in which the 70% correspond to point mutations in the X-linked $gp91^{phox}$ gene, 25% for $p47^{phox}$ gene and 5% between $p67^{phox}$ and $p22^{phox}$ genes, according to the study done by Pao et al. (Pao et al. 2004). Electrophysiological analyses on the knockout mice $gp91^{phox\ -/-}$ and $p47^{phox\ -/-}$ shown that both genotypes develop a decreased LTP response compared to wild type animals, which propose that the lack of NOX proteins affects memory and learning consolidation processes in a murine model (Kishida et al. 2006). However, we must be careful before to do any correlation between the impaired LTP response on mice and the cognitive impairments detected in human CGD cases, because the models of study, the assays and both phenotypic and genetic contexts are quietly different.

Synaptic plasticity and dendritic spine remodeling are both actin and microtubules dependent processes (Conde and Caceres 2009; Hotulainen and Hoogenraad 2010; Jausoro et al. 2013; Jaworski et al. 2009; Kapitein et al. 2010; Merriam et al. 2013). Despite these, it remains unclear whether dendritic spine remodeling depends on redox signaling through actin dynamics regulation. Interestingly, glutamate signaling activated by NMDA agonist induce superoxide synthesis via NOX2 (Brennan et al. 2009; Reyes et al. 2012), which may suggest a link between glutamatergic activity, synaptic plasticity, dendritic spine remodeling and actin cytoskeleton dynamics on a redox dependent manner. Further analyses will be required to dissect this issue.

A recent report suggests that NOX proteins have a dynamic expression pattern during the early development of the nervous system of zebrafish (0-2 post fertilization) (Weaver et al. 2015). In situ hybridization coupled to qPCR approaches revealed that nox1, nox5 and duox enzymes have a dynamic expression pattern, whereas cybb2/NOX2 was stable on the first 2 days post fertilization (Weaver et al. 2015). This is the first comprehensive analysis about NOX expression pattern done in vertebrates and further studies will be required to reveal the expression of NOX proteins in superior animals. The acquisition of neuronal morphology occurs after the last post-mitotic division of the neural precursor, in a process called the establishment of neuronal polarity (Caceres et al. 2012). Here, axonal specification occurs approximately 1 day after neuronal differentiation and it rules the break of the cellular symmetry through an actin dependent mechanism (Bradke and Dotti 1999; Dotti et al. 1988). Inhibition of the NOX complex activity through both genetic manipulation and pharmacological agents inhibits neuronal polarization and axonal growth (Wilson et al. 2015). Neurons that expressed $p22^{phox}$ P156Q shown a decreased on filopodium dynamics at the axonal growth cone (Wilson et al. 2015). In the same line, neurite outgrowth was also inhibited after NOX inhibition at both cerebellar granule neurons of mice and Aplysia neurons (Munnamalai and Suter 2009; Olguin-Albuerne and Moran 2015). However, cultured cerebellar granule neurons isolated from the NOX2 null exhibited normal neurite outgrowth compared with the wild type mice. These animals also exhibited normal ROS levels, which may suggest compensative mechanisms and/or that mice also expresses other NOX isoforms, like NOX1 and NOX4, according to previous reports (Olguin-Albuerne and Moran 2015; Sorce et al. 2012). The phenotypes here discussed are directly correlated with actin dynamics. Considering these, it is plausible to suggest that

redox balance exerts a global regulation of neuronal function, in which cytoskeleton and particularly F-actin is tightly linked to changes on redox homeostasis.

Neurological disorders like Alzheimer's disease usually develops actin rods at neurites (Minamide et al. 2000). Actin rods consist on cofilin and actin aggregation on a stoichiometric ratio (1: 1 = actin: cofilin) (Minamide et al. 2010). Rods may induce loss of synaptic function by blocking vesicle trafficking across dendrites or by sequestering cofilin at the somato-dendritic compartment, affecting LTP response (Cichon et al. 2012). The amyloid-β (Aβ) peptide, as well the proinflammatory cytokines TNF-α and IL-6, induce actin rods formation through a NOX dependent mechanism in both cortical and hippocampal cultured neurons (Walsh et al. 2014). Superoxide produced by NOX (and further interconversion into other ROS) oxidizes cofilin's cysteines, triggering disulfide bridge formation and cofilin aggregation (Bernstein and Bamburg 2010; Bernstein et al. 2012; Klamt et al. 2009). In addition, sustained excitotoxic glutamatergic activity, as well as energy deprivation (i.e.: mithocondrial dysfunction), also induce rod formation but through a NOX independent mechanism (Walsh et al. 2014). However, authors do not discard a redox effect through a non enzymatic source of ROS, like mitochondrion or Fenton reaction at cytoplasm (Nunez et al. 2012), like occurs after the sciatic nerve crush in mice, in which mithocondrial dysfunction plays a key role on the neurodegenerative process (Villegas et al. 2014), and motoneuron degeneration on Amiotrophic Lateral Sclerosis (ALS)'s disease (Rojas et al. 2015). On the other hand, NOX-derived ROS are also involved on neuronal degeneration of spinal cord neurons at the peripheral nervous system (Kuhn 2014). Proinflammatory cytokines TNF-α and IL-1β induce the collapse of the growth cone of spinal cord neurons through a NOX2 dependent mechanism. Elevated ROS synthesis triggers aberrant actin reorganization on the affected growth cones after spinal cord injury (Kuhn 2014). The growth cone morphology is mainly composed by actin microfilaments and microtubules, suggesting that neurodegeneration of after spinal cord injury migh be directly linked to NOX and actin cytoskeleton, according to Walsh et al. paper (Walsh et al. 2014). Together, these findings support the notion that actin oxidation impacts on the physiology and pathology of neurons.

Conclusions

The evidence here summarized intends to strength out the point that neuronal actin cytoskeleton is a target for redox species. Structural motif of actin (cysteines and methionines residues exposed to the cytoplasm susceptible to oxidative modifications) as well as redox signaling (at both control and stress conditions) affects F-actin dynamics in a positive and/or negative way, which depends on the cellular demands, spatio-temporal context, the source of ROS synthesis and the mechanism of the cell to remove properly the accumulated oxidative molecules. Whereas high ROS levels, usually associated with oxidative stress, induce F-actin severing and aberrant actin aggregation (like actin rods), the loss of ROS levels under physiological concentrations also decreases actin polymerization and it reduces the F-actin content in neurons. It seems that a proper balance of redox species is required to maintain physiological F-actin dynamics which in turns sustain a healthy morphology of neurons and a normal performance of neurons.

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Chapter 5

A feed-forward mechanism involving the NOX complex and ER calcium release mediated by the ryanodine receptor promotes axonal growth

A feed-forward mechanism involving the NOX complex and ER calcium release mediated by the ryanodine receptor promotes axonal growth

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Abbreviated title: NOX promotes axonal growth through ER Ca^{2+} release

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Highlights

- NOX2 promotes axonal growth through a RyR-mediated Ca^{2+} release mechanism
- RyR-mediated Ca^{2+} release activates Rac1 through a NOX2 dependent mechanism
- A feed-forward mechanism between NOX2-RyR-Rac1 promotes axonal growth

In brief

Wilson et al. show through pharmacological and genetic approaches, including the null mice of the $p47^{phox}$ protein ($Ncf1^{-/-}$), that NOX2 complex promotes axonal growth through a positive feedback mechanism involving NOX2, endoplasmic reticulum Ca^{2+} release mediated by the RyR and Rac1 in hippocampal neurons.

Summary

Physiological levels of ROS promote neurite outgrowth and axonal specification, but the mechanisms by which ROS contribute to shape neurons remains unknown. Ca^{2+}, a wide intracellular messenger, promotes both Rac1 activation and axonal growth. Ca^{2+} release from endoplasmic reticulum (ER), mediated by both IP3R1 and RyR channels, needs physiological ROS levels that are mainly sustained by the NOX complex. In this work we explored the contribution of the link between NOX and RyR-mediated Ca^{2+} release to the axonal growth of hippocampal neurons. Using genetic approaches we found that NOX activation promotes both axonal growth and Rac1 activity through a RyR-mediated Ca^{2+} release mechanism, which in turn led to NOX activation through Rac1, one of the NOX subunits. Collectively, these data suggest a feed-forward mechanism that integrates both NOX activity and RyR-mediated Ca^{2+} release to support cellular mechanisms involved in axon development.

Introduction

Reactive oxygen species (ROS) may exert both physiological and pathological effects on neurons. The increase on ROS levels above physiological concentrations is associated with oxidative stress and neurodegeneration at both central and peripheral nervous system (Lambeth, 2004; Villegas et al., 2014; Walsh et al., 2014). In contrast,

downregulation of ROS synthesis below physiological levels is also deleterious for neurons (Wilson and Gonzalez-Billault, 2015). The specific inhibition of the NADPH oxidase (NOX) complex through either genetically or pharmacological strategies, leads to an abnormal neuronal morphology evidenced by the loss of polarity and reduced axonal growth in cultured cerebellar and hippocampal neurons (Olguin-Albuerne and Moran, 2015; Wilson et al., 2015). Previous reports support the notion that NOX activity is required to maintain a basal ROS concentration needed to support neurite outgrowth (Munnamalai and Suter, 2009; Munnamalai et al., 2014; Olguin-Albuerne and Moran, 2015; Wilson et al., 2015). We recently showed that NOX loss of function impairs actin dynamics by inhibiting the activity of the small Rho GTPases Rac1 and Cdc-42 (Wilson et al., 2015). Both GTPases and proper F-actin dynamics are required for neuronal polarization (Bradke and Dotti, 1999; Gonzalez-Billault et al., 2012; Stiess and Bradke, 2011), i.e. the morphological transformations by which a neuron reach a mature phenotype characterized by the presence of a single axon and several dendrites (Caceres et al., 2012; Dotti et al., 1988; Namba et al., 2015). While we demonstrated that NOX loss of function impairs axon elongation (Wilson et al., 2015), the precise contribution of NOX gain of function and ROS generation to axonal growth remains unknown. Since ROS production is usually paralleled as a negative signal for neurons, it is relevant to carefully scrutinize whether physiological ROS production may support normal neuronal functions.

ROS are highly dynamic molecules with an average lifetime on the nanosecond time range. In contrast, neuronal polarization and axonal growth occur after hours and even days in culture. This feature poses the question about how short-live species like ROS regulate both axonal development and the establishment of neuronal polarity. We hypothesize that ROS may act as signaling molecules involving downstream mediators. Previous reports suggest that the basal NOX activity is required to promote Ca^{2+} release from the endoplasmic reticulum (ER), particularly mediated by the Ryanodine Receptor (RyR) (Bedard and Krause, 2007; Espinosa et al., 2006; Riquelme et al., 2011; Zhang and Forscher, 2009). Interestingly, RyR stimulation with ryanodine on the agonist concentration range (10 nM), promotes the exchange of GDP by GTP of both Rac1 and Cdc-42 in HEK293 cells and cerebellar granule neurons, suggesting that the activity of these GTPases depends on the RyR-mediated Ca^{2+} release (Jin et al., 2005). Activation of Rac1 and Cdc-42 promotes axonal growth in neurons (Gonzalez-Billault et al., 2012),

which allow us to hypothesize a functional coupling between NOX and ER Ca^{2+} release as a novel regulation point for actin cytoskeleton. However, ER Ca^{2+} release induced by NOX activity has not been reported in polarizing (immature) neurons. Ca^{2+} release from the ER has been previously associated with neuritogenesis, growth cone motility and neuronal differentiation (Gomez et al., 2001; Gomez and Zheng, 2006; Holliday et al., 1991; Mhyre et al., 2000; Nakamuta et al., 2011; Zhang and Forscher, 2009), but the precise contribution of ER Ca^{2+} release to the axonal growth and neuronal polarity acquisition needs further evaluation.

