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Rotenone inhibits axonogenesis via an Lfc/RhoA/ROCK pathway in cultured hippocampal neurons

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Abbreviations

Cdc42: Cell division cycle 42
CFP: Cyan Fluorescent Protein
DIV: Days *In Vitro*
DMSO: Dimethyl Sulfoxide
DSU: Disk Spinning Unit
FRET: Forster Resonance Energy Transfer
GEF: Guanine Nucleotide Exchange Factor
LUT: Look Up Table
MT: Microtubule
PBS: Phosphate-Buffered Saline
Rac1: Ras-related C3 Botulinum substrate 1
RhoA: Ras homology member A
RhoA2G: RhoA second generation biosensor
ROCK: Rho-associated protein kinase
SEM: Standard Error of the Mean
Tau-1 +: Tau-1 positive
Tiam1: T-cell lymphoma invasion and metastasis-inducing protein 1
YFP: Yellow Fluorescent Protein

ABSTRACT

Rotenone, a broad-spectrum insecticide, piscicide and pesticide, produces a complete and selective suppression of axonogenesis in cultured hippocampal neurons. This effect is associated with an inhibition of actin dynamics through activation of Ras homology member A (RhoA) activity. However, the upstream signaling mechanisms involved in rotenone-induced RhoA activation were unknown. We hypothesized that rotenone might inhibit axon growth by the activation of RhoA/ROCK pathway due to changes in microtubule (MT) dynamics and the concomitant release of Lfc, a MT-associated Guanine Nucleotide Exchange Factor (GEF) for RhoA. In the present study we demonstrate that rotenone decreases MT stability in morphologically unpolarized neurons. Taxol (3 nM), a drug that stabilizes MT, attenuates the inhibitory effect of rotenone (0.1 µM) on axon formation.

Radiometric Forster Resonance Energy Transfer (FRET), revealed that this effect is associated with inhibition of rotenone-induced RhoA and ROCK activation. Interestingly, silencing of Lfc, but not of the RhoA GEF ArhGEF1, prevents the inhibitory effect of rotenone on axon formation. Our results suggest that rotenone-induced MT de-stabilization releases Lfc from MT thereby promoting RhoA and ROCK activities and the consequent inhibition of axon growth.

INTRODUCTION

Rotenone, a naturally occurring toxin, commonly used as a broad-spectrum piscicide, pesticide and insecticide, crosses the blood-brain barrier and the cell membranes because it is a highly lipophilic compound (Betarbet *et al*. 2002; Bové *et al.* 2005). It has been reported that rotenone has a toxicity on dopaminergic neurons *in vivo* and *in vitro* (Sherer *et al*. 2003; Liu *et al.* 2005; Radad *et al.* 2006; Shaikh and Nicholson 2008). More specifically, the chronic administration of rotenone in rats causes systemic mitochondrial impairment, oxidative damage, selective nigrostriatal dopaminergic degeneration and α-synuclein and ubiquitin positive Lewy body-like inclusions within Tyroxine Hydroxylase-positive neurons (Cannon *et al.* 2009).

Although the mechanism underlying the toxicity upon dopaminergic neurons has been deeply studied, few studies have been performed to uncover additional targets and/or its effects on developing neurons. In a previous study, we evaluated the effects of rotenone in primary cultures of hippocampal and dopaminergic neurons in order to obtain novel insights into the mechanisms underlying the neurotoxic effects of this pesticide. From that work we concluded that exposure to rotenone produces a complete and selective suppression of axon outgrowth and these effects were partially associated with an inhibition of actin dynamics through modifications of Rho-GTPases activities (Sanchez *et al.* 2008).

It is well known that Ras-related C3 Botulinum substrate 1 (Rac1), Cell division cycle 42 (Cdc42) and Ras homology member A (RhoA) regulate actin and MT dynamics (Conde and Cáceres 2009; Gonzalez-Billault *et al.* 2012; Bentley and Banker 2016). In the case of neurons, Rho-GTPases regulate axonal growth, dendritic arborization, spine morphogenesis, growth cone development, and axon guidance (Dickson 2001; Wojnacki *et al.* 2014). There is now a considerable body of evidence showing that MT are key regulators of Rho-GTPases activity, and that

positive and negative feed-back loops exist between them, in both neuronal and non-neuronal cells (Gonzalez-Billault *et al.* 2012; Wojnacki *et al.* 2014). Our previous data showed that rotenone inhibits axon formation by RhoA-mediated Rac1 inactivation. Besides, we demonstrated that either inhibition of the RhoA downstream effector, ROCK or the overexpression of T-cell lymphoma invasion and metastasis-inducing protein 1 (Tiam1), a Rac1 GEF, revert the inhibitory effect of rotenone on axon formation (Sanchez *et al.* 2008). However, whether or not these effects reflect RhoA activation due to changes in MT organization and/or dynamics remain unexplored.

