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Dynein c1h1, dynactin and syntaphilin expression in brain areas related to neurodegenerative diseases following exposure to rotenone

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Neurodegeneration is often accompanied by protein inclusions which may interfere with cell physiology. On the other hand, alteration in intracellular trafficking may precede impairment of neurotransmission and therefore trigger cell death. In view of this, it is hypothesized that changes in mitochondrial traffic may occur before neurodegeneration triggered by rotenone exposure and could favor this process. The effects of low concentrations of rotenone on the expression of dynein c1h1, dynactin and syntaphilin, which are proteins related to mitochondria transport and anchoring, were evaluated in cell cultures of substantia nigra, locus coeruleus and hippocampus as well as in these same brain areas in Lewis aged rats. The results indicate that low concentrations of rotenone decrease dynein c1h1 protein levels in cell cultures and brain areas of aged rats. Dynactin is decreased after exposure to 0.1 and 0.3 nM of rotenone, and increased after exposure to 0.5 nM of rotenone in cell cultures. Aged rats present increased dynactin expression. Syntaphilin expression decreased *in vitro* and increased *in vivo* after rotenone exposure. These findings suggest that changes in protein expression related to mitochondrial retrograde transport and anchoring occur before neurodegeneration induced by rotenone exposure, which may be a primary factor to trigger neurodegenerative mechanisms.

Key words: protein aggregation, rotenone, neurodegeneration, mitochondria, retrograde trafficking, anchoring

INTRODUCTION

Mitochondria are essential organelles for neuronal survival and function; they are highly dynamic and need to be specifically transported to cell body, dendrites and axon. Mitochondrial biogenesis and recycling is mainly performed in neuron body at the central nervous system, although it may occur in axons and dendrites of peripheral neurons at lower rates (Amiri and Hollenbeck 2008).

Distribution of mitochondria in neurons is regulated by specific motor and accessory proteins that support mitochondrial functions and biogenesis. The organelle goes from neuronal periphery to cell body through retrograde trafficking carried out by dynein and dynactin complex (Hollenbeck and Saxton 2005). Disruption and pathogenic mutations in this complex are related to models of neurodegenerative diseases (Braunstein et al. 2010, Deng et al. 2010, Eschbach and Dupuis 2011).

Mitochondria also can stay anchored at sites of high energetic demand, through their association with the cytoplasmic protein syntaphilin (Kang et al. 2008). Recently Zhu and Sheng (2011) showed that deleting of syntaphilin gene results in increase of mobile mitochondria but cannot rescue the decrease of mitochondrial transport and neurodegeneration in amyotrophic lateral sclerosis mice model.

Extensive evidences from animal and clinical studies suggest that mitochondria dysfunction, including in their traffic, has a critical role in neurodegenerative diseases. (reviewed by Cho et al. 2010, Arduino et al. 2011, Schon and Przedborski 2011).

Neurodegeneration is often characterized by formation of extra and intracellular protein aggregates dis-

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tributed throughout the central nervous system (Ross and Poirier 2005), which may also interfere with cell physiology and intracellular trafficking. We have recently demonstrated that rotenone was able to promote *in vitro* aggregation of alpha-synuclein, amyloid beta peptide and hyperphosphorylated tau (Chaves et al. 2010) revealing an important tool to study intracellular events preceding and during protein deposit.

Rotenone is a natural pesticide that acts with high affinity as a specific inhibitor of mitochondrial NADH dehydrogenase within complex I of the respiratory chain and is used to elucidate the mechanisms underlying the neurodegeneration *in vitro* and *in vivo* (Radad et al. 2008, Cannon et al. 2009, Drolet et al. 2009, Chaves et al. 2010).

Rotenone can alter microtubules dynamics, causing centrosome disorganization (Diaz-Corrales et al. 2005), microtubule depolymerization (Choi et al. 2011) and inhibition of microtubule assembly (Srivastava and Panda 2007), which can modify intracellular transport of mitochondria in neurons.

In agreement with this Borland and colleagues (2008) demonstrated that SH-SY5Y cell lineage differentiated to neural cells and exposed to rotenone showed reduced mitochondria transport velocities while Arnold and collaborators (2011) recently showed an increase in mitochondria transport velocities and retrograde/anterograde transport ratio in cortical cell cultures chronic exposed to low dose of rotenone.