In this work we explored the contribution of gain of function of the NOX complex and ROS synthesis to the axonal growth of cultured hippocampal neurons. In addition, we studied the potential coupling between NOX and RyR-mediated Ca^{2+} release to both activation of Rac1 and axonal growth of hippocampal neurons.

Materials & Methods

Primary culture of hippocampal and cortical neurons from rat brain embryos. Pregnant Sprague-Dawley rats were sacrificed, both female and male embryos (E18.5) were removed and neurons were cultured as described (Kaech and Banker, 2006). All experiments were previously approved by the Bioethical Research Committee of Universidad de Chile and conducted following the guidelines of CONICYT manual for animal experimentation.

Primary culture of hippocampal neurons from wild type and Ncf1-/- mouse postnatal (P0) brains. Wild type C57Bl6/J (Harlan, UK) and B6(Cg)-Ncf1^{m1J}/J (Ncf1$^{-/-}$, Jackson) newborn pups (P0) were obtained from The Central Biomedical Service (CBS) of Imperial College London (UK). Pups were sacrificed and then both dissection and culture of hippocampal neurons were done according to (Kaech and Banker, 2006). Animal work was carried out in accordance to regulations of the UK Home Office.

Transient transfection of cDNA coding vectors. Neurons were transiently transfected with Lipofectamine 2000 (Life Technologies, CA, USA) in Neurobasal medium following manufacturer's instructions. After 2 h of transfection, neurons were supplemented with B27, Glutamax, sodium pyruvate and antibiotics. Experiments were performed between 18 and 72 h after cDNA transfection.

HyPer H_2O_2 measurement. Neurons ($4x10^4$ cells/well) were cultured on glass coverslips pre-treated with poly-D-lysine (1 mg/mL). Immediately after plating, neurons were transiently transfected with the HyPer biosensor (Evrogen, Moscow, Russia) to detect local H_2O_2 production (Belousov et al., 2006; Lukyanov and Belousov, 2014). H_2O_2 analysis was done as described (Wilson et al., 2015). The distal/proximal axonal ratio was estimated by measuring HyPer-H_2O_2 levels on the first third of the axon (proximal segment) and the last third of the axon (distal segment).

Hippocampal and cortical lysates and immunoblotting for RyR2 and IP3R1 detection. After dissection from embryonic brains, hippocampi and cerebral cortices were collected into 1.5 mL tubes and washed three times with PBS. After this, hippocampi and cortices were incubated in RIPA (from radio immune precipitation assay) buffer for 15 min at 4°C. Tissue lysates were centrifuged at 14,000 g for 15 min at 4°C. Supernatants were recovered, and aliquots were taken for protein quantification using the Bradford method. The supernatant was diluted with Laemmli buffer and denatured for 5 min at 95°C. Proteins were separated by SDS-PAGE and transferred to a nitrocellulose membrane for 90 min at 380 mA and 4°C. Membranes were blocked with 3% non-fat milk dissolved in TBS with 0.05% w/v Tween-20 (0.05% TBST) for 1 h at room temperature. Primary antibody anti Ryanodine Receptor 2 (RyR2, 1:1000, rabbit) was purchased from Millipore (AB9080). The antibody anti α-tubulin (1:10,000, mouse) was acquired from Sigma (MO, USA). The antibody anti-IP3R1 is a homemade antibody which was kindly gifted by Dr. Manuel Estrada (Faculty of Medicine, University of Chile). For antibody references please see (Choe et al., 2004; Estrada et al., 2006). Primary antibodies were diluted in 1% non-fat milk dissolved in 0.05% TBST and incubated with the membrane overnight at 4°C with agitation. Membranes were washed three times with 0.05% TBST, and appropriate secondary antibodies conjugated with HRP (1:2,000 for RyR2 and IP3R1 receptors, 1:5,000 for α-tubulin, Sigma) were incubated for 1 h at room temperature with agitation in primary antibody incubation solution. Protein detection was done using the ECL Western Blotting substrate (Pierce).

Immunofluorescence for RyR2 and IP3R1. Neurons (10^4 cells/well) were cultured in 24 multi-well plates on glass coverslips pre-treated with poly-D-lysine (1 mg/mL) for 18 and 48 h (for stage 2 and 3 neurons, respectively). Neurons were fixed with 4% of paraformaldehyde/sucrose for 30 min at 37°C, washed with PBS and permeabilized

with PBS containing 0.1% Triton X-100 for 10 min at room temperature. Neurons were then blocked with 5% BSA for 1 h at room temperature followed by primary antibody incubation. Anti-RyR2 (1:100), anti-IP3R1 (1:100) and anti-Tau-1 (Sigma, 1:400) were incubated overnight at 4°C. Coverslips were washed three times with PBS, and appropriate Alexa Fluor–conjugated secondary antibodies (1:400; Life Technologies) were incubated for 1 h at room temperature. Phalloidin-Alexa Fluor 633 was co-incubated with secondary antibodies. Coverslips were washed three times with PBS and mounted on glass slides with FluorSave reagent (Merck, Germany).

ER Ca^{2+} release assays. Hippocampal neurons ($1.5x10^5$) were cultured in 35 mm dishes containing 25 mm glass coverslips pre-treated with poly-D-lysine (1 mg/mL). After 2 days of culture, neurons were loaded with FLUO4-AM (5 μM) for 20 min in Hank's balanced salt solution supplemented with Hepes (HBSS) at 37°C and 5% CO_2. Then, FLUO4-AM containing medium was replaced by neurobasal medium supplemented with B27, Glutamax, sodium pyruvate and antibiotics for 40 min at the same incubation conditions. Coverslips were mounted into a video-microscopy chamber and perfused with HBSS without Ca^{2+} in order to discard the influx from extracellular medium. Images were acquired every 2 seconds for 5 min using a time-lapse recording. Imaging was done with a Zeiss LSM 710 microscope (Oberkochen, Germany) using a 20x magnification. To induce RyR-mediated Ca^{2+} release, neurons were stimulated with 4-Chloro-m-cresol (4-CMC, Sigma, 750 μM) after 1 min of baseline recording (first 30 images). Previous reports suggest that 4-CMC is a specific agonist of RyR (Adasme et al., 2015; Westerblad et al., 1998). The average intensity of the baseline fluorescence was noted as "Fo". Fluorescence intensity after 4-CMC addition (or vehicle in Fig. 3A) was noted as "F". Both "Fo" and "F" quantifications were done selecting a ROI at the soma of neurons. The ratio F/Fo was used to show the fold change on FLUO4-AM fluorescence intensity after RyR stimulation with 4-CMC. The analyses were done using the Fiji-ImageJ plug-in *Time series analyzer*.

FRET analysis of the Raichu-Rac1 probe to measure Rac1 activity. Neurons ($4x10^4$ cells/well) were transfected with the Raichu-Rac1 FRET biosensor (provided by Dr. Alfredo Cáceres, IMMF, Córdoba, Argentina) to measure the Rho GTPase activity (Nakamura et al., 2006). Raichu-Rac1 expression, image acquisition and FRET efficiency estimation were performed as described (Wilson et al., 2015).

Rac1 activity pull down assay in embryonic cortical neurons. Cortical neurons (E18.5) were cultured for 2 days in 100 mm diameter plastic plates (10^7 neurons / plate) pre-treated with poly-D-lysine (1 mg/mL) and then were stimulated with 4-CMC (750 μM) for 0-30 min at 37°C and 5% CO_2. To inhibit NOX activity, neurons were treated with VAS2870 (5 μM) for 1 h before 4-CMC stimulation. To evaluate Rac1 activity we addressed a pull down assay (Henriquez et al., 2012). Briefly, the CRIB domain of p21-activated kinase (Pak1) that binds specifically to the Rac1-GTP, but not to the inactive form of Rac1 (Rac1-GDP), was expressed and purified from BL21 (DE3) *E.coli* strain carrying the pGEX-CRIB-GST plasmid. The strain was grown overnight (ON) in LB-ampicillin medium. Overnight cultures were diluted 1:100 and grown in fresh medium until OD 0.6 at 37°C. Then, 0.1 mM isopropyl-b-D-thiogalactopyranoside (IPTG) was added. 2 h after induction cells were collected and lysed by sonication (Branson 450 digital sonifier, Millipore) in lysis buffer A (50 mM Tris-HCl pH 8.0, 1%Triton X-100, 1 mM EDTA, 0.15 M NaCl, 25 mM NaF, 0.5 mM PMSF and 1x of protease inhibitor complex (Roche)). Cleared lysate was then purified by affinity with glutathione-Sepharose beads (Amersham). Loaded beads were washed 10 times with lysis buffer B (lysis buffer A plus 300 mM NaCl) at 4°C.The GST fusion proteins were quantified and visualized in SDS-PAGE gels stained with Coomassie brilliant blue (CBBS) or immunoblotted using the anti-GST antibody. For the Rac1 assay activity, cortical neurons were cultured for 2 days in 100 mm diameter plastic plates pre-treated with poly-D-lysine (1 mg/mL) and then were stimulated with 4-CMC (750 μM) for 0-30 min at 37°C and 5% CO_2. To inhibit NOX activity, neurons were treated with VAS2870 (5 μM) for 1 h before 4-CMC stimulation. Then, neurons were lysed with fishing buffer (50 mM Tris-HCl pH 7.5, 10% glycerol, 1% Triton X-100, 200 mM NaCl, 10 mM $MgCl_2$, 25 mM NaF and 1x protease inhibitor complex) for 5 min at 4°C. Loaded beads were incubated for 1 h at 4°C with 1 mg of cortical neurons proteins using fishing buffer. The beads were then washed three times with washing buffer (50 mM Tris-HCl pH 7.5, 30 mM $MgCl_2$, 40 mM NaCl) and the washed beads were suspended in SDS–PAGE sampling buffer. The bound Rac1-GTP was subjected to immunoblot analysis and quantified with respect to total Rac1 with the Fiji-ImageJ program.

Image acquisition and analysis. All images were obtained with an LSM 710 confocal microscope (Zeiss, Oberkochen, Germany). Imaging of results illustrated in figure 7 was done according to the equipment of the microscopy facility of the Imperial College

London. Briefly, axonal length measurement and Ca^{2+} imaging were done using a Nikon eclipse TE 2000-u coupled to a CoolLED pE-4000 illumination system. Imaging for FRET experiments was done using a Leica TCS SP5 II confocal microscope. Processing, measurement and quantification of images were done with Fiji-ImageJ.

Statistics. Results are the mean of three independent cultures (N = 3) ± s.e.m. The number of neurons analyzed for each experiment (n) is indicated in the figure legends. ANOVA, Dunnett's post-test and t-student tests were done with the GraphPad Prism 5 software.