Previous results from our laboratory showed that Lfc, a specific GEF for RhoA (Glaven *et al.* 1996; Ren *et al.* 1998; Krendel *et al.* 2002), promotes the activity of RhoA, impairing neurite sprouting and axon formation (Conde *et al.* 2010). To go further into the mechanism of RhoA-mediated rotenone neurotoxicity, we have now explored the involvement of Lfc, since it is activated upon MT-depolymerization (Birkenfeld *et al.* 2008). The results obtained show that both Taxol-induced MT-stabilization or Lfc suppression reverse rotenone-induced axon growth inhibition caused by the activation of the RhoA/ROCK pathway.

METHODS

Animals use and care

Pregnant Wistar rats born in the vivarium of INIMEC-CONICET-National University of Córdoba (UNC, Córdoba, Argentina) whose founding colony came from Charles River (Charles River, Cologne, RRID: RGD_737929) were used. The Pregnant rats of two months old and weighing between 220 and 250 gr were housed (1 per cage) and maintained in air-conditioned room (about 20–24 ºC) under a 12 h light–dark cycle (lights on at 06:00 am) with free access to food and water and killed 18 days post coitus. Pregnant rats were killed by cervical dislocation, the embryos were taken immediately and killed by decapitation in order to minimized animal´s suffering. All animal procedures were performed following approved protocols of the National Institute of Health Guide for the Care and Use of Laboratory Animals. Approval to conduct the study was granted by the Animal Care and Ethics Committee (CICUAL) of INIMEC-CONICET-UNC (Resolution numbers 014/2017 B and 006/2017 A).

Both protocols are in compliance with the general guidelines of the National Institute of Health (NIH, USA; https://grants.nih.gov/grants/olaw/Guide-for-the-Care-and-Use-of-Laboratory-Animals.pdf). Efforts were made to minimize the number of manipulations and animals used.

Neuronal cultures

Hippocampal cultures were prepared as described previously (Rosso *et al.* 2004; Sosa *et al.* 2006; Sanchez *et al.* 2008). Briefly, hippocampi from 18 days of male and female fetal rats were dissected and treated with trypsin (0.25% for 15 min at 37°C), (Thermo Fisher Gibco, Waltham, MA, USA; Cat Number: 15090-046) and mechanically dissociated by trituration with a Pasteur pipette. Cells were plated on Cover Glasses, Circles, 12 mm (Marienfeld Superior; Cat Number: 633029) coated with 1 mg/ml poly-L-lysine (Sigma Chemical Co. St Louis, MO; Cat Number: P2636) at a density of 2000 cells/cm^2 in minimum essential medium (MEM, Thermo Fisher Gibco, Waltham, MA, USA; Cat Number: 61100-061) supplemented with Penicillin-Streptomycin (Thermo Fisher Gibco, Waltham, MA, USA; Cat Number: 15140122), CTS GlutaMAX I Supplement (Thermo Fisher Gibco, Waltham, MA, USA; Cat Number: A1286001), Sodium Piruvate (Thermo Fisher Gibco, Waltham, MA, USA; Cat Number:11360070) and 10% horse serum (Thermo Fisher Gibco, Waltham, MA, USA; Cat Number:16050122). After 2 h, the coverslips were transferred to dishes containing serum-free Neurobasal (Thermo Fisher Gibco, Waltham, MA, USA; Cat Number: 21103049) with B-27 Plus Supplement (Thermo Fisher Gibco, Waltham, MA, USA; Cat Number: A3582801) and CTS GlutaMAX I Supplement (Thermo Fisher Gibco, Waltham, MA, USA; Cat Number: A1286001).

Drug treatment

Rotenone (Sigma-Aldrich; Cat Number: R8875, PubChem CID: 6758) 0.1 µM was added to the culture medium. This dose was chosen from our previous studies where we demonstrated that at this concentration does not induce apoptosis (Sanchez *et al.* 2008). For some experiments Taxol (3 nM; Sigma-Aldrich; Cat Number: T7191, PubChem CID: 36314) was also added to the culture medium, as described by Witte *et al.* (2008). Rotenone and Taxol were dissolved in dimethyl sulfoxide (DMSO, 0.01%; Sigma-Aldrich; Cat Number: D5879). Control cultures were treated with DMSO (0.01%) alone.

Primary antibodies

The following antibodies were used: a monoclonal antibody (mAb) against Tau-1 protein (MilliporeSigma, Cat Number: MAB3420, RRID: AB_94855) diluted 1:2000, a mAb against tyrosinated-α-Tubulin (Tyr-Tub; MilliporeSigma Antibody; Cat Number: MAB1864, RRID: AB_2210391) diluted 1:1000, a mAb against detyrosinated-α-Tubulin (Glu-Tub Sigma Aldrich Antibodies, Cat Number: AB3201, RRID: AB_177350) diluted 1:1000, and a mAb against acetylated-α-Tubulin (Acet-Tub; Sigma Aldrich, Cat Number: T6793, RRID: AB_477585) diluted 1:1000. For Western blot analysis the primary antibodies used were: anti rabbit-Lfc (Santa Cruz Biotechnology, INC; Cat Number: sc-49682, RRID: AB_2243051) and anti α-Tubulin (Sigma Aldrich, Cat Number T6199, RRID: AB_477583) diluted 1:200 and 1: 2000, respectively.

