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

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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 molecular mechanisms involved in neuronal polarity and axon elongation is not well understood. In this work, we found that loss of function of the NOX complex altered neuronal polarization and decreased axonal length by a mechanism that involves actin cytoskeleton dynamics. Together, 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. Basal ROS levels, from NADPH oxidase (NOX) complex, are 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 NOX 1-5 and Duox 1-2 (Bedard and Krause, 2007), with NOX1, 2 and 4 being the main enzymes expressed in the CNS (Sorce et al., 2012). NOX2 interacts with five regulatory proteins (p22phox, p40phox, p47phox, p67phox and Rac) (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 gp91phox, p47phox, p67phox and p22phox 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 Cdc-42, 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; Hung et al., 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 cross-link 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 development of neuronal polarity. Inhibition of the NOX complex affected polarity acquisition and reduced axonal length of cultured neurons. NOX inhibition also targeted actin organization and decreased both filopodial dynamics and the activity of Rac1 and Cdc-42. 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\textsubscript{phox} (DNp22\textsubscript{phox} in this text), which has a dominant-negative effect on ROS production, affecting NOX 1-3 enzymes (Kawahara et al., 2005). Transfected neurons were fixed after 24 h in culture (Fig. 1). DNp22\textsubscript{phox} expression delayed neuronal polarity acquisition (Fig. 1A, B). To evaluate the contribution of NOX in axonal growth, neurons transfected with DNp22\textsubscript{phox} were cultured for 3 DIV and then fixed for MAP2 and Tau1 immunostaining (somato-dendritic and axonal markers, respectively) (Fig. 1C). DNp22\textsubscript{phox} expression decreased axonal, but not minor neurites length (Fig. 1D, E). In addition, MAP2 was detected in axons after DNp22\textsubscript{phox} expression (Fig. 1C), suggesting that NOX inhibition disrupted neuronal polarization. To confirm that DNp22\textsubscript{phox} indeed reduced ROS content, neurons were co-transfected with the genetically encoded biosensor Hyper, which detects intracellular H\textsubscript{2}O\textsubscript{2} (Lukyanov and Belousov, 2014), an indicator of NOX activity. DNp22\textsubscript{phox} expression
(48 h) significantly reduced H$_2$O$_2$ content compared with control neurons (Fig. 1F,G). The Hyper-H$_2$O$_2$ map revealed that the highest H$_2$O$_2$ production was at the periphery of the soma as well as at the axonal tip (Fig. 1F, arrows), whereas DNp22$^{\text{phox}}$ expression abolished this pattern. Expression of DNp22$^{\text{phox}}$ was confirmed in N1E115 cells and cultured neurons (Supplementary Figure 2). Together, these results suggest that NOX inhibition altered neuronal polarity acquisition and axonal growth.

As a second strategy to reduce NOX activity, neurons were treated with NOX inhibitors at 6h after plating. Those chosen were gp91 ds-tat (5 µM), a peptide that inhibits p47$^{\text{phox}}$ association with gp91$^{\text{phox}}$ (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$^{\text{phox}}$ translocation to the plasma membrane (Ximenes et al., 2007). Neurons were fixed at 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. 2 B-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 the gp91 scrambled (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 3DIV to quantify axon and minor neurites length (Supplementary figure 1 and Fig. 2F-H). After 3 DIV, most of neurons were fully polarized (stage 3) (92% ±1). Such percentage decreased after NOX inhibition (gp91 ds-tat: 62% ± 13, VAS2870: 35% ± 15, p<0.01 and apocynin: 30% ± 3.5, p<0.01). Remaining neurons did not develop an axon, resembling stage 2 of polarity. Control neurons exhibited somatic MAP2 and axonal Tau1 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 axon but not minor neurites length (Fig. 2G, H). These results are consistent with DNp22$^{\text{phox}}$-dependent NOX inhibition.
NOX complex expression and cellular localization in embryonic brain and hippocampal neurons

The NOX complex have 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. gp91\textsuperscript{phox}, p22\textsuperscript{phox}, p47\textsuperscript{phox} and p67\textsuperscript{phox} subunits were detected 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\textsuperscript{phox}, p22\textsuperscript{phox} and p47\textsuperscript{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.