Results

Our previous work showed that NOX loss-of-function inhibits axon elongation in a mechanism involving ROS production, Rac1 activation and F-actin dynamics (Wilson et al., 2015). On the first set of experiments of this work, we explored a NOX gain-of-function approach to scrutinize its effects upon axonal growth. As gain-of-function paradigm we choose to co-transfect neurons immediately after plating with a vector encoding the wild type $p47^{phox}$ subunit ($p47^{phox}$ WT) and GFP as a transfection control. A previous report has shown that the expression of the $p47^{phox}$ WT enhances ROS production through the NOX complex (Roepstorff et al., 2008) (Figure 1). Control neurons were only transfected with GFP. After 3 days in vitro (3 DIV) neurons were fixed for morphology analysis (axon and minor neurite length measurements), according to our previous report (Wilson et al., 2015). Neurons expressing the $p47^{phox}$ WT exhibited axons 50% longer than control neurons (Figure 1A-B, black arrows). Minor neurites were also enlarged after the $p47^{phox}$ WT expression, although such increment was milder than observed in axons (Figure 1A and C, red arrows). Both control and $p47^{phox}$WT neurons display axons that were in average 9-10 times longer than minor neurites, suggesting that neuronal polarity acquisition was not affected, and that an axon was clearly distinguishable from minor neurites (Figure 1B-C). To evaluate ROS production, neurons were transfected with the genetically encoded probe HyPer to measure H_2O_2 levels after $p47^{phox}$WT expression (Figure 1D-F). To this aim, neurons were co-transfected with both $p47^{phox}$WT and HyPer after 1 day of culture and later fixated at 2 days *in vitro*. Control neurons were only transfected with HyPer. Neurons expressing the $p47^{phox}$ WT exhibited a global increase on H_2O_2 levels (Figure 1D-F). In addition, we detected that H_2O_2 was enriched on the distal segment of the axon of these

neurons (Figure 1D, arrows in the H_2O_2 map and quantification in Figure 1F). To discard non-specific cellular responses, neurons expressing the $p47^{phox}$WT were treated with the NOX inhibitor VAS2870 (5 μM), a drug that blocks the translocation of $p47^{phox}$ to the plasma membrane (Altenhofer et al., 2012). The treatment with VAS2870 was done after 1 day of culture (when most of neurons only exhibit minor neurites) and then neurons were fixed after 2 and 3 days for both H_2O_2 measurement and neurite growth analysis, respectively (Figure 1G-L). The increase on the axonal length observed after $p47^{phox}$WT expression was abolished after the VAS2870 treatment (Figure 1G-H), suggesting a NOX-dependent axonal growth mechanism. On the same line, VAS2870 treated-neurons did not increase H_2O_2 levels after $p47^{phox}$ WT expression (Figure 1J-L), suggesting that NOX gain of function leads to an increased axonal H_2O_2 content mediated by the expression of the $p47^{phox}$ subunit. Together, these data supports our hypothesis that ROS are crucial for the proper polarization, axonal growth and further development of hippocampal neurons in culture.

Having shown that NOX activation promotes axonal growth, we sought to define a molecular mechanism that relates the generation of short-live species (i.e. H_2O_2) with a long-lasting process such as axonal growth. We brought our attention into Ca^{2+} release from the endoplasmic reticulum (ER) as a cellular intermediate. This hypothesis was based on two main evidences: 1) Ca^{2+} is a second messenger that activates several signaling cascades, some of which have been linked earlier to axon and neuritic elongation (Davare et al., 2009; Estrada et al., 2006; Henley and Poo, 2004; Wayman et al., 2004; Zheng and Poo, 2007) and 2) previous reports suggest that ER Ca^{2+} release, which is mediated by both the inositol 1,4,5-triphosphate receptor (IP3R) and the ryanodine receptor (RyR) (Bardo et al., 2006), is regulated by NOX activity (Bedard and Krause, 2007). The RyR presents several cysteine residues sensitive to redox species that need to be oxidized to support normal activity of the receptor (Aracena-Parks et al., 2006; Donoso et al., 2011; Espinosa et al., 2006; Hidalgo et al., 2006). We first designed experiments to reveal the contribution of both IP3R and RyR to the establishment of polarity and axonal growth. Previous reports suggest that the main embryonic isoforms expressed in neurons are IP3R1 and RyR2 (Bardo et al., 2006). Accordingly, we confirmed that both RyR2 and IP3R1 are expressed at both embryonic (E18.5) hippocampus and cerebral cortex by western blot analysis (Figure 2A, C). We detected a high-molecular weight protein (close to 500 kDa) that specifically reacts with

a RyR2 antibody and also a protein that migrated at 230 kDa corresponding to IP3R1 (Choe et al., 2004; Estrada et al., 2006). In addition, we found both RyR2 and IP3R1 in the soma and minor neurites (white arrows) of stage 2 cultured neurons (Figure 2B, D). Moreover, both receptors were also detected at the somato-dendritic compartment of stage 3 neurons (white arrows), the growing axon (yellow arrows, Tau-1 positive neurites) and the axonal growth cone (asterisks) (Figure 2B, D). Together, these data suggest that both RyR2 and IP3R1 are expressed embryonically on rat hippocampus and both receptors segregate to the emerging axon at the initial stages of neuronal polarization. We then sought to address the contribution of Ca^{2+} release mediated by these receptors to axonal growth. To this aim 2 parameters were evaluated: the proportion of neurons at stages 1-3 of polarity after 1 day of culture and the axonal growth after 3 days of culture (Wilson et al., 2015). To evaluate neuronal polarity acquisition, neurons were treated with xestospongin C (3 μM) or ryanodine (25 μM) after plating to block IP3R and RyR, respectively (Figure 2E and G). After 24 h, neurons were fixed to evaluate the population of them at stage 1, 2 or 3 of polarity (Wilson et al., 2015). We found that both xestospongin C and ryanodine treatments induced a significant increase in the number of neurons at stage 1 of polarity (Figure 2E, G). Moreover, xestospongin C treatment also decreased the proportion of neurons at stage 3 compared to the untreated neurons (Figure 2G). We then evaluated the contribution of RyR2 and IP3R1 receptors to the axonal growth. Neurons were treated with xestospongin C or ryanodine after 1 day of culture (when most of cultured neurons were already at stage 2) and fixed after 3 days in vitro to evaluate axonal growth (Figure 2F, H). Both ryanodine and xestospongin C treatment decreased axonal growth compared to control neurons, suggesting that both channels contribute to the axonal development in culture. Together, these data support the notion that both IP3R and RyR2 promote the development, polarization and axonal growth of hippocampal neurons.

In order to check whether NOX activity was coupled to RyR activity in our experimental paradigm, we designed experiments to evaluate the potential dependence of Ca^{2+} release mediated by RyR with NOX activity. Neurons of 2 days in culture were loaded with Fluo4-AM (a Ca^{2+} sensitive probe) to measure cytoplasmic Ca^{2+} levels after RyR stimulation with the agonist 4-Chloro-m-cresol (4-CMC) (Westerblad et al., 1998). After Fluo4-AM loading, neurons were mounted on a video-recording chamber

HBSS/Hepes. Figure 3A-E shows representative Ca^{2+} signals in neurons before (0-1 min) and after (1-5 min) 4-CMC (750 μM) stimulation. Yellow arrows of each panel indicate the time point at which 4-CMC was added to neurons (1 min). Figure 3A shows that the vehicle (ethanol) did not change Fluo4-AM intensity at any time of the recording. Then, neurons were stimulated with 4-CMC (Figure 3B). White arrows show representative neurons that experiment an increase on the Fluo4-AM intensity after 4-CMC addition (Figure 3B, arrows). In order to control that Ca^{2+} release was mediated by RyR after 4-CMC stimulation, neurons were incubated by 1 h with ryanodine (25 μM) before Ca^{2+} recording (Figure 3C). These neurons did not show any change on Fluo4-AM intensity after 4-CMC additions, reinforcing the idea that 4-CMC specifically induces ER Ca^{2+} release mediated by RyR. Figure 3F and G show the quantification of the ER Ca^{2+} release of both control and ryanodine-treated neurons after 4-CMC additions. We observed that ryanodine completely abolished Ca^{2+} release from ER (Figure 3F). We compared the F/Fo ratio in both control and ryanodine conditions after 5 min of 4-CMC stimulation (t=5 min) in which it is possible to observe that ryanodine-treated neurons do not release Ca^{2+} compared to control neurons in a statistically significant way (Figure 3G, ***$p<0.001$ vs control). Next, we explored the link between NOX and Ca^{2+} release mediated by RyR. To this aim, neurons were incubated with two NOX inhibitors, the peptide gp91 ds-tat (5 μM) (Rey et al., 2001) (Fig. 3D) and VAS2870 (5 μM) (Fig. 3E) for 1 h before Ca^{2+} recording. After NOX inhibition, 4-CMC failed to induce Ca^{2+} release as observed in control condition (Figure 3H-I). Quantitative analyses show that gp91 ds-tat decrease in 50% the Ca^{2+} release from ER compared to control neurons, whereas VAS2870 completely abolished RyR dependent Ca^{2+} release (Figure 3H). The F/Fo ratio at t=5 min suggests that both gp91 ds-tat and VAS2870 significantly decreased Ca^{2+} release from ER mediated by the RyR (Figure 3I, ***$p<0.001$ vs control). Collectively, these data support the notion that the basal activity of the NOX complex is important to support Ca^{2+} release from the endoplasmic reticulum mediated by RyR in polarizing neurons.

Previous reports suggest that Ca^{2+} release mediated by RyR activates both Rac1 and Cdc-42 in cerebellar granule neurons, reaching a peak after 3 min of RyR stimulation (Jin et al., 2005). Considering that axonal growth is a Rac1 dependent process (Gonzalez-Billault et al., 2012), we evaluated whether the coupling between NOX and RyR may regulate the activity of Rac1 in our model. To this aim, neurons were

transfected after 1 day of culture with the FRET probe Raichu-Rac1 (Nakamura et al., 2006). After 1 day of expression, neurons were treated with 4-CMC (750 μM) for 1, 5, 15, 30 min and then neurons were fixed to evaluate the FRET efficiency using confocal microscopy (Figure 4A-C). Neurons reached a 2-fold peak on the FRET efficiency after 5 min stimulation with 4-CMC when we analyzed both the whole neuron and axonal compartment (Figure 4B-C). On the same line, pull down analysis performed on primary cultured cortical neurons confirm that after 5 min of 4-CMC stimulation, neurons exhibit an enrichment in the pool of the Rac1-GTP protein (Figure 4F-G). Next, we designed experiments to down-regulate NOX activity, with the purpose of scrutinize whether the NOX-RyR coupling was involved in Rac1 activation after 4-CMC stimulation. To this aim, hippocampal cultured neurons were transfected after 1 day of culture with a cDNA encoding the P156Q $p22^{phox}$ protein ($DNp22^{phox}$), a mutant version of $p22^{phox}$ that decreases ROS production mediated by NOX (Kawahara et al., 2005; Wilson et al., 2015). These neurons were co-transfected with the Raichu-Rac1 FRET probe to study Rac1 activity after NOX loss of function (Figure 4D-E). After 1 day of expression of both $DNp22^{phox}$ and Raichu-Rac1 probe, neurons were stimulated with 4-CMC for 5 min in order to compare the Raichu-Rac1 FRET efficiency between the $DNp22^{phox}$ and control neurons. In addition, transfected neurons with the FRET probe were treated with VAS2870 (5 μM) and then were stimulated with 4-CMC for 5 min (Figure 4D and E). Both the genetic ($DNp22^{phox}$) and pharmacological (VAS2870) inhibition of NOX blocked 4-CMC-induced Rac1 activation (Figure 4D and E). On the same line, pull down assays in cortical cultured neurons suggest that 4-CMC stimulation did not increase the amount of the Rac1-GTP protein after VAS2870 treatment (Fig. 4H and I). Together, these results suggest that the activity of Rac1 depends on the functional coupling between the NOX complex and the RyR in cultured neurons.