Immunofluorescence

Two different procedures were used to prepare cells for immunofluorescence: 1) Cells were fixed at room temperature with 4% (weight/volume) paraformaldehyde (Sigma, Aldrich; Cat Number: 441244) in phosphate-buffered saline (PBS) containing 4% (weight/volume) sucrose (Anedra; Cat Number: AN00711809) for 20 minutes. Cultures were washed with PBS, permeabilized for 5 minutes with 0.2% (volume/volume) Triton X-100 (Bio-Rad; Cat Number: 1610407) in PBS, and again washed in PBS (Sanchez *et al.* 2008). 2) Extraction with detergent prior to fixation to prepare “cytoskeletons fractions”. For this procedure, the cells were washed for 30 s with buffer PHEM [60 mM piperazine-N, N′-bis(2-ethanesulfonic) acid (Sigma, Aldrich; Cat Number: P6757), 25 mM HEPES (Sigma, Aldrich; Cat Number: H3375), 10 mM EGTA (Sigma, Aldrich; Cat Number: H3889), 2 mM MgCl2 (Biopack; Cat Number: 2000962000), pH 6.9] followed by extraction (2 min) with 0.2% saponin (Sigma, Aldrich; Cat Number: 47036) in buffer PHEM containing 10 μM taxol (Gonzalez–Billault *et al.* 2001). After extraction the cells were fixed for 20 min with warmed 2% paraformaldehyde-0.05% glutaraldehyde (Sigma, Aldrich; Cat Number: G5882) in buffer PHEM, pH 6.9 and quenched in 50 mM NH4Cl for 10 min. After fixation cells were incubated in blocking buffer (BSA 5%/PBS) for 1 h and incubated for 1 hour at room temperature with primary antibodies, washed with PBS, and then incubated with secondary antibodies conjugated with Alexa 488 (Molecular Probes, Thermo Fisher Scientific; Cat

Numbers: A11029 and A11006, dilution 1:1000), or Alexa 568 (Molecular Probes, Thermo Fisher Scientific; Cat Numbers: A11077 and A11004, dilution 1:1000) or Alexa 633 (Molecular Probes, Thermo Fisher Scientific; Cat Number: A21070; dilution 1:1000) (1 hour at 37°C), then washed with PBS and the coverslips mounted using FluorSave (Millipore Calbiochem; Cat Number: 34-578-920ML).

Detergent extracted cytoskeletal preparations were used to evaluate whether or not rotenone affects MT stability in neurons arrested at stage 2 of neuronal development. The Glu-MT/Tyr-MT and Acet-MT/Tyr-MT ratios were calculated from fluorescent images obtained by confocal microscopy of rotenone-treated neurons for 24 or 48 hours and compared with the values obtained in stage 2 control (non-treated) neurons. The relative intensities of Tyr-Tub, Acet-Tub and Glu-Tub were evaluated using quantitative fluorescence techniques as described previously (Gonzalez-Billault *et al.* 2001). Cells were visualized on either a spinning disk [Olympus, Disk Spinning Unit (DSU)] confocal microscope equipped with a ANDOR iXon3 DU-888E-C00-#BV camera, controlled by Cell Sense software or an inverted epifluorescence microscope (Zeiss Axiovert 200) equipped with an ORCA-ER camera (Model C4742-80-12AG S.Nº 242662), controlled by μManager software (Edelstein *et al.* 2010).

Measurements of fluorescence intensity were performed pixel by pixel and then these data used to calculate the average fluorescence intensity expressed in pixels (0 = black/255 = white). Measurements were made using Fiji-Image J software (Schindelin *et al.* 2012). Blind tests were not conducted in this set of experiments.

DNA constructs

The Lfc short hairpin interference RNA (sh-Lfc-GFP, target sequence 5′-GGGCTGCGGTTGCTTCTGTAA-3′) and the corresponding scrambled (SC-sh-Lfc-GFP, target sequence 5′-GGGTATTCGGTTGACCCTGTG-3′) were generated and validated by Conde *et al.* (2010). The ArhGEF1-shRNA (sh-ArhGEF1, target sequence 5′-GGGCTGAGCAGTATCCTAG-3′) and its scrambled control sequence (SC-sh-ArhGEF1, target sequence 5′-GGTGAGGCGACGACATTCT-3′) were gently provided Dr. Lei Shi (Xiang *et al.* 2016; 2017). The RhoA biosensor was generously provided by Dr. Olivier Pertz (Pertz 2010; Fritz *et al.* 2013) and the Eevee-ROCK was a kind gift of Dr. Matsuda (Li *et al.* 2017). Both constructs are unimolecular

FRET-based biosensor, which consists of genetically encoded fusions of a sensing module flanked with a donor fluorophore (a cyan fluorescent protein) and an acceptor fluorophore (a yellow fluorescent protein) that allow ratiometric measurements (Fritz *et al.* 2013). These resulting FRET biosensors are expected to increase its FRET signals upon an increase in a RhoA activity (RhoA-GTP) and in an increment in phosphorylation by ROCK.