Recently (Melo et al. 2012) demonstrated alteration in protein and messenger RNA expression levels of KIF1B and KIF5, proteins related to mitochondria anterograde transport, associated with decrease in mitochondria mobility in locus coeruleus, hippocampus and substantia nigra of aged rats and cell culture, from these same brain areas, exposed to low doses of rotenone.

However to our knowledge the influence of rotenone effects in mitochondrial retrograde transport and anchoring in neurons of brain areas affected by neurodegenerative diseases remains to be elusive. Considering this, the aim of the present study is to analyze the expression of dynein c1h1, dynactin and syntaphilin in the hippocampus, substantia nigra and locus coeruleus, which are some of the most neurodegeneration-susceptible areas associated to protein aggregation, of aged Lewis rats as well as primary cell cultures after exposure to low concentrations of rotenone.

METHODS

All the procedures were performed in strict accordance with Institutional and International Guidelines for animal care and use (Demers et al. 2006), as well as respecting the Brazilian federal law 11794/08 for animal welfare. Special attention was taken to minimize the number and discomfort of the animals used in the present research.

Cell culture

Methodology employed for cell culture was a modification of the previously described protocol (Kivell et al. 2001). Briefly, 20 neonatal (1 day) Lewis rats, per culture, had their brains dissected out to acess the hippocampus, locus coeruleus and substantia nigra. Blood and epithelial cells were removed from the areas of interest in sterile cold solution consisting of NaCl 120 mM, KCl 5 mM, KH_2PO_4 1.2 mM, $MgSO_4$ 1.2 mM, $NaHCO_3$ 25 mM, glucose 13 mM, pH 7.22. Subsequently, the tissues were physically and chemically dissociated. After the total decoupling cell solution was centrifuged at 300 g for 5 minutes. The supernatant was discarded and cells were suspended in Neurobasal A medium (Gibco) supplemented with Glutamax (Gibco) 0.25 mM, B27 (Gibco) 2%, L-Glutamine (Sigma) 0.25 mM and Gentamicin (Gibco) 40 mg/L.

Cells were plated on 35 mm nunclon (Nunc) dishes coated with poli-$_D$-lysine, at the concentration of 1 800 cells/mm^2. Cultures were kept in a humidified incubator with 5% CO_2 at 37°C for nine days. Culture medium was changed three hours after plating the cells and every three days of cultivation.

Cell culture characterization

Cell cultures were washed in PBS, fixed in 4% paraformaldehyde for 10 minutes and permeabilized with PBS containing 0.2% Triton for 30 minutes both at room temperature. Unspecific binding sites were blocked with PBS containing 2% NGS (Vector Laboratories), 0.2% Triton and 4% bovine serum albumin (BSA, Sigma) for 30 minutes at room temperature.

Cells from hippocampus, locus coeruleus and substantia nigra were incubated, independently, with mouse polyclonal antibodies against microtubule associated protein 2 (MAP2) (1/1 000, M4403, Sigma) and tyrosine

hydroxylase (TH) (1/1 500, MAB138, Millipore) for 24 hours at 4°C, followed by incubation with anti-mouse immunoglobulin conjugated to FITC (Jackson, 1/120) for 45 minutes at room temperature protected from light.

Culture dishes were mounted with mounting medium containing DAPI (4',6-diamidino-2-phenylindole, Vector Laboratories) to visualize cell nuclei. Immunolabeled cells were analyzed using a fluorescence microscope Axiophot 2 (Zeiss) equipped with Axio Cam MRm and appropriated filters using a 20× objective lens. Quantification was done by comparing images taken of 2 fields per culture plate, in the total of 3 plates, using filters to visualize the label generated by FITC and DAPI. Cell culture characterization was repeated twice.

Cell culture and exposure to rotenone

At the ninth day of cell culture rotenone was prepared with DMSO (stock solution of 1 mM) and diluted in culture medium and firstly applied to cell cultures in concentrations of 0.5 and 10 nM for 48 hours for cell death analysis and finally in concentrations of 0.1, 0.3 and 0.5 nM for 48 hours for dose-response analysis and 0.3 nM for 12, 24 and 48 hours for time-response analysis.

Control cultures were exposed to less than 0.001% DMSO diluted in culture medium. After rotenone exposure cells were then subjected to protein extraction for mitochondrial retrograde molecular motors and anchoring proteins analysis through Western blot.