Based on our data, we explored the contribution of the functional coupling between NOX and RyR to the axonal growth of hippocampal neurons. Recent reports described that sustained endoplasmic reticulum Ca^{2+} release, as well as massive Ca^{2+}influx, lead to neurodegeneration (Bernard-Marissal et al., 2015; Vargas et al., 2015; Villegas et al., 2014). Accordingly, we explored different time points for 4-CMC stimulation to induce Ca^{2+} release from the endoplasmic reticulum specifically mediated by RyR. After 1 day of culture, neurons were treated with 4-CMC (750 μM) for times ranging from short

term stimulation (0, 1, 5, 15 and 30 min) to long term stimulation (1 and 24 h) (Figure 4J). 4-CMC was then washed and neurons were fixed at day 3 of culture. We found that after 15 min of 4-CMC stimulation, neurons developed longer axons compared to the non-stimulated neurons (Figure 4J). However, this phenotype disappeared after 30 min of treatment. Even more, after 24 h of stimulation axons were even shorter than those of control neurons, suggesting a toxic effect after long-term stimulation (Figure 4J). The next set of experiments was conducted using 15 minutes of stimulation with 4-CMC (Figure 4J). Neurons cultured for 1 day were treated with NOX inhibitors gp91 ds-tat (5 μM) and VAS2870 (5 μM) for 1 h and then were stimulated with 4-CMC for 15 min. After 4-CMC withdrawal we re-added the corresponding NOX inhibitors and neurons were fixed at day 3 of culture to measure neurites length (Fig.4K and L). NOX inhibitors blocked the axonal growth induced by 4-CMC (Figure 4K) without affecting the length of minor neurites (Figure 4L). Neurons were also treated with ryanodine (25 μM) to inhibit RyR activity before 4-CMC stimulation, which blocked the axonal growth induced by 4-CMC (Figure 4K and L), suggesting an effect mediated by RyR. In addition, neurons were transfected immediately after plating with the $DNp22^{phox}$ construct to induce the loss of function of the NOX complex with a genetic tool (Figure 4M-N). After 1 day, neurons were stimulated with 4-CMC for 15 min and then were cultured to complete 3 days in vitro to measure neurites length. Neurons that expressed $DNp22^{phox}$ did not exhibit longer axons after 4-CMC stimulation (Figure 4N) whereas minor neurites length was not affected (Figure 4N). Together, these results suggest that Ca^{2+} release from the ER mediated by RyR requires a basal NOX activity to promote axonal growth.

Rac1 is one of the subunits of the NOX complex (Lambeth, 2004). Considering our data, we wondered whether Ca^{2+} release mediated by RyR could stimulate ROS production by NOX through a Rac1 dependent mechanism. Previous evidence suggests that the electrical stimulation of the RyR produces H_2O_2 through NOX2 (Riquelme et al., 2011), but the link with Rac1 has not been explored. To this aim, neurons were transfected after 1 day of culture with the HyPer sensor to evaluate H_2O_2 levels after 4-CMC stimulation in control and both $DNp22^{phox}$ and Rac1T17N (dominant negative form of Rac1) (Ridley et al., 1992) transfected neurons. After transfection, neurons were cultured for 1 day and later on we re-stimulated with 4-CMC. Neurons were then fixed to evaluate H_2O_2 levels using confocal microscopy (Figure 5). After 15 min of 4-

CMC stimulation an increase on H_2O_2 levels was detected. However, this increase was blocked after both $DNp22^{phox}$ and Rac1T17N expression (Figure 5B). These results suggest that both RyR stimulation and Ca^{2+} release promote H_2O_2 synthesis through the NOX complex in a mechanism that depends on Rac1, suggesting a feed-forward mechanism between NOX and Ca^{2+} release mediated by RyR in polarizing neurons.

In order to check whether ROS produced by the NOX complex increase the axonal length through RyR activity, hippocampal neurons were transfected immediately after plating with $p47^{phox}$ WT (Figure 6A-C). After 1 day of expression (when most of neurons are at stage 2, Wilson et al., 2015), neurons were treated with ryanodine (25 μM) to block RyR activity and then were fixed at day 3. Ryanodine inhibition blocked the increase on the axonal growth induced by the $p47^{phox}$ WT expression (Figure 6B) without affecting minor neurites length (Figure 6C). Finally, we used the same experimental paradigm shown earlier in this work to evaluate Rac1 activity. NOX gain-of-function induced by the expression of the $p47^{phox}$ increased the FRET efficiency of the Raichu-Rac1 probe, which was blocked after VAS2870 or ryanodine treatments (Figure 6D-F), suggesting that NOX activates Rac1 via a mechanism that involves the RyR.

Finally, we evaluated axonal growth, Ca^{2+} release mediated by RyR and Rac1 activity in hippocampal neurons derived from the B6(Cg)-Ncf1m1J/J ($Ncf1^{-/-}$) mouse (Figure 7). Ncf1 corresponds to the $p47^{phox}$ subunit of the NOX2 complex. To this aim, wild type and $Ncf1^{-/-}$ postnatal (P0) neurons were cultured for 2 days to measure axonal length. In order to perform rescue experiments, neurons were transfected immediately after plating with the $p47^{phox}$ WT construct and GFP to check transfection. Neurons were fixed and axonal length was measured. We found that $Ncf1^{-/-}$ neurons have shorter axons compared to wild type neurons, which were restored close to wild type values after $p47^{phox}$ WT expression (Figure 7A). To check Ca^{2+} release mediated by the RyR, wild type and $Ncf1^{-/-}$ neurons were cultured for 2 days and then were loaded with Fluo4-AM (Figure 7B-D). Using the experimental approach described in Figure 3, we found that $Ncf1^{-/-}$ neurons have impaired endoplasmic reticulum Ca^{2+} release after RyR stimulation with 4-CMC (750 μM). Wild type P0 neurons showed a different pattern of Ca^{2+} release compared to embryonic (E18.5) hippocampal neurons (Figure 3). The expression of the ryanodine receptors, as well as that of the molecular regulators of their activity, like voltage-dependent Ca^{2+} channels and SERCA ATPase, among others, change across the

development of the brain (Giannini et al., 1995; Hertle and Yeckel, 2007; Lanner et al., 2010; Mori et al., 2000; Seymour-Laurent and Barish, 1995). Considering this fact, we attribute to the developmental stage the differences between the E18.5 neurons and P0 wild type neurons of figures 3 and 7, respectively. In addition, both wild type and $Ncf1^{-/-}$ neurons of 2 DIV were loaded with Fluo4-AM to evaluate Ca^{2+} influx after 90 mM KCl depolarization. To this aim, the recording solution (HBSS-Hepes) was supplemented with 2 mM Ca^{2+}. Wild type neurons exhibited massive Ca^{2+} influx after KCl treatment, which did not occur in $Ncf1^{-/-}$ neurons (Figure 7E), suggesting that depolarizing stimuli fail to induce Ca^{2+} influx in $Ncf1^{-/-}$ neurons. Finally, we also measured Rac1 activity expressing the Raichu-Rac1 FRET in wild type and $Ncf1^{-/-}$ neurons based on the protocol described in Figure 4. In addition, $Ncf1^{-/-}$ neurons were co-transfected with the FRET probe to check Rac1 activity after rescue (Figure 7F-G). The representative FRET maps (Figure 7F) and quantification of FRET efficiency (Figure 7G) suggest that $Ncf1^{-/-}$ neurons present a decrease on Rac1 activity compared to wild type neurons, which was reverted after the expression of the $p47^{phox}$ WT. Together, the data obtained from the $Ncf1^{-/-}$ mice led us to propose that the absence of a functional $p47^{phox}$ protein impairs axonal growth, RyR-mediated Ca^{2+} release and Rac1 activity in neurons.

Collectively, these data allow us to propose that the NOX complex promotes the axonal growth of neurons through a mechanistic feed-forward mechanism that links the NOX complex and Ca^{2+} release from the endoplasmic reticulum mediated by RyR with the activity of Rac1.

Discussion

Our data led us to propose that the NOX complex promotes Rac1 activation and axonal growth of hippocampal neurons through the regulation of the ER Ca^{2+} release mediated by the RyR. The gain of function of NOX after $p47^{phox}$ expression enhanced both ROS levels and axonal growth. The NOX inhibition with VAS2870 decreased both ROS levels and axonal growth after $p47^{phox}$ WT expression, suggesting that increasing ROS concentrations enhance the axonal growth of hippocampal neurons. Previously we showed that the loss of function of the NOX complex negatively regulate neuronal polarity and axonal growth (Wilson et al., 2015). In this work we also found that $Ncf1^{-/-}$ neurons have shorter axons compared to wild type neurons as well as a reduction on

basal Rac1 activity, which is consistent with the notion that NOX activity promotes axonal growth involving actin cytoskeleton regulation. Together, these evidences suggest that ROS derived from the NOX complex are needed for proper neuronal development, polarization and axonal specification in cultured hippocampal neurons. Similar results in different neuronal models suggest that NOX and ROS synthesis are required for neurite outgrowth (Munnamalai and Suter, 2009; Munnamalai et al., 2014; Olguin-Albuerne and Moran, 2015). Moreover, the differentiation of neuronal stem cells into neurons also depends on ROS signaling (Dickinson et al., 2011; Forsberg and Di Giovanni, 2014; Forsberg et al., 2013). The reduction of the oxidative power commits neural precursors to differentiate into non-neuronal phenotypes like oligodendrocytes at the central nervous system, whereas increasing ROS levels promotes early neurogenesis (Dickinson et al., 2011; Forsberg et al., 2013). Based on these findings it seems that physiological ROS levels are needed to regulate both neuronal differentiation and polarization at central nervous system. A recent report proposes that NOX genes are expressed immediately after fecundation in zebrafish larvae, which also occurs throughout the emerging nervous system in this model (Weaver et al., 2015). Further comprehensive studies are required to correlate both gene and protein expression with ROS synthesis and neuronal functions in higher animals.

We asked how ROS could regulate axonal growth. Considering that ROS are short life-time species, we conceived ROS as signaling molecules that regulate downstream pathways. For this aim we constructed on 2 set of evidences; firstly, ROS regulate Ca^{2+} release from endoplasmic reticulum in muscle and neuronal cells (Aracena-Parks et al., 2006; Donoso et al., 2011; Espinosa et al., 2006; Hidalgo et al., 2006; Riquelme et al., 2011; Zhang and Forscher, 2009) and second, Ca^{2+} release from the endoplasmic reticulum, principally mediated by RyR, promotes Rac1 activation in HEK293 cells, cerebellar granule neurons, Swiss 3T3 fibroblasts and PC3 cells (Fleming et al., 1999; Jin et al., 2005; Price et al., 2003). On the other hand, Rac1 is a key molecule for neuronal polarization and axonal growth (Gonzalez-Billault et al., 2012). High levels of intracellular Ca^{2+}, due to a massive influx from extracellular space or sustained ER Ca^{2+} release, leads to the collapse of the growth cone, whereas physiological levels are needed to maintain healthy growth cones and the navigation properties of neurites (Gomez et al., 2001; Gomez and Zheng, 2006). In this work we found that both IP3R and RyR are needed for proper neuronal polarity acquisition and axonal growth. Both

receptors were detected in neurons during early stages of neuronal polarization and both segregated to the emerging axon. RyR is also localized in mature axons of postnatal neurons (Hertle and Yeckel, 2007), suggesting a local role within axons in both embryonic and postnatal stages. We also found that RyR is functional at this polarization stage and depends on NOX basal activity. Ncf1$^{-/-}$ neurons also exhibited impairment on RyR-mediated Ca^{2+} release. This evidence is consistent with previous reports that suggest that RyR is expressed in an active conformation during the early differentiation of hippocampal neurons in culture (Seymour-Laurent and Barish, 1995; Sukhareva et al., 2002). Previous work showed that electrical stimulation of mature hippocampal neurons promotes ER Ca^{2+} release mediated by RyR, which is abolished in the presence of the general anti-oxidant N-acetyl-cysteine (NAC) (Riquelme et al., 2011). Our data specify that NOX, the main enzymatic source of ROS, regulates ER Ca^{2+} release by RyR in polarizing (immature) neurons, according with previous experiments on muscle and cardiac cells.

The KCl treatment did not result in depolarization of Ncf1$^{-/-}$ neurons. The endogenous agonist of RyR is Ca^{2+} that may come from the extracellular medium or endoplasmic reticulum (Lanner et al., 2010). Voltage-dependent Ca^{2+} channels located at the plasma membrane are also susceptible to redox balance and NOX activity (Bedard and Krause, 2007). Considering this aspect, we speculate that Ca^{2+} influx may also be impaired in a context of NOX-loss of function, which could impact directly on RyR signaling and further endoplasmic reticulum Ca^{2+} release.