**Contribution of the NOX complex to actin cytoskeleton dynamics**

Based on our findings, we hypothesized that the NOX complex targets 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\textsuperscript{phox} and GFP immediately after plating, and fixed after a short time in culture to measure the area of the lamella. F-actin and tubulin cytoskeleton were detected with phalloidin-Alexa 546 and \(\beta3\)-tubulin immunolabeling, respectively (Fig. 4A). DNp22\textsuperscript{phox} expression significantly reduced the lamellar area compared with control neurons (Fig. 4B). Moreover, gp91ds-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).

Secondly, 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\textsuperscript{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.

Thirdly, and considering that both lamellar and filopodial dynamics were altered after NOX inhibition, we sought to measure Rac1 and Cdc-42 activities after DNp22^{phox} expression. To this end, 1 DIV neurons were transfected with the Raichu FRET biosensors for Rac1 or Cdc-42 in control and DNp22^{phox} expression conditions (Nakamura et al., 2006). Representative FRET maps for Rac1 and Cdc-42 are shown to indicate their local activity (Fig. 4G-J). Quantification of FRET efficiency was performed at the soma and the whole, proximal and distal axon. The expression of DNp22^{phox} decreased Rac-1 FRET globally (Fig. 4H), which is consistent with the decrease in axonal length and lamellar area (Figs 1D, 4A). Cdc-42 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 observed 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 this possibility besides the regulation of the Rho GTPase protein activity.

gp91^{phox} and p47^{phox} KO 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 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 polarity and axonal growth in vitro through the regulation of Rac1, Cdc-42 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 sacrificed, embryos (E18.5) were removed and neurons were cultured according to (Kaech and Banker, 2006).

N1E115 neuroblastoma cell culture

N1E115 cells (ATCC, VA, USA) were cultured in 5% FBS DMEM to check DNp22\textsubscript{phox} expression.

Transient transfection of cDNA coding vectors

Neurons were transiently transfected with Lipofectamine 2000 (Life Technologies, CA, USA) 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

gp91\textsuperscript{phox}(ab109366, lot: YH081212C) (1:1,000 for immunoblotting (IB) and 1:100 for immunofluorescence (IF), mouse), p67\textsuperscript{phox}(ab80897, lot: GR23630-9)(1:500 for IB, rabbit) and p22\textsuperscript{phox}(ab75941, lot: GR83982-1)(1:1,000 for IB and 1:100 for IF, rabbit) were purchased from Abcam (MA, USA). α-tubulin (1:10,000, mouse) was from Sigma (MO, USA) and p47\textsuperscript{phox} (sc-14015, lot: A2113)(1:500 for IB and 1:100 for IF, rabbit) from Santa Cruz Biotechnology (TX, USA). MAP2 (1:500, rabbit) and Tau1 (1:500, mouse) were from Merck Millipore (Darmstadt, Germany). β3-tubulin (1:1,000, mouse) was from Promega (WI, USA). For solutions and general considerations for IB and IF experiments, please see (Henriquez et al., 2012).

Hyper H\textsubscript{2}O\textsubscript{2} measurement

Neurons (4x10\textsuperscript{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\textsubscript{2}O\textsubscript{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$_2$O$_2$ content (Belousov et al., 2006).

**DCFH-DA ROS measurement**

Neurons were incubated with 1 µM DCFH-DA (Sigma, MO, USA) 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 in (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, similarly to (Munnamalai and Suter, 2009).

**Measurement of lamellar area**

Neurons (24 h in culture) were fixed and immunostained against β3-tubulin. Phalloidin-Alexa 546 was incubated for 1h at RT 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 $A_{\text{Area lamella}} = A_{\text{Area F-actin}} - A_{\text{Area β3-tubulin}}$ (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, USA). 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 emerge and disappears.

**Measurement of Rac1 and Cdc-42 activity**

Neurons (4x10$^4$ cells/well) were transfected with the Raichu-Rac1 and Raichu-Cdc42 FRET biosensors (provided by Dr. Alfredo Cáceres, IMMF, Córdoba, Argentina) to measure Rho GTPase activity. Raichu probes expression and FRET efficiency measurements were performed accordingly to (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
acceptor/donor emission was established as the FRET efficiency. The FRET map was achieved by dividing the acceptor/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, whole axon and proximal and distal axon.

Statistics

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

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Competing interests

The authors declare no competing interests

Author contributions

CW, MTN and CG-B conceived and designed the experiments. CW performed the experiments. CW and CG-B analyzed the data. CW, MTN and CG-B wrote the paper.