Transient electroporation

Neurons were electroporated with the constructs using the Amaxa Nucleofector II device (Lonza, Cat Number: AAD-1001N), according to the manufacturer's instructions. The program used was O-003 for Primary Rat Hippocampal Neurons. 24 hours post-electroporation the neurons received the corresponding treatment and fixed 48 hours later.

Measurements of RhoA and ROCK activities

RhoA or ROCK activities were analyzed by FRET using a second generation RhoA biosensor (Pertz 2010; Fritz *et al.* 2013; Quassollo *et al.* 2015) or the Eevee ROCK biosensor (Li *et al.* 2017). The neurons were electroporated with 4 µg of RhoA or Eevee ROCK biosensors. 24 hours later, DMSO, rotenone, Taxol or rotenone + Taxol were added to the incubation medium.

FRET imaging was performed in arbitrary selected cells as previously described (Quassollo *et al.* 2015). An Olympus IX81 inverted microscope equipped with a DSU device, fluorescence illumination (100 W mercury arc lamp), and a microprocessor were used. For ratio imaging FRET calculation, donor channel (donor emission) and FRET channel (acceptor emission) images where smoothed with a median filter (1.5 pixel ratio), background subtracted (50.0 pixels rolling back radius) and aligned. FRET map images where generated dividing processed FRET channel images over donor channel images. To exclude from the analysis out-of-cell pixels a 0-1 intensity binary mask was created using FRET channel images and multiplied by the FRET map images. Finally, the intensity levels on FRET maps images where color coded using a custom Look Up Table (LUT) based on the Metamorph® pseudocolor LUT. FRET maps were generated and analyzed using

ImageJ (http://imagej.nih.gov/ij/). Blind tests were not conducted in this set of experiments.

Morphometric analysis of neuronal shape parameters

To determine whether the inhibitory effect of rotenone in hippocampal neuron is mediated by the expression of Rho-GEF proteins the neurons were electroporated with 3 µg of Sh-Lfc-GEF or SC-Sh-Lfc-GEF (Conde *et al.* 2010) or co-electroporated with 3 µg of Sh-Arhgef1 or SC-Sh Arhgef1 plus 1 µg YFP (Xiang *et al.* 2017). 24 hours post-electroporation, DMSO or rotenone were added to the culture medium; electroporated cells were fixed 48 hours later and immunolabeled using the Tau-1 mAb. The length and number of neuritic processes were quantitated as described previously (Sanchez *et al.* 2008; Conde *et al.* 2010).

Immunoblotting of Cell Lysates

Levels of Lfc and tubulin in control- and rotenone-treated cells were evaluated by Western blot as described previously (Conde *et al.* 2010). Briefly, the cultures were treated with DMSO or rotenone 0.1 µM 24 hs after plating. 48 hours later the cell monolayers were rinsed with cold PBS, scraped into ice-cold lysis buffer [50 mM HEPES (pH 7.4), 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 2 mM $MgCl_2$, and protease inhibitor mixture (1 mM phenylmethylsulfonyl fluoride, 0.7 µg/ml pepstatin, 2 µg/ml aprotinin, and 2 µg/ml leupeptin. All drugs were purchased from Sigma), and then dispersed by extensive pipetting. The suspension was incubated at 4 °C for 15 min and centrifuged at 13,000 × *g* for 15 min in a microcentrifuge to remove insoluble material. The lysates were separated by SDS-polyacrylamide gel electrophoresis and blotted onto nitrocellulose membranes (Li-cor; Cat Number: 926-31092). Subsequently, the nitrocellulose membranes were incubated with either anti-Lfc or DM1A anti-α-tubulin at 4 °C overnight and then washed and incubated with the corresponding Horseradish Peroxidase conjugated secondary antibody (Jackson Immunoresearch Labs INC, Cat Number 705-035-003, RRID: AB_2340390 and 115-035-003, RRID: AB_10015289) for 1 hour at a dilution of 1:2000. Immunodetection was performed with WesternSure ECL Substrat from Li-Cor (Catalogue Number: 926-80200) and quantified with ImageJ (http://imagej.nih.gov/ij/). Values were

expressed as fold change with respect to the control group after normalization to α-tubulin. Blind tests were not conducted in this set of experiments.