Finally, we explored the contribution of the link between NOX and RyR-dependent Ca^{2+} release to the activity of Rac1 and axonal growth. We found that stimulation of RyR with 4-CMC for 5 min increased Rac1-GTP levels in neurons, suggesting that Rac1 activity depends on RyR activity in our model. A similar time frame has been described earlier to enhance both Rac1 and Cdc-42 activities in HEK293 cells and cerebellar granule neurons (Jin et al., 2005). In addition, Rac1 activation after 4-CMC treatment was blocked by NOX inhibition (Figure 4D-E, H-I), suggesting that ER Ca^{2+} release mediated by RyR depends on NOX and this affects the activity of Rac1 in neurons. *In vitro* assays suggest that ER Ca^{2+} release induces the phosphorylation and further activation of Tiam1 (one of the Guanine Exchange Factor of Rac1) through a PKC and CaMKII dependent mechanisms (Fleming et al., 1999; Price et al., 2003). Moreover, both protein kinases are involved in neurite extension and axonal growth

(Gomez and Zheng, 2006; Nakamuta et al., 2011). In this work we found that the functional coupling between NOX and RyR regulates axonal growth. The stimulation of RyR with 4-CMC enhanced the axonal length of hippocampal neurons which was abolished after NOX loss of function. Our data also suggest that the activation of Rac1 induced by RyR stimulation increases ROS synthesis derived from the NOX complex. Ca^{2+} release mediated by RyR also promotes H_2O_2 synthesis through NOX in hippocampal mature neurons (Riquelme et al., 2011). Considering both our and previous evidences, we speculate the existence of a feed-forward mechanism between NOX, Ca^{2+} release mediated by RyR and Rac1 needed for both axonal growth and neuronal functions. Finally, the gain of function of the NOX complex through the expression of the $p47^{phox}$WT construct did not increase the axonal growth on neurons treated with 25 μM ryanodine, suggesting that NOX requires the RyR to promote axonal growth of hippocampal neurons.

The evidence here discussed supports the notion that ROS regulate Rac1 activation and axonal growth with the participation of RyR. However, we cannot discard other signaling pathways that might be regulated by redox signaling. In fact, both F-actin and microtubules are susceptible to redox post-translational modifications which affect the dynamic properties of the cytoskeleton (Chowdhury et al., 2009; Dalle-Donne et al., 2003; Dalle-Donne et al., 2002; Dalle-Donne et al., 2001; Hung and Terman, 2011; Landino et al., 2002; Landino et al., 2004a; Landino et al., 2004b; Terman and Kashina, 2013). This property may impact directly on the morphology of neurons during the early stages of the establishment of neuronal polarity (Wilson and Gonzalez-Billault, 2015). On the same line, the PI3K/Akt pathway, which is involved on axonal extension (Cosker et al., 2008), depends on ROS levels (Forsberg et al., 2013), which allows to hypothesize that other pathways involved on axonal growth may be regulated by the redox balance in neurons.

In summary, based on our data we suggest that ROS are signaling molecules that regulate pathways involved on axonal growth, like endoplasmic reticulum Ca^{2+} release, without discarding other pathways or different levels of cellular regulation. It seems that ROS synthesis, when it is well regulated, is instrumental to support neuronal polarization and axonal growth, which is crucial for the development of mature neuronal functions, like synapses and neurotransmission.

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Figure legends

Figure 1. The gain of function of the NOX complex after $p47^{phox}$ wild type (WT) expression increases the axonal growth of hippocampal neurons. Embryonic (E18.5) hippocampal neurons were cultured and transfected immediately after plating with GFP alone (control) or co-transfected with both GFP the $p47^{phox}$ WT constructs. ***A,*** Representative control and $p47^{phox}$WT neurons of 3 days in vitro (DIV). ***B-C,*** Quantification of both axonal (black arrows) and minor neurites (red arrows) length of panel ***A***. **p<0.01 vs control (Student's t-test,). ***D,*** 1 DIV neurons were transfected with the HyPer biosensor to measure local H_2O_2 production. Representative H_2O_2 maps of both control and $p47^{phox}$ WT neurons. ***E-F,*** Quantification of the HyPer-H_2O_2 levels from neurons of panel ***D***. *p<0.05 vs control, **p<0.01 vs control, (Student's t-test). ***G,*** Control and $p47^{phox}$ WT neurons were treated with VAS2870 (5 μM) after 1 DIV and then were fixed at 3 DIV. ***H-I,*** Quantification of both axonal (black arrows) and minor neurites (red arrows) length of panel G. H, ***p<0.001 vs control (ANOVA, Dunnett's post-test). ***I,*** ns = non significant (ANOVA, Dunnett's post-test). ***J,*** Representative HyPer-H_2O_2 maps of both control and $p47^{phox}$ WT neurons after NOX inhibition with VAS2870. HyPer was expressed as in ***D*** and then neurons were treated with VAS2870 (5 μM) for 1 day (2 DIV in total). ***K-L,*** Quantification of the HyPer-H_2O_2 levels from neurons of panel ***J***. **p<0.01 vs control (ANOVA, Dunnett's post-test). Results are from 3 different independent cultures (N=3). A total of 45 transfected neurons were analyzed by each condition.

Figure 2. Contribution of ER Ca^{2+} channels to the establishment of neuronal polarity in culture. ***A, C,*** RyR2 and IP3R1 detection by western blot on both embryonic (E18.5) hippocampus and cerebral cortex lysates. ***B, D,*** RyR2 and IP3R1 distribution on both stage 2 and stage 3 hippocampal cultured neurons by using immunofluorescence and confocal microscopy. Tau-1 detection was used as an axonal marker and Phalloidin-Alexa Fluor 633 to stain F-actin. White arrows: minor neurites; yellow arrows: axons; asterisks: axonal growth cones. ***E, G,*** Quantification of the neuronal polarization of hipoccampal neurons after RyR and IP3R inhibition with ryanodine (Rya, 25 μM) and xestospongin C (3 μM). ***E,*** **p<0.01 vs control stage 1. ***G,*** *p<0.05 vs control stage 1; $^{\#}$p<0.05 vs control stage 3. ***F, H,*** Quantification of the axonal length after after RyR and IP3R inhibition. After plating, neurons were cultured for 1 day (when most of them are already at stage 2, (Wilson et al., 2015)) and then were treated with ryanodine (Rya, 25

μM, ***F***) and xestospongin C (3 μM, ***H***). Neurons were fixed after a total of 3 DIV. ***p<0.001 vs control, Student's t-test. Results are from 3 different independent cultures (N=3). A total of 90 neurons were analyzed for both neuronal polarity and axonal length measurements.

Figure 3. Functional coupling between NOX and RyR-mediated Ca^{2+} release in young hippocampal neurons. Neurons were cultured for 2 days on 35 mm dishes with a glass coverslip (pre-treated with poly-D-lysine) and then were loaded with FLUO4-AM to visualize cytoplasmic accumulation of Ca^{2+} in live neurons in Ca^{2+} free HBSS. ***A-B,*** Time-lapses imaging before and after the addition of the 4-CMC vehicle (ethanol, yellow arrow in ***A***) and 4-CMC (750 μM, ***B***). ***C,*** Neurons were pre-treated with ryanodine (25 μM) for 1 h before 4-CMC addition in order to block RyR. ***D-E,*** Neurons were treated with gp91 ds-tat (5 μM) and VAS2870 (5 μM) for 1h before 4-CMC addition. ***F,*** Quantification of the ER Ca^{2+} release of ***B*** and ***C***. ***G,*** Quantification of the F/Fo ratio after 4 min of 4-CMC addition (t=5 min of total register) in both control and ryanodine-pretreated neurons of ***B*** and ***C***. Arrow indicates the time at which 4-CMC was added. ***p<0.001 vs control, Student's t-test. ***H,*** Quantification of the ER Ca^{2+} release after 4-CMC stimulation to control and both gp91 ds-tat (5 μM) and VAS2870 (5 μM) of ***B***, ***D*** and ***E***. ***H,*** Quantification of the F/Fo ratio after 4 min of 4-CMC addition (t=5 min of register) of control, gp91 ds-tat (5 μM) and VAS2870 (5 μM) neurons of ***B, D*** and ***E***. Arrow indicates the time at which 4-CMC was added. ***p<0.001 vs control, Student's t-test. Results are from 3 different independent cultures (N=3). A total of 60 neurons were analyzed for each condition.

Figure 4. The NOX complex promotes both Rac1 activation and axonal growth through a RyR-dependent mechanism. ***A-E,*** Hippocampal neurons of 1 DIV were transfected with the FRET probe Raichu-Rac1 to measure the FRET efficiency after RyR stimulation in control and NOX-inhibited neurons. After this, neurons were fixed at 2 DIV to visualize the FRET efficiency using a confocal microscopy according to (Wilson et al., 2015). ***B-C,*** Quantification of the FRET efficiency in neurons stimulated with 4-CMC (750 μM) for 0, 1, 5 15 and 30 min at the whole neuron (***B***) and whole axon (***C***). ***p<0.001 vs 0 min, ANOVA, Dunnett's post-test, 21 neurons analyzed from 3 independent cultures (N=3). ***D-E,*** For NOX inhibition neurons were co-transfected with both the FRET probe and the P156Q $p22^{phox}$ ($DNp22^{phox}$) construct or just treated with VAS2870 (5 μM) after transfection of the FRET Raichu-Rac1 probe.

Ryanodine (25 μM) treatment was done as with VAS2870. Analyses were done at whole neuron (***D***) and axon (***E***) levels. ***p<0.001 vs control (ANOVA, Dunnett's post-test). ***F,*** Pull down analysis were performed to check the Rac1-GTP levels after 4-CMC stimulation in embryonic cortical neurons of 2 DIV. Neurons were treated with 4-CMC (750 μM) for 0, 1, 5 15 and 30 min. ***G,*** Densitometry of western blots of Rac1-GTP done in ***F,*** N=3. ***H,*** Representative western blots of the pull down analysis done to check Rac1-GTP levels after 4-CMC stimulation in cortical neurons of 2 DIV in control and NOX inhibited (VAS2870, 5 μM) conditions. N=3. ***I,*** Densitometry of western blots of Rac1-GTP done in ***H***, N=3. ***J,*** Hippocampal neurons were cultured for 1 day and then were stimulated with 4-CMC for 0, 1, 5, 15, 30, 60 min and 24 h. Then, neurons were fixed at day 3 of culture to measure axonal length. *p<0.05 vs 0 min, ***p<0.001 vs 0 min. ANOVA Dunnett's post-test. ***K-L,*** Hippocampal neurons were treated with gp91 ds-tat (5 μM), VAS2870 (5 μM) and ryanodine (25 μM) after 1 day of culture and then neurons were stimulated with 4-CMC (750 μM) for 15 min. Then, 4-CMC was removed and inhibitors re-added to the culture medium. Neurons were fixed at day 3 of culture for both axonal length (***K***) and minor neurites length quantification (***L***). **p<0.01 vs control, ***p<0.001 vs control, ns= non significant. ANOVA, Dunnett's post-test, N=3. 90 neurons were analyzed by condition. ***M-N,*** Neurons were transfected after plating with $DNp22^{phox}$ and after 1 day of culture were treated with 4-CMC for 15 min. After 4-CMC withdrawal, neurons were fixed at day 3 of culture to measure axonal and minor neurite lengths. *p<0.05 vs control, ANOVA, Dunnett's post-test, N=3. 45 neurons analyzed per condition.