Aged rats and exposure to rotenone

Aged (12 months old) Lewis rats were anesthetized with ketamine (1.25 mL/kg) and xylazine (0.5 mL/kg) to have osmotic minipumps (Alzet) implanted subcutaneously on their back. Minipumps were filled either with rotenone (Sigma, USA) dissolved in equal volumes of dimethyl sulfoxide (DMSO, Sigma, USA) and polyethylene glycol (PEG, Sigma, USA) which was delivered at the rate of 1 mg/kg/day for 4 weeks, or only DMSO:PEG (1:1) as control.

After treatment the animals were euthanized and their brains removed for analysis of hippocampus, locus coeruleus and substantia nigra by Western blot and real time PCR.

Cell death analysis in cell cultures exposed to rotenone

Hippocampal, locus coeruleus and substantia nigra cell cultures after exposure to rotenone were stained with trypan blue stain solution (Gibco), through of the addition of 10 µl of it in cell culture medium. Trypan blue stains in blue the cytoplasm of cells with damaged plasma membrane and does not stain live cells, allowing cell death analysis. Immediately after the addition of trypan blue, the cells were examined under a microscope (Olympus) using an objective lens of 40× and photographed to detect stained cells, this experiment was repeated twice.

Western blot analysis of mitochondrial molecular motors

Cultured cells and brain tissue were homogenized in PBS, pH 7.4, containing 1% NP40, 0.5% sodium deoxycholate, 1% SDS, 1 mM EDTA, 1 mM EGTA and 1% protease inhibitor cocktail (Sigma). After centrifugation at 14 000 rpm for 20 minutes, the resulting supernatant

Table I

Cell culture characterization

	DAPI	% MAP2 positive cells	% TH positive cells
Locus coeruleus	337	54	23
Substantia nigra	394	59	42
Hippocampus	222	60	–

Quantification of the percentage of cells immunopositive to MAP2 and tyrosine hydroxylase (TH) in relation to the total number of cells indicated by DAPI, in cells cultures of locus coeruleus, substantia nigra and hippocampus. Count was performed in 2 fields of each culture plate of a total of 3 plates per experiment.

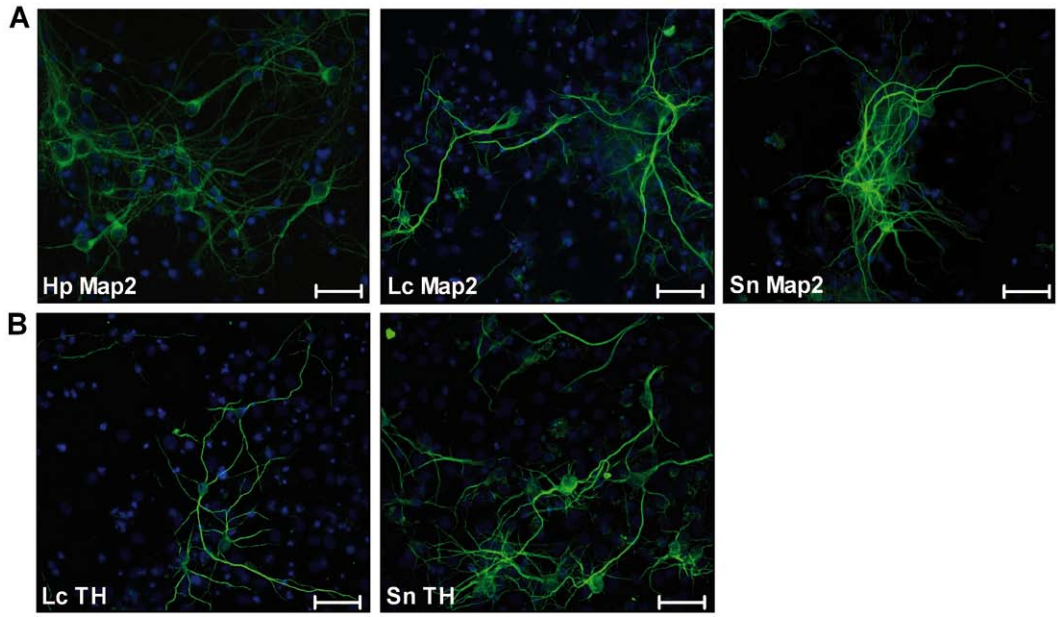

Fig. 1. Cell cultures characterization. Illustrative digital images showing the immunoreactivity of microtubule associated protein 2 (MAP2) in cultured hippocampal, locus coeruleus and substantia nigra cells (A) and tyrosine hidroxilase (TH) immunoreactivity in locus coeruleus and substantia nigra cultured cells (B). Scale bar is 50 µm.

was fractionated by SDS-PAGE (10 µg of protein/lane) using a 12% Tris-HCl gel at 100V for 1 hour. Proteins were transferred to nitrocellulose membrane for 1 hour at 100V. Blots were incubated in blocking solution containing 5% milk/TBS-T for 1 hour at room temperature.