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Fig. 1. **DNp22**<sub>phox</sub> expression altered neuronal polarity acquisition and axonal growth. Neurons were transfected with GFP (control) or co-transfected with GFP and DNp22<sub>phox</sub>. A. Representative images of control and DNp22<sub>phox</sub> neurons at stage 1, 2 and 3. F-actin was labeled with phalloidin-Alexa 546 (red) and tubulin (blue). B. Neuronal polarity after DNp22<sub>phox</sub> expression; *p<0.05 vs control stage 1. C. Representative 3 DIV control and DNp22<sub>phox</sub> neurons stained with MAP2 and Tau1. White arrows indicate transfected neurons. D. Axonal length (yellow arrows in C) of control and DNp22<sub>phox</sub> neurons of 3 DIV; ***p < 0.001 vs. control, t-student. E. Minor neurites length (arrow heads in C) in control and DNp22<sub>phox</sub> neurons of 3 DIV; ns: not significant, t-student. F. H<sub>2</sub>O<sub>2</sub> content evaluated with Hyper in control and DNp22<sub>phox</sub> neurons. Magnifications 1 and 2 corresponds to soma and axonal tips, respectively. G. Quantification of H<sub>2</sub>O<sub>2</sub> in control and DNp22<sub>phox</sub> neurons from images in F; ***p <
0.001 vs. control, t-test. 40 transfected neurons were analyzed per condition. Scale bar: 20μm.
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 A. B.*p < 0.05 vs. stage 1 control, ANOVA, Dunnett’s post-test, N = 4. C.**p < 0.01 vs. stage 1 control, ##p < 0.01 vs. stage 2 control, t-student. D. **p < 0.01 vs. stage 1 control, #p < 0.05 vs. stage 3 control, t-student. E. Intracellular ROS content measured with DCFH-DA. *p < 0.05, **p < 0.01, ***p < 0.001 vs. control, ANOVA, Dunnett’s post-test. F. 3 DIV neurons stained with MAP2 and Tau1 after NOX inhibition. Neurons were treated at 18 h of culture and fixed after 3 DIV to evaluate polarity and both axonal and minor neurites length. G. Axonal length; ***p < 0.001 vs. control, ANOVA, Dunnett's post-test, N=3. H. Minor neurites length; ns: not significant, ANOVA, Dunnett's post-test, N=3. 80–120 neurons were analyzed for each treatment. Scale bar: 20 µm.
Fig. 3. Expression and cellular localization of the NOX complex in hippocampal neurons. A. Immunoblotting of gp91\textsubscript{phox}, p67\textsubscript{phox}, p47\textsubscript{phox}, p22\textsubscript{phox} and α-tubulin in E18.5 hippocampus and cerebral cortex. B. Stage 2 and 3 cultured neurons were assessed for NOX complex proteins gp91\textsubscript{phox}, p67\textsubscript{phox}, p47\textsubscript{phox}, p22\textsubscript{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 bar: 20µm.
Fig. 4. Contribution of the NOX complex to actin cytoskeleton dynamics. A. Stage 1 neurons transfected with GFP alone or with GFP and DNp22\textsuperscript{phox}. B. Quantification of the lamellar area of control and DNp22\textsuperscript{phox} neurons at stage 1; *p < 0.01 vs. control, t-student. C. Time-lapse with Lifeact in control and DNp22\textsuperscript{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 vs. control, t-student. G and I. Rac1- and Cdc42-FRET map in control and DNp22\textsuperscript{phox} neurons. H and J. Local quantification of Rac1 and Cdc42 activity using the FRET biosensors in control and DNp22\textsuperscript{phox} neurons; H. **p < 0.01 vs. control, ***p < 0.001 vs. control, t-student. J *p < 0.05 vs. control, t-student. (20 transfected neurons per condition). Scale bar: 20 µm, inset 5 µm.
References


Figure S1. 2 DIV axonal lengths after NOX inhibition. A. Neurons were transfected with DNp22phox and fixed after 2 DIV. B, C. Neurons were treated with NOX inhibitors at stage 2 (18 h in culture) and fixed after 30 h of treatment. A, C. ***p<0.001 vs. control, t-student, N=3. B. ***p<0.001 vs. control, ANOVA, Dunnet's post-test.
Figure S2. DNp22<sup>phox</sup> control expression. A. Immunoblot of p22<sup>phox</sup> after DNp22<sup>phox</sup> expression in N1E115 cells. B. Immunofluorescence detection of p22<sup>phox</sup> in cultured hippocampal neurons after DNp22<sup>phox</sup> expression. ***p<0.001 vs GFP.