Figure 5. RyR stimulation induces H_2O_2 production through the NOX complex by a Rac1 dependent mechanism. Neurons were transfected after 1 day of culture with the HyPer biosensor alone (control) or co-transfected with the $DNp22^{phox}$ or the Rac1T17N (dominant negative version of Rac1). After 1 day of expression neurons were treated with 4-CMC. ***A,*** H_2O_2 maps after 750 μM 4-CMC stimulation for 5 and 15 min in control, $DNp22^{phox}$ and Rac1T17N transfected neurons. ***B***, Quantification of the HyPer-H_2O_2 levels of panel A. *p<0.05, **p<0.01 and ***p<0.001 vs control (white column), ANOVA, Dunnett's post-test, N=3. 15 neurons analyzed per condition.

Figure 6. NOX gain of function increases both axonal growth and Rac1 activity trough a RyR dependent mechanism. ***A-C,*** Neurons were cultured and transfected immediately after plating with GFP alone (control) or co-transfected with both GFP the $p47^{phox}$ WT

constructs. After 1 day of culture, neurons were treated with ryanodine (25 μM) and fixed at day 3 of culture to measure neurites length. ***A,*** Representative neurons after $p47^{phox}$ WT expression and ryanodine treatment. ***B-C,*** Quantification of both axonal length (***B***) and minor neurites length (***C***) after $p47^{phox}$ WT expression in control and ryanodine treated neurons. ***B,*** **p<0.01 vs control, ANOVA, Dunnett's post-test, N=3. ***C,*** ns = non significant, ANOVA, Dunnett's post-test, N=3. 45 were neurons analyzed by each condition. ***D-E,*** Neurons were transfected after 1 day of culture with the Raichu Rac1 probe alone (control) or together with the $p47^{phox}$ WT. After 1 day of expression, neurons were treated with VAS2870 (5 μM) or ryanodine (25 μM) for 1 h. Neurons were then fixed to evaluate the FRET efficiency of the Raichu-Rac1 probe. ***D,*** FRET maps. ***E-F,*** Quantification of the FRET efficiency in control, VAS2870 and ryanodine-treated neurons on both whole neuron (E) and axonal compartment (F). *p<0.05, ***p<0.001 vs control (first column of each graph), ANOVA, Dunnett's post-test, N=4. 20 neurons analyzed per condition.

Figure 7. Ncf1 ($p47^{phox}$) null neurons exhibit reduced axonal growth, inhibited RyR-mediated Ca^{2+} release and Rac1 activity impairment. Postnatal (P0) neurons isolated from $Ncf1^{-/-}$ hippocampi were cultured for 2 days in vitro to address axonal length, Ca^{2+} release and Rac1 activity experiments. ***A,*** Axonal length measurement at 2 days in vitro of wild type and $Ncf1^{-/-}$ neurons. For rescue experiments, $Ncf1^{-/-}$ cultured neurons were transfected immediately after plating with the $p47^{phox}$ WT construct together with GFP (transfection control). ***p<0.001 vs wild type neurons, ANOVA, Dunnett's post-test, N=3. 21 neurons were analyzed per condition. ***B,*** Representative time-lapse imaging of both wild type and $Ncf1^{-/-}$ neurons loaded with FLUO4-AM to measure Ca^{2+} release from the endoplasmic reticulum mediated by the RyR after 4-CMC (750 μM) stimulation. Yellow arrow indicates the time at which 4-CMC was added in each case. White arrows show representative neurons to follow FLUO4-AM fluorescence intensity throughout the time of the recording (5 min). ***C,*** Quantification of F/Fo ratio at t= 68 s, corresponding to the maximum amplitude achieved after 4-CMC addition in wild type neurons. Arrow indicates the time at which 4-CMC was added. ***p<0.001 vs Ncf1 -/- neurons, Student's t-test. N=3, 45 neurons analyzed per condition. ***D,*** Depolarization with 90 mM KCl in wild type and Ncf1-/- neurons. Arrow indicates the time at which KCl was added N=3, 45 neurons analyzed per condition. To this aim, recording was performed using HBSS medium supplemented with both Hepes buffer and 2 mM

$CaCl_2$. ***E-F,*** Wild type and $Ncf1^{-/-}$ neurons of 1 day of culture were transfected with the Raichu-Rac1 FRET probe. For rescue experiment, the Raichu-Rac1 probe was co-transfected with the $p47^{phox}$ WT construct in $Ncf1^{-/-}$ neurons. In all cases, neurons were fixed after 1 day of the FRET probe expression (2 days in culture). ***E,*** Representative FRET maps of wild type, Ncf1 -/- and Ncf1 -/- rescued neurons ($p47^{phox}$ WT transient transfection). ***F,*** Quantification of the Raichu-Rac1 FRET efficiency. N=3. 21 neurons analyzed per condition.