Primary antibodies against dynein c1h1 (Santa Cruz, R-325, sc-9115, 1/200) dynactin (Santa Cruz, H-300, sc-11363, 1/400) and syntaphilin (Santa Cruz, H-250, sc-33824, 1/200) were diluted in TBS-T containing 3% milk and incubated at room temperature for 1 hour.

Horseradish peroxidase-conjugated anti-rabbit 1/10 000 (Amersham) antibody incubation was performed at room temperature for 1 h.

Development was done after 5 minute of incubation with enhanced chemiluminescence reagent (Millipore) and exposure to chemoluminescence sensitive films (Hyperfilm ECL, Amersham Biosciences). After development, blots were incubated with anti-beta-actin antibody 1/1 000 (Santa cruz, C4, sc-47778) for 1 hour at room temperature, horseradish peroxidase conjugated anti-mouse (Amersham) diluted 1/6 000, incubated for 1 hour also at room temperature and developed as previously described.

Films were quantified using Image J software (NIH). Normalization was done by dividing the values corresponding to the bands relative to proteins of interest by beta-actin value.

Real time PCR

Total RNA was extracted from brain tissue using InviTrap® Spin Tissue RNA Kit 09808 (Invitek Gmbh, D13125 Berlin). RNA was transformed in cDNA after incubations of 10 min at 25°C followed

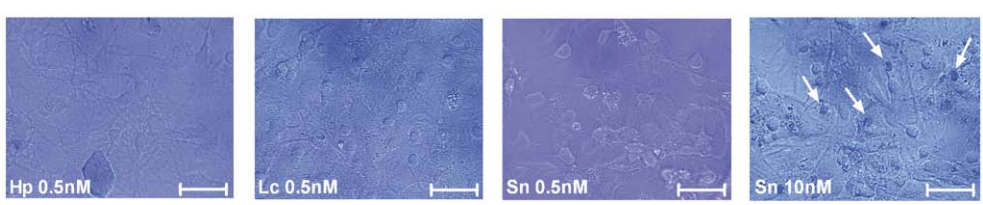

Fig. 2. Cell death analysis in cell cultures exposed to rotenone**.** Illustrative digital images demonstrating tripan blue staining in hippocampal, locus coeruleus and substantia nigra cell cultures exposed to different concentrations of rotenone for 48 hours. Cells stained in blue (arrows) are under death process. Scale bar is 50 µm.

by 30 min at 48°C and 5 min at 95°C in a thermocycler using the reverse transcription reagents (Applied Biosystems). Briefly, TaqMan buffer (1×), MgCl2 (5.5 µM), dNTPs (500 µM), randomic hexamers (2.5 µM), RNAse inhibitor (0.4 U/µL) and the MultiScribe Reverse Transcriptase (1.25 U/µL) were added to 1 µg of total RNA to a final volume of 50 µL. Two control tubes were added to the assay: one without the template and another without reverse transcriptase. cDNA was kept at −80°C until its use for Real Time PCR.

Dynein (Rn00570138), dynactin (Rn01421080) and syntaphilin (Rn01536119) expression were evaluated using primers, probes and reagents for Real Time PCR commercially available at Applied Biosystems (Foster City, CA). The protocol was rigorously followed where 12.5 µl of 2× PCR Master Mix, 1.25 µl of primers/probe and 6.25 µl of DEPC water were added to 5 µl of experimental cDNA. The real-time PCR system was from Applied Biosystems (Applied Biosystems 7300 Real Time PCR System). The results were normalized using 18S rRNA (Applied BiosystemsFoster City, CA) and the logarithmic equation $2^{-\Delta\Delta CT}$.

Statistical analysis

Results were analyzed by either one-way ANOVA followed by Tukey post-test for *in vitro* analysis or unpaired Student's *t* test for *in vivo* experiments. Statistics were accessed through GraphPad Prism (GraphPad Software Inc., version 4.00, CA). A *P*-value ≤0.05 was considered to indicate statistically significant differences. Data are expressed as percent of control ± standard deviation (SD).