Statistical analyses

All data are presented as mean ± SEM. Comparisons of quantitative variables were performed by one-factor analysis of variance (ANOVA), and the Duncan test was used for multiple comparisons $*P < 0.01$. Comparisons between the two evaluations within each group were performed by Student's t-test for dependent variables $*P < 0.1$. Normality of the variables was tested by the Shapiro-Wilks test. Statistical significance was set at $p < 0.05$. Outliers were not tested since all the values were included when performing the statistical analysis. Analyses were performed using InfoStat statistical software using the generalized linear model procedure (Di Rienzo *et al.* 2012).

Data availability

The datasets generated and/or analyzed during the current study are available from the corresponding author on request.

RESULTS

Rotenone Inhibits Axonal Elongation by altering MT stability

It is now well established that changes in MT and actin dynamics are crucially required for neuronal polarization (Wiggin *et al.* 2005; Arimura and Kaibuchi 2007; Conde and Caceres 2009). Since previous biochemical studies have demonstrated that rotenone inhibits MT assembly (Brinkley *et al.* 1974; Marshall and Himes 1978) and that some of its toxic effects in cells (Ren et al. 2005; Ren and Feng 2007; Srivasta and Panda 2007) involve MT depolymerization, it is possible that rotenone-induced axon growth arrest is a direct consequence of alterations in MT assembly, organization, or dynamics.

Although we previously showed that a low percentage of neurons, which despite the presence of the agrochemical, were able to develop an axon and multiple minor processes displaying unaltered MT organization and dynamics (Sanchez *et al.* 2008), in this study we evaluated if rotenone-treated neurons arrested at stage 2 of neuritic development display alterations in MT dynamics. To this end, we examined if

minor neurites formed in the presence of the agrochemical differ from control ones in their relative content of dynamic and stable MT. Cultures were fixed after detergent extraction performed under MT-stabilizing conditions and processed for immunofluorescence with antibodies against Tyr-Tub, Acet-Tub and Glu-Tub. This method removes unassembled tubulin from the cell, so that the remaining tubulin staining is attributable to MT (Brown *et al.* 1992; Gonzalez-Billault *et al.* 2001; Witte *et al.* 2008). It is now well established that the relative abundance of Tyr, Acet and Glu-Tub in MTs are associated with its dynamic properties, such that Tyr-Tub is enriched in the more dynamic and recently assembled MT, whereas Glu- and Acet-Tub are enriched in the more stable (long-lived) one (Brown *et al.* 1992; Baas *et al.* 1993; Li and Black 1996; Gonzalez-Billault *et al.* 2001). Thus, the ratio of these posttranslational modifications of α-tubulin can be used as an indirect, but reliable, assay of MT dynamics. Figure 1 (A-E) shows that rotenone significantly decreases the Glu-MT/Tyr-MT and Acet-MT/Tyr-MT ratios calculated from confocal fluorescent images obtained from neurons treated with rotenone for 24 (Fig. 1B, D and E) or 48 hours (Fig. 1C, D and E) compared with the values obtained from stage 2 control cells neurons (Fig. 1A, D and E). The results obtained show that in rotenone-treated neurons MT dynamics is increased, raising the possibility that these neurons fail in generating an axon because of an impairment in their ability to generate stable MT.

Microtubule stabilization reverses the inhibitory effect of rotenone on axonogenesis and Rotenone-enhanced RhoA activity.

Since RhoA activity is regulated by MT depolymerization and rotenone-mediated neurotoxicity involves MT destabilization (Fig. 1; Ren *et al.* 2005) it became of interest to explore the relationship between these two events. To this end, we evaluated whether or not MT stabilization itself is sufficient to protect axons from rotenone-induced toxicity. 1 DIV (day *in vitro*) cultured hippocampal neurons were treated with Taxol (3 nM) in the presence or absence of rotenone for 48 hours. As expected, rotenone-treated neurons display a significant inhibition of axon formation, and failed to extend Tau-1 + axon-like neurites (Fig. 2B, E, F). The results obtained also show that Taxol-treated neurons display a dramatic increase in the number and length of Tau-1 + axon-like neurites (Fig. 2C, E, F) with respect to control (Fig. 2A, E, F); more importantly, Taxol significantly reduced the inhibitory effects of rotenone on

axon formation with treated-neurons displaying several Tau-1 + neurite (Fig. 2D, E, F).

Pull-down assays have yielded opposing results regarding rotenone-induced RhoA activation (Sanchez *et al.* 2008; Fujimura and Usuki 2012). Therefore, we re-evaluated this phenomenon using a second generation RhoA biosensor (RhoA2G; Fritz *et al.* 2013; Quassollo *et al.* 2015). Neurons were electroporated with RhoA2G at the time of plating and treated with DMSO, rotenone, Taxol or rotenone + Taxol as described (see Materials and Methods). Radiometric FRET revealed that rotenone-treated neurons (Fig. 3B, E), display significantly higher RhoA activity than control ones (Fig. 3A, E). Interestingly, Taxol treatment significantly reduced RhoA activity in rotenone-treated neurons (Fig. 3D,E) without having an effect per se (Fig. 3C,E). Fluorescent images from the donor emission channel (CFP excitation and emission) shows that RhoA2G biosensor distributes throughout the cell in DMSO (Fig. S1A), rotenone (Fig. S1B), Taxol (Fig. S1C) and rotenone + Taxol (Fig. S1D) treated neurons. Together, these observations confirmed that rotenone-induced axon growth arrest is paralleled by increased RhoA activity and show that a MT-stabilizing agent could attenuate this activation and the inhibitory effect on axon formation, supporting the existence of a mechanistic link between them.