Figure 1

Wilson et al. 2016

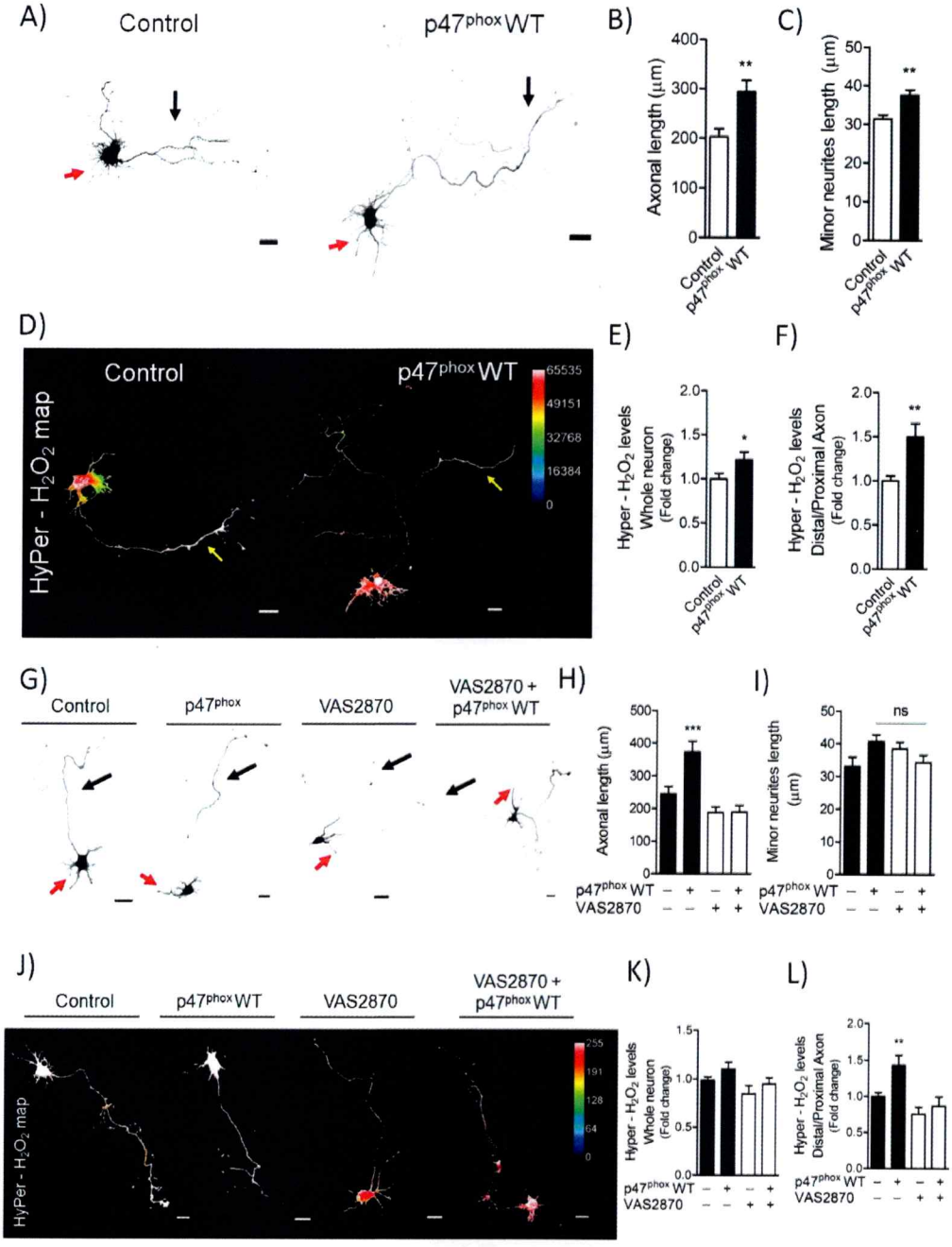

Figure 2

Wilson et al. 2016

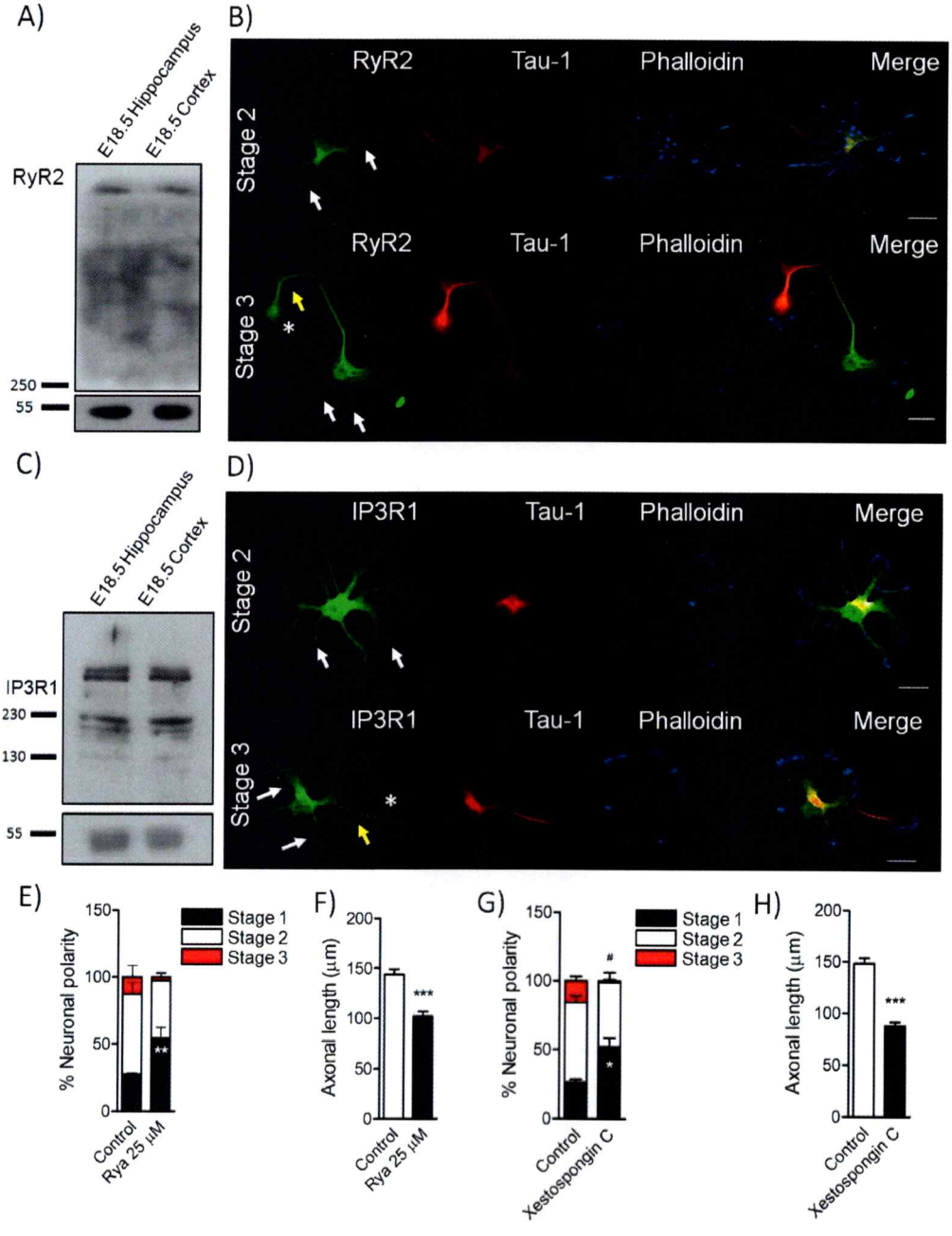

Figure 3 Wilson et al. 2016

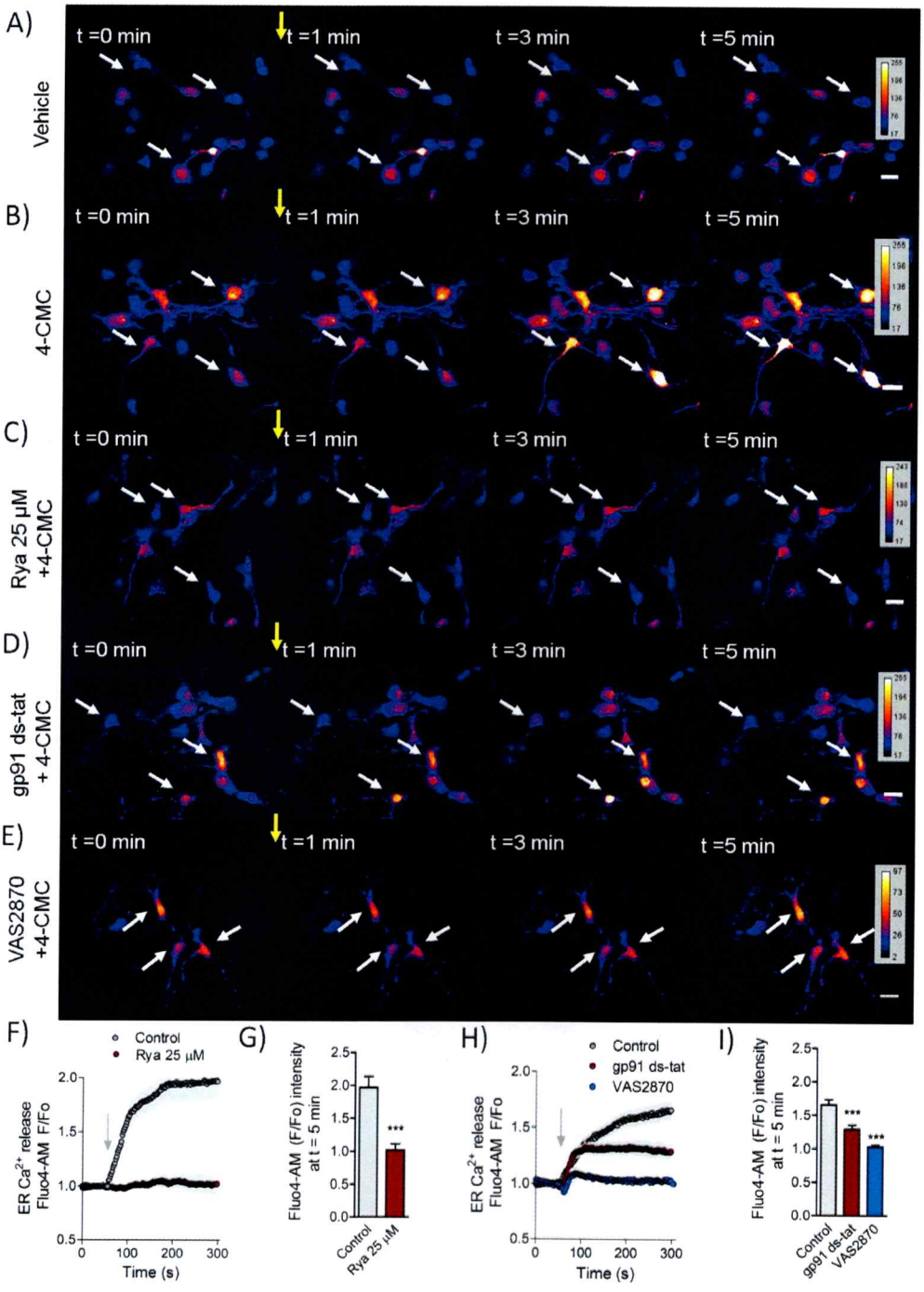

Figure 4

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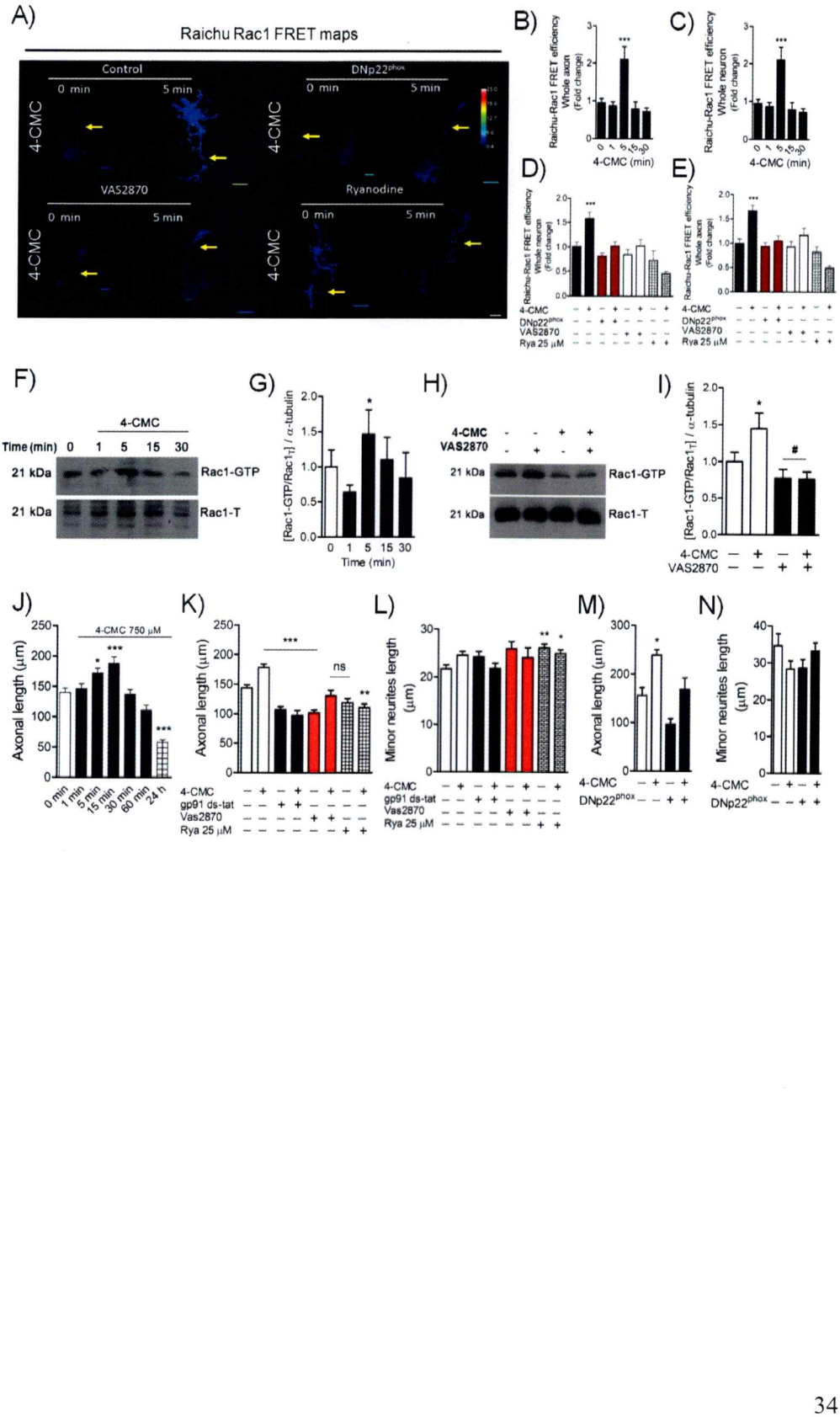

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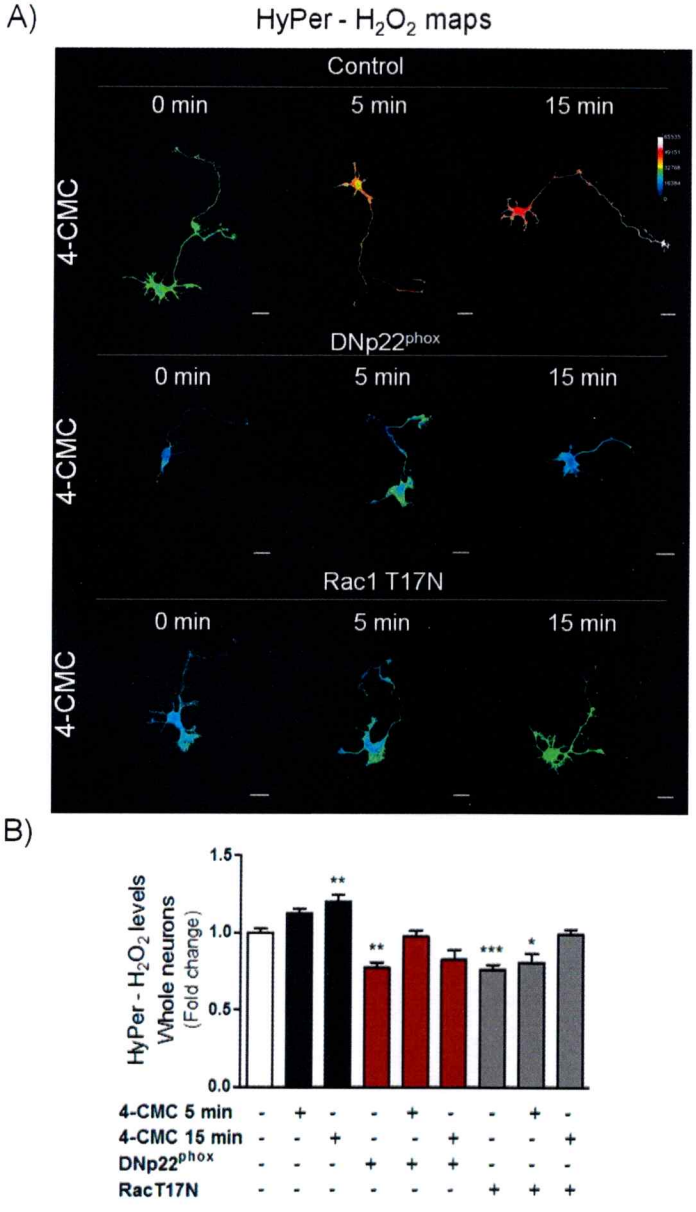