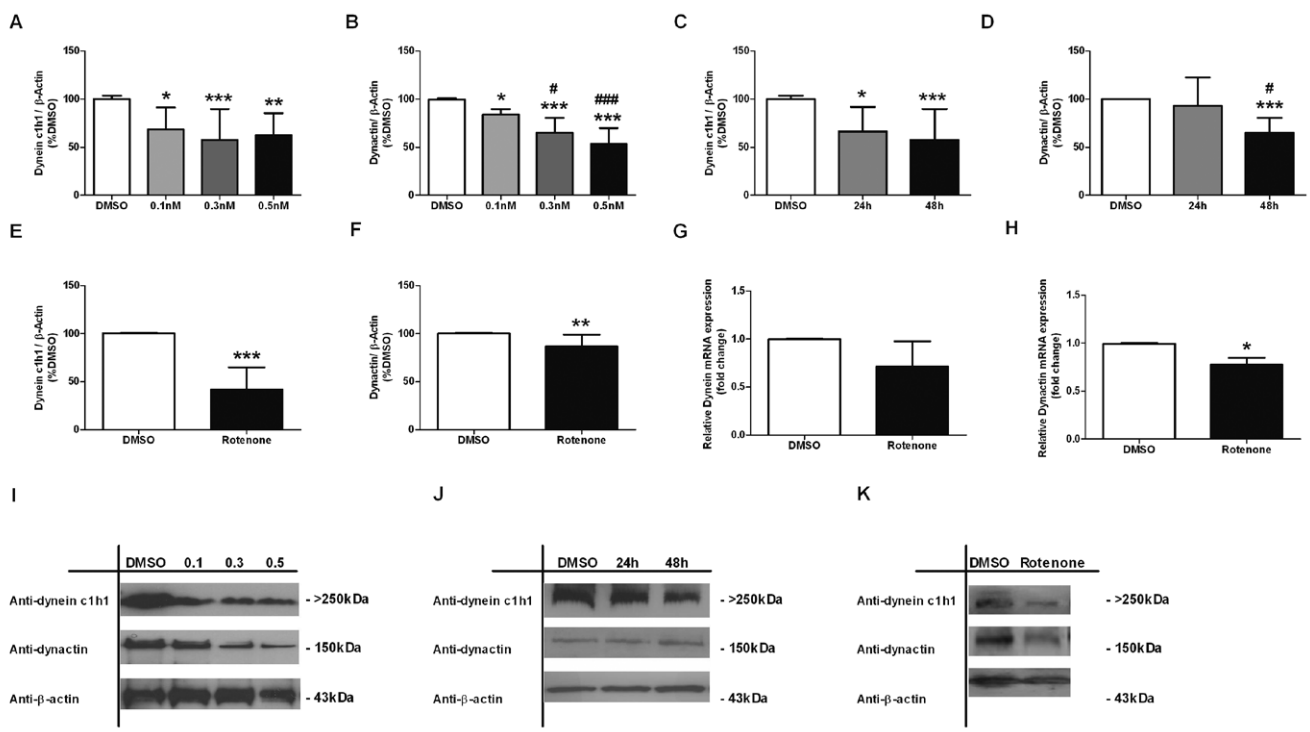

Fig. 3. Hippocampal dynein c1h1 and dynactin expression in cell culture and aged rats exposed to rotenone. Dose-response (A and B) and time-course (C and D) of dynein c1h1 (A, C) and dynactin (B, D) protein expression in hippocampal cell cultures exposed to 0.1, 0.3 or 0.5 nM of rotenone for 48 hours (dose-response); or 0.3 nM for 24 or 48 hours (time-course). Dynein c1h1 and dynactin protein levels (E, F) and mRNA expression (G, H) in hippocampus of 12 months-old rats following exposure to 1 mg/kg/day of rotenone for 4 weeks. Illustrative images of the pattern of bands corresponding to dose-response (I) and time-course (J) of dynein c1h1 and dynactin protein expression in hippocampal cell cultures and in hippocampus of 12 months-old rats (K) after rotenone exposure. Normalization was performed by beta-actin (43 kDa) signal. Data are shown as percent of control (DMSO) ± SD. **P*<0.05; ***P*<0.01; ****P*<0.001 as compared to DMSO, #*P*<0.05; ### *P*<0.001 as compared to 0.1 nM (dose-response), according to one-way ANOVA followed by Tukey post-test to cell cultures and Student's *t*-test for aged animals analysis. Experiments were repeated twice, each run was performed in sample triplicates.

In vivo experiments were done with a minimum of 5 animals per experimental group (n=5), using triplicates. *In vitro* experiments were repeated twice independently, unless stated otherwise, using three dishes per experimental group each time (n=3).

RESULTS

Cell culture characterization

Quantification of neurons using MAP2 labeling in cell cultures from hippocampus, locus coeruleus and substantia nigra showed that 60%, 54% and 59%, respectively, of these cells express MAP2 being considered as neurons (Table I, Fig. 1A). Analysis of tyrosine hydroxylase expression in locus coeruleus and substantia nigra cell cultures demonstrated that 23% and 42%, respectively, of these cells in culture are catecholaminergic (Table I, Fig. 1B). Results demonstrate the suitability of this cell culture method to study hippocampal, locus coeruleus and substantia nigra cell cultures.

Cell death analysis in cell cultures exposed to rotenone

Exposure to 0.5 nM of rotenone for 48 hours, the higher concentration applied in this study, did not induce significant cell death in hippocampal, locus coeruleus and substantia nigra cell cultures (Fig. 2), similar to described in our previous study (Chaves et al. 2010). However exposure to 10 nM of rotenone promoted a massive cell staining with trypan blue in substantia nigra cell cultures illustrating rotenone capability to induce cell death process at high concentration (Fig. 2), similarly hippocampus and locus coeruleus cell cultures also presents the same response pattern (data not shown).

Rotenone decreased dynein c1h1 and dynactin expression in hippocampus

Hippocampal cells exposed to 0.1, 0.3 or 0.5 nM of rotenone for 48 hours showed a significant decrease of dynein c1h1 and dynactin levels (Fig. 3A and 3B, respectively). Time-course experiment also revealed a decrease in dynein c1h1 levels after exposure to 0.3 nM of rotenone for 24 and 48 hours (Fig. 3C). Dynactin levels also decreased however only after 48 hours of rotenone exposure (Fig. 3D).

Aged rats exposed to 1 mg/kg/day of rotenone showed a significant down-regulation of dynein c1h1 and dynactin protein expression in hippocampus as compared to DMSO-exposed age-matched rats (Fig. 3E and 3F, respectively). Dynein c1h1 mRNA did not vary (Fig. 3G) but dynactin mRNA decreased after rotenone exposure in aged rats after rotenone (Fig. 3H). Figures 3I and 3J show illustrative images of the pattern of bands of anti-dynein c1h1 and anti-dynactin in dose-response and time-course experiments of dynein c1h1 and dynactin protein expression in hippocampal cell cultures and aged rats (Fig. 3K).

Rotenone decreased syntaphilin expression in hippocampal cell culture and increased it in hippocampus of aged rats

The imunoblots labeling pattern of syntaphilin presented one band in the predicted size (70 kDa) and a extra band of 65 kDa which was considered an alternative syntaphilin gene product or a stable degradation product according to Lao and coworkers (2000). *In vitro* dose-response analysis demonstrated a significant decrease in 70 kDa syntaphilin isoform levels after exposure to 0.3 and 0.5 nM of rotenone (Fig. 4A), expression of the 65 kDa syntaphilin also decreased after exposure to 0.1, 0.3 and 0.5 nM of rotenone (Fig. 4B). Time-response experiments of rotenone exposition corroborate dose-response results illustrating also a decrease in both syntaphilin isoforms levels after 48 hours of exposure to 0.3 nM of rotenone (Fig. 4C, D).

Aged rats presented a significant increase in the expression of 70 kDa syntaphilin isoform (Fig. 4E) while no alteration was observed in 65 kDa syntaphilin isoform (Fig. 4F). Syntaphilin mRNA was largely up-regulated in aged-rats hippocampus after rotenone exposure (Fig. 4G).

Representative images of immunoblots of dose-response and time-response experiments of syntaphilin expression in cell cultures and aged rats experiments are shown in Figures 4H, 4I and 4J, respectively.

Rotenone decreased dynein c1h1 and differentially influences dynactin expression in locus coeruleus cell culture and of aged rats

Locus coeruleus cell culture exhibited a decrease in dynein c1h1 levels after exposure to 0.1, 0.3 and 0.5 nM of rotenone as compared to DMSO cells (Fig. 5A), as well as

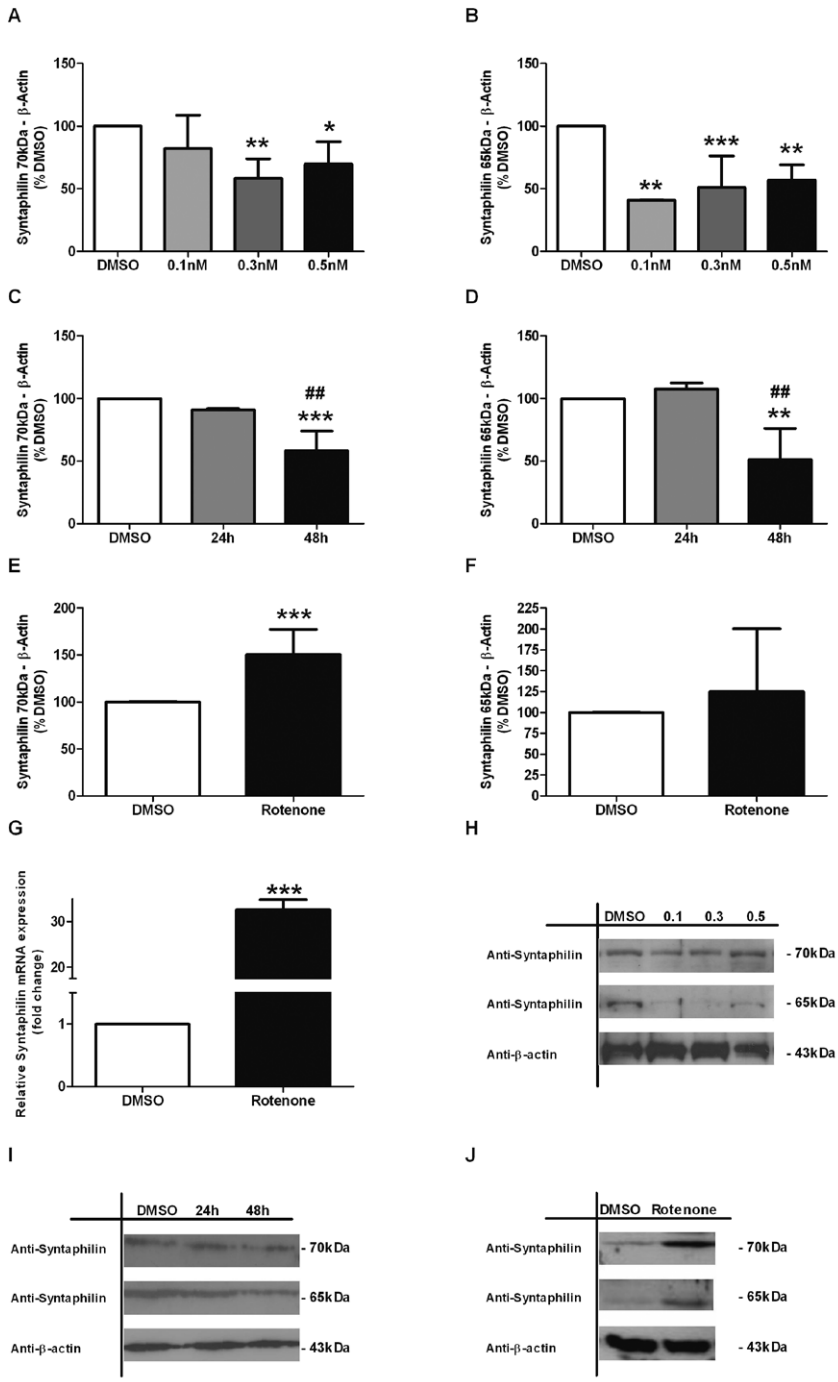

Fig. 4. Hippocampal syntaphilin expression in cell culture and aged rats exposed to rotenone. Dose-response (A and B) and time-course (C and D) of syntaphilin isoforms of 70 and 65 kDa expression in hippocampal cell cultures exposed to 0.1, 0.3 or 0.5 nM for 48 hours (dose-response); or 0.3 nM for 24 or 48 hours (time-course). Syntaphilin isoforms of 70 (E) and 65 kDa (F) protein levels and mRNA expression (G) in hippocampus of 12 months-old rats following exposure to 1 mg/kg/day