A RhoA/ROCK signaling pathway is involved in rotenone-induced neurotoxicity

It has been proposed that RhoA acts as a negative regulator of axon formation through its downstream effector ROCK (Gonzalez-Billault *et al.* 2012). Previously, we have demonstrated that Y-27632, a ROCK inhibitor (Narumiya *et al.* 2000), reduced rotenone-induced axon growth arrest (Sanchez *et al.* 2008). Therefore, to examine this with more detail we evaluated whether or not rotenone-induced RhoA activation involves an increase in ROCK activity using the Eevee-ROCK biosensor (Li *et al.* 2017).

Radiometric FRET revealed that rotenone-treated neurons displayed significantly higher ROCK activity (Fig. 4B, E) than DMSO-treated ones (Fig. 4A, E). Furthermore, while Taxol per se did not affect ROCK activity (Fig. 4C, E), it significantly reduced rotenone-induced ROCK activation (Fig. 4D, E). Fluorescent images from the donor emission channel (CFP excitation and emission) showed that Eevee-ROCK biosensor distributes throughout the cell in DMSO (Fig. S2A),

rotenone (Fig. S2B), Taxol (Fig. S2C) and rotenone + Taxol (Fig. S2D) treated neurons.

Lfc mediates RhoA activation during rotenone-induced axon growth inhibition

Lfc, also known as GEF-H1, is a MT-associated RhoA GEF whose activity is triggered by MT depolymerization/destabilization (Gonzalez-Billault *et al.* 2012; Pathak and Dermardirossian 2013). It has recently been shown that an Lfc-RhoA pathway exerts a negative control on axonal growth (Conde *et al.* 2010). Therefore, we evaluated a possible relationship between rotenone, RhoA activity, Lfc expression and axon growth, by testing (a) whether the agrochemical modifies Lfc expression and (b) the response of Lfc-suppressed (RNAi-treated) neurons to rotenone. As a first test of this idea we examined Lfc protein levels in cell extracts from control and rotenone-treated hippocampal cell cultures. The results obtained show that the agrochemical increases Lfc protein levels (Fig. S3A, B). In the second set of experiments, we examined if silencing of Lfc was able to reduce rotenone-enhanced RhoA activity and its inhibitory effect on axonal growth. To this end, cultures were electroporated at the time of plating with control scrambled SC-sh-Lfc-GFP or sh-Lfc-GFP. One day later the neurons were treated with DMSO or rotenone and cultured for 2 additional days. As expected control SC-sh-Lfc-GFP transfected cells treated with rotenone (Fig. 5B) failed to elaborate an axon or to segregate Tau-1+ to a single neurite (Fig. 5E). By contrast, rotenone has little if any effect on axon formation after Lfc silencing (Fig. 5D). The majority of Lfc-suppressed neurons reached stage 3 of neurite development (Fig. 5C), displaying much longer and branched axon-like neurites than control cells (Fig. 5A, E, F); besides, multipolar neurons with 2 or 3 Tau-1+ axon-like neurites were also detected (Fig. 5E).

To test if rotenone specifically targets Lfc we examined if silencing another Rho-GEF, such as ArhGEF1, also known as p115 RhoGEF, also reduces its toxicity. For this, neurons were co-electroporated with 3 µg of control scramble SC-sh-ArhGEF1 or sh-ArhGEF1 plus 1 µg of YFP (Xiang *et al.* 2016). One day later the neurons were treated with DMSO or rotenone and fixed 2 days after treatment. The results showed that sh-ArhGEF1 transfected neurons treated with rotenone failed to segregate Tau-1+ to a single neurite (Fig. 5G). Besides, these neurons were not able to polarize and generate a Tau-1+ axon-like neurite in (Fig. 5G, H). Together,

our results support a model in which rotenone-induced axon growth arrest involves MT destabilization and activation of the Lfc-RhoA-ROCK signaling pathway.

DISCUSSION

Previously, we provided evidence about the participation of Rho-GTPases on rotenone-induced neurotoxicity (Sanchez *et al.* 2008). Our results showed that rotenone-induced axon growth arrest is mediated by an increase in RhoA activity, paralleled by a decrease in Cdc42 and Rac1 activities. Changes in the dynamic properties of the MT and actin cytoskeleton are crucial for the establishment of neuronal polarity, as increases in stable MT and actin dynamics paralleled the transformation of an undifferentiated neurite (e.g. minor process) into an axon (Bradke and Dotti 1999; Witte and Bradke 2008; Li and Gundersen 2008; Conde and Cáceres 2009). In accordance with this, it has been shown that treatment of cultured neurons with low doses of either Taxol, which promotes MT assembly and stabilization (Witte *et al.* 2008) or cytochalasin D (Sanchez *et al.* 2008; Bradke and Dotti 2000; Kunda *et al.* 2001; Schwamborn and Puschel 2004), which increases actin dynamics, are sufficient to induce axon formation.

A direct interaction of rotenone with tubulin and the subsequent inhibition of MT assembly has been proposed as a possible mechanism responsible for at least some of the neurotoxic effects of the pesticide (Ren *et al.* 2005; Ren and Feng 2007; Srivasta and Panda 2007). In this direction, it has already been shown that microtubule-depolymerizing agents, such as colchicine or nocodazole produced an inhibition of axon growth and differentiation (Ren *et al.* 2005; Witte *et al.* 2008). In this study we have extended these observations by showing that rotenone alters MT dynamics by significantly decreasing the ratio of stable to dynamic MT in those neurons that remain arrested at stage 2 of neuritic development. Interestingly, these changes were not detected in the low percentage of neurons, which despite the presence of the agrochemical, were capable of developing an axon and multiple minor processes.

In agreement with these observation, our results also show that Taxol reduces the inhibitory effect of rotenone on axon growth. This effect can be explained by considering that Taxol selectively compromises catastrophe events, thereby favoring the overall polymerization of MT at their plus ends. In the case of neurons, this

phenomenon leads to MT protrusion and invasion of the growth cone peripheral domain, which results in axon outgrowth (Witte *et al.* 2008). It has been proposed that these events result in Rac1 activation with a concomitant increase in actin dynamics and the generation of positive feed-back loops required for sustained axon growth (Conde and Cáceres 2009).

Opposite to our previous findings, Fujimura and Usuki (2012) reported that rotenone affects cortical neurons only by suppressing the expression and activity of Rac1, without changes in RhoA activity. Surprisingly, in the same paper the authors pointed out that either ROCK inhibition by Y-27632 or RhoA knockdown in neuronal cultures, protects neurons from the effect of rotenone, which clearly suggest a role of a RhoA signaling pathway in the neurotoxic effect of the agrochemical. In the present study, we verified and confirmed the critical role of RhoA in axonal development in cells treated with rotenone using a FRET-based second generation RhoA biosensor (Fritz *et al.* 2013; Pertz *et al.* 2006). Our results showed a significant increase in the FRET efficiency, indicative of enhanced RhoA activity, in rotenone-treated neurons as it was previously observed using pull-down assays (Sanchez *et al.* 2008). Our data, as well as those obtained by Sengottuvel *et al.* (2011) in retinal ganglion cells indicate that Taxol does not affect RhoA activity in control neurons. While the aforementioned group found that Taxol was able to reverse the inhibition of myelin-induced neuritic growth in retinal ganglion cells without affecting RhoA activity, we observed that in cultured hippocampal neurons Taxol reduces rotenone-induced RhoA activation. The reasons for this difference are not known at present, but might reflect differences in cell type or signaling pathways upstream of RhoA.

Further evidence for the involvement of RhoA in rotenone-induced axon growth arrest come from our experiments with the Eevee-ROCK biosensor, showing that rotenone increases ROCK activity and Taxol reduces rotenone-induced ROCK activation. This result fully agrees with our previous observations revealing that Y27632 reverts the inhibitory effect of rotenone on axon formation (Sanchez *et al.* 2008).

Finally, the present observations raised the possibility that the increase in RhoA-ROCK activity of rotenone-treated neurons is mediated specifically by enhanced Lfc activity as a result of MT destabilization. The fact that Lfc but not Arhgef1 suppression reduces rotenone-induced RhoA activation and axon growth arrest fully supports this idea. This is further sustain by our observations showing

that: 1) Rotenone induces MT destabilization, a phenomenon that most likely release Lfc from MT, leading to RhoA activation; 2) Taxol mimics the effects of Lfc suppression; 3) The agrochemical increases Lfc protein levels; and; 4) ArhGEF1 suppression does not reverse the inhibitory effect of rotenone. Together, our results involve specifically and for the first time, the Lfc/Rho/ROCK signaling pathway on the inhibition of axon formation caused by rotenone. In future studies will be of interest to explore whether or not rotenone affects other neuronal domains (e.g. dendritic spine) where this signaling pathway has been implicated (Ryan *et al.* 2005; Kang *et al.* 2009).

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Conflict of interest

The authors declare that there are no conflicts of interest. The present work is not pre-registered.

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Legends of Figures

Figure 1. Differential distribution of Tyr, Glu and Acet MTs in hippocampal neurons in stage II. (A–C) Rat hippocampal neurons (DIV1) were treated with DMSO (A), rotenone (0.1 µM) by 24 h (B) or 48 h (C). Rat hippocampal neurons stained for Tyr (red), Glu (blue) and Acet (green) -tub. Cells were permeabilized during fixation to remove unpolymerized tubulin subunits, therefore only tubulin incorporated in MTs was assessed. Ratio quantification of fluorescence intensities of Glu / Tyr (D) or Acet / Tyr (E) tub in MTs of neurons. *p ≤ 0.01 was considered statistically significant (ANOVA and Duncan test). Control n = 21 neurons, rotenone by 24 h n = 20 and rotenone by 48 h n = 20

Figure 2. Taxol reverts the inhibitory effect of Rotenone on axon formation. Rat hippocampal neurons (DIV1) were treated with DMSO (A), rotenone 0.1 µM (B), Taxol 3 nM (C), Taxol + rotenone (D). Neurons were fixed at DIV3 and stained for Tyr-tub (green) and for the axonal marker Tau-1 (red). (E) Quantification of number of Tau-1+ neurites per cell and (F) mean length of Tau-1+ neurites per cell (µm). The data represent the mean ± SEM. $^{\#}$p ≤ 0.01 was considered statistically different to all other treatments; *p ≤ 0.01 was considered statistically significant (ANOVA and Duncan test). Control n= 20 neurons, rotenone n= 20, Taxol n= 20 and Taxol + rotenone n= 20.

Figure 3. Increased RhoA activity caused by rotenone is blocked by Taxol in hippocampal neurons, as revealed by radiometric FRET. Hippocampal neurons were electroporated with a RhoA2G biosensor (see materials and methods). Representative images showing FRET maps of neurons treated 24 h after plating with DMSO (A), rotenone (B), Taxol (C), and rotenone plus Taxol (D). The images were obtained at DIV3. (E) Quantification of RhoA activity, as defined by relative

FRET efficiency (FRET channel over donor channel images). *p≤ 0.01 was considered statistically significant (ANOVA and Duncan test). Control n= 14 neurons, rotenone n= 11, Taxol n= 11 and Taxol + rotenone n= 11.

Figure 4. Rotenone increase ROCK activity in hippocampal neurons, as revealed by radiometric FRET. Hippocampal neurons were electroporated with Eevee-ROCK biosensor (see materials and methods). Representative images showing FRET maps of neurons treated 24 h after plating with DMSO (A), rotenone (B), Taxol (C), and rotenone plus Taxol (D). The images were obtained at DIV3. (E) Quantification of ROCK activity, as defined by relative FRET efficiency (FRET channel over donor channel images). *p≤ 0.01 was considered statistically significant (ANOVA and Duncan test). Control n= 35 neurons, rotenone n= 37, Taxol n = 14 and Taxol + rotenone n= 14.

Figure 5. Knock-down of Lfc reverses the inhibitory effect of rotenone on axonal formation. (A-F) Hippocampal neurons were electroporated with SC-sh-Lfc-GFP and sh-Lfc-GFP. Representative images of 3 DIV neurons electroporated with SC-sh-Lfc-GFP and treated with DMSO (A) or rotenone (B) and 3 DIV neurons electroporated with sh-Lfc-GFP treated with DMSO (C) or rotenone (D). (E) Quantification of number of Tau-1+ neurites per cell and (F) mean length of Tau-1+ neurites per cell (µm). The data represent the mean ± SEM. $^{\#}$p≤ 0.01 was considered statistically different to all other treatments; *p≤ 0.01 was considered statistically significant (ANOVA and Duncan test). SC-sh-Lfc-GFP n= 34, SC-sh-Lfc-GFP + rotenone n= 30, sh-Lfc-GFP n= 30 neurons, SC-sh-Lfc-GFP + rotenone n= 27. Note that Lfc suppression increases axonal length, collateral branching and the formation of supernumerary axons in both control and rotenone-treated neurons (arrowhead). (E) Quantification of number of Tau-1+ neurites per cell and (F) mean length of Tau-1+ neurites per cell (µm) of 3 DIV. Neurons co-electroporated with 3 µg of SC-sh-Arhgef1 and sh-Arhgef1 + 1 µg of YFP followed by treatment with rotenone or control vehicle at DIV1. (G) Quantification of number of Tau-1+ neurites per cell and (H) mean length of Tau-1+ neurites per cell (µm) of 3 DIV. The data represent the mean ± SEM. $^{\#}$p≤ 0.01 was considered statistically different to all other treatments; *p≤ 0.01 was considered statistically significant (ANOVA and Duncan test). SC-sh-ArhGEF1+YFP n= 10, SC-sh-ArhGEF1 + YFP + rotenone n =10, sh-ArhGEF1 + YFP n= 10, sh-ArhGEF1 + YFP + rotenone n= 10.