Figure 6

Wilson et al. 2016

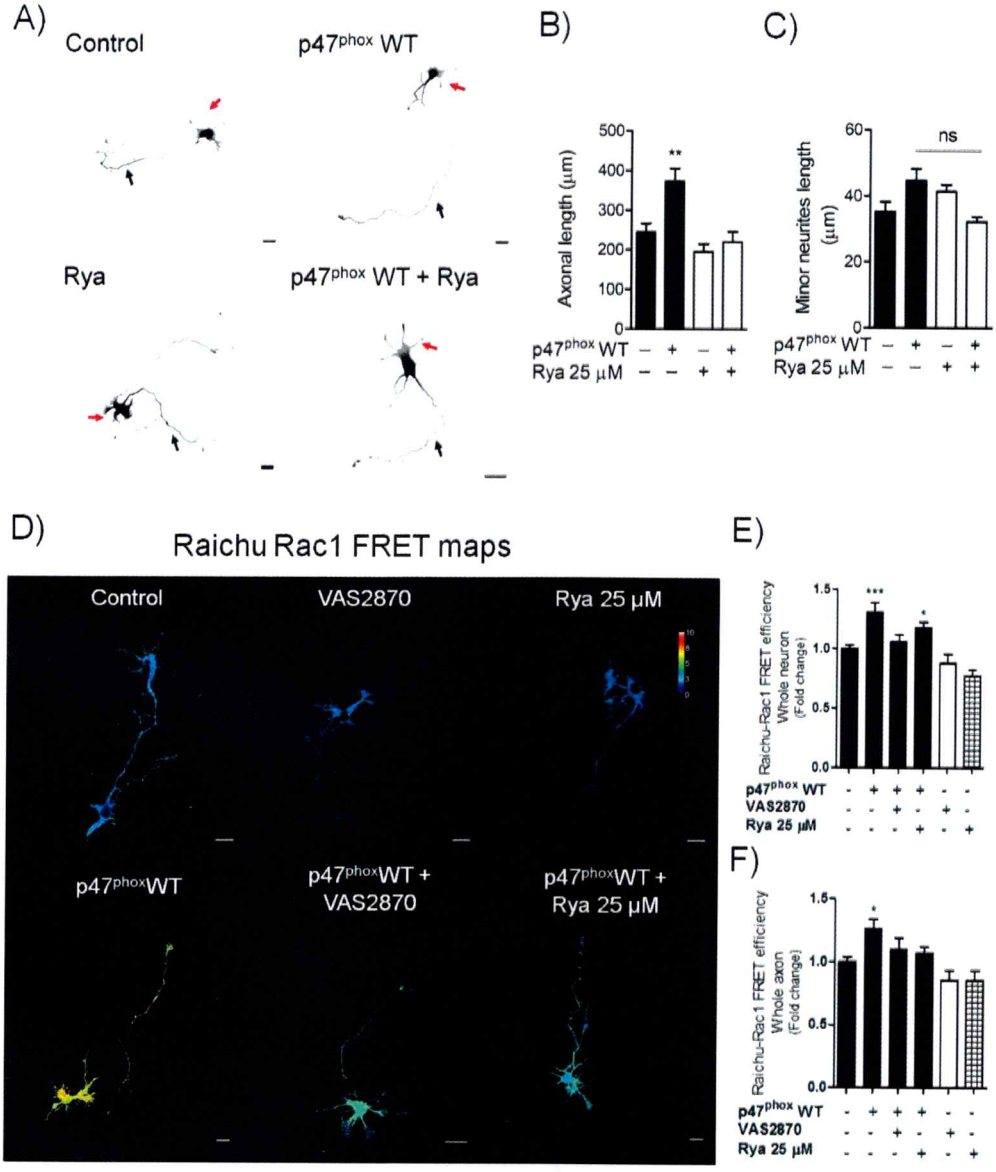
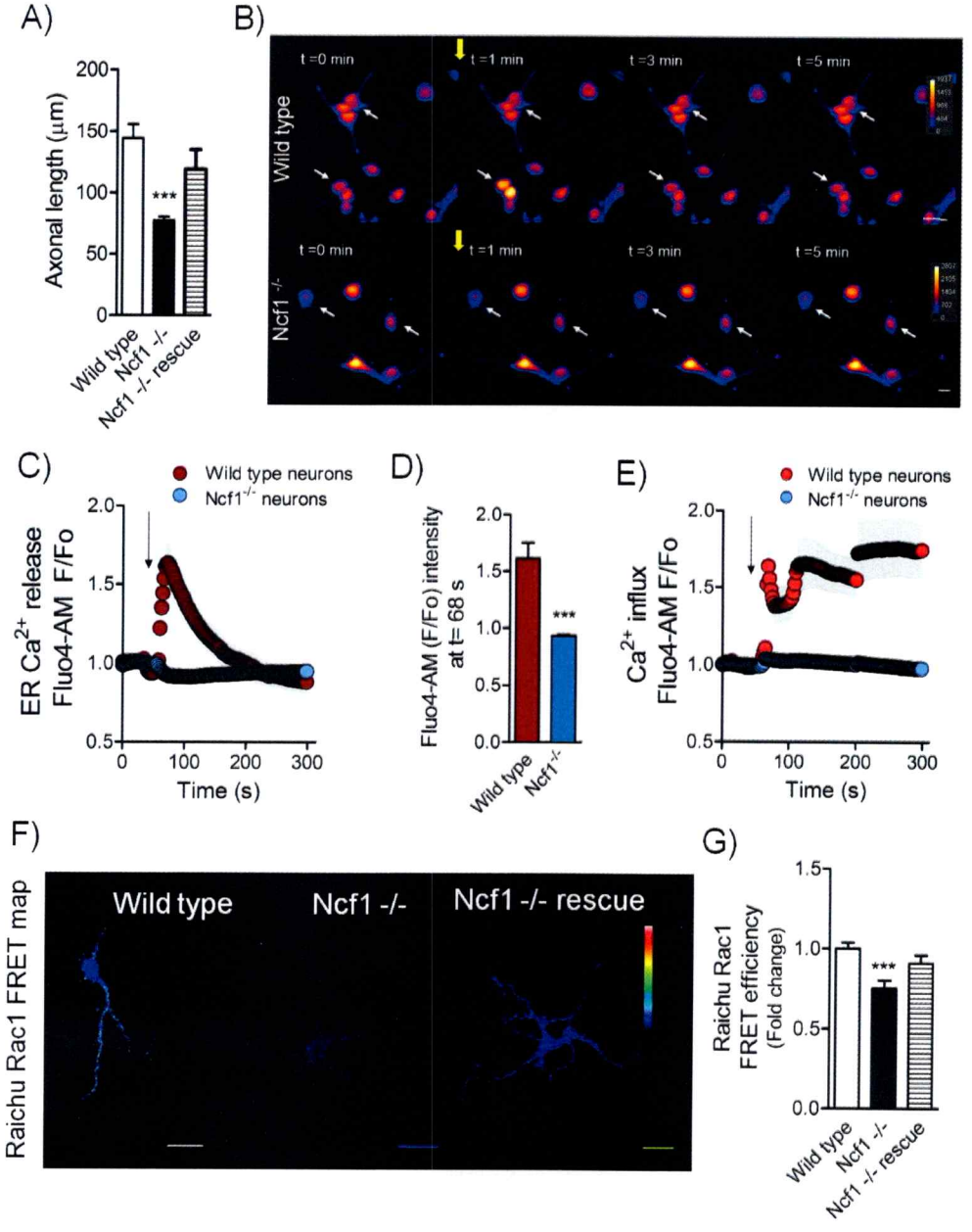


Figure 7

Wilson et al. 2016



Concluding Remarks and Future Perspectives

Evidence presented in this work support the hypothesis that ROS contribute positively to the development and polarization of neurons. In addition, this thesis suggests a mechanism by which ROS are able to shape neurons, involving actin cytoskeleton dynamics and ER Ca^{2+} release, mainly mediated by the ryanodine receptor. Moreover, these data suggest that genetic activation of the NOX complex allows increasing ROS levels that enhance both the polarization and axonal growth of neurons in culture. These findings are particularly relevant considering that ROS production has been usually associated with oxidative stress and it is common to refer to ROS and stress as synonymous terms. Our data support an alternative point of view that conceives ROS as signaling molecules when are produced within the physiological range. Considering this, genetic activation of NOX complex (e.g.: ectopic expression of NOX proteins) may enhance ROS levels within the physiological range of concentration, supporting signaling functions in neurons. Chapters 1 and 3 discuss the contribution of redox balance to the differentiation of neuronal progenitors. Handling NOX activity with genetic tools might be a promising strategy to fine tune ROS levels needed to promote neurogenesis and further neuronal development, in which both polarization and neurite outgrowth are central issues. Further research will be necessary to understand the contribution of the NOX complex to neuronal development, polarization and neurite outgrowth *in vivo*, which may have direct implications in the field of restorative neuroscience at both central and peripheral nervous system.

Published articles

1. Borquez DA, Urrutia P, Wilson C, van Zundert B, Nuñez MT and Gonzalez-Billault C. Dissecting the role of redox signaling in neuronal development. J. Neurochem. 2016. Feb 14. doi: 10.1111/jnc.13581. [Epub ahead of print]

2. Wilson C and González-Billault C. Regulation of cytoskeletal dynamics by redox signaling and oxidative stress: implications for neuronal development and trafficking. Front. Cell. Neurosci. 2015. 9:381.doi: 10.3389/fncel.2015.00381

3. Wilson C, Núñez MT., González-Billault C. Contribution of NADPH-oxidase to the establishment of hippocampal neuronal polarity. J Cell Sci. 2015. 128(16):2989-95.

Submitted articles

1. Wilson C, Muñoz E, Henríquez DR, Palmisano I, Di Giovanni S, Núñez MT, González-Billault C. A feed-forward mechanism involving the NOX complex and ER calcium release mediated by the ryanodine receptor promotes axonal growth. Submitted to Cell Reports. March, 2016.

2. Wilson C, Terman J, González-Billault C. Neuronal actin microfilaments: a target for redox species. Submitted to Cytoskeleton. March, 2016.

Articles in collaboration

1. Muñoz-Llancao P, Henríquez DR, Wilson C, Bodaleo F, Boddeke EW, Lezoualc'h F, Schmidt M, González-Billault C. Exchange Protein Directly Activated by cAMP (EPAC) Regulates Neuronal Polarization through Rap1B. J Neurosci. 2015. 35(32):11315 29.

2. Quintá HR, Wilson C, González-Billault C,Pasquini LA,Rabinovich GA, Pasquini JM.Ligand-mediated Galectin-1 endocytosis prevents intraneural H2O2 production promoting F-actin dynamics reactivation and axonal re-growth. Submitted to Neurobiology of Disease. February, 2016.

Scientific meetings

1. Wilson C., Núñez MT., González-Billault C. Contribution of NADPH-oxidase and endoplasmic reticulum Ca^{2+} release in the establishment of hippocampal neuronal polarity and axonal growth. EMBO workshop - Emerging concepts on neuronal cytoskeleton. Puerto Varas, Chile. March 22-26, 2015.

2. Wilson C., Núñez MT., González-Billault C. Contribution of NADPH-oxidase and endoplasmic reticulum Ca^{2+} release in the establishment of hippocampal neuronal polarity and axonal growth. Gordon Research Conference of Oxidative Stress & Disease. Ventura, California, USA. March 1-6, 2015.

3. Wilson C., Núñez MT., González-Billault C. Contribution of Reactive Oxygen Species to the Establishment of Hippocampal Neuronal Polarity. American Society for Cell Biology (ASCB)/International Federation for Cell Biology (IFCB) Meeting. Philadephia, Pennsylvania, USA. December 6-10, 2014.

4. Wilson C., Núñez MT., González-Billault C. Contribution of Reactive Oxygen Species to the establishment of hippocampal neuronal polarity.XXVIII Annual Meeting Chilean Society for Cell Biology, Puerto Varas, Chile. October 26-30, 2014.

Outreach

"El científico va a la calle". Faculty of Sciences, University of Chile – Explora Conicyt Program– Comisión Nacional de Investigación Científica y Tecnológica2015 (100 hours).

Honors

2015. Whood Whelan Research Fellowship – International Union of Biochemistry and Molecular Biology (IUBMB).

2014. Travel award of the American Society of Cell Biology (ASCB). ASCB/IFCB meeting. Dec 6-10 - Philadelphia, USA.

2014. Travel award - Consejo Nacional de Ciencia y Tecnología (Conicyt), Santiago, Chile.

2013. IBRO fellowship. Summer course "Dynamic Imaging in Neuroscience". Dec 2-13 - Valdivia, Chile.

2012. PhD fellowship - Consejo Nacional de Ciencia y Tecnología (Conicyt), Santiago, Chile.

Internships

Imperial College London. Division of Brain Sciences, Faculty of Medicine. London, United Kingdom. Supervisor: Prof. Simone Di Giovanni. January 6th – March 5th, 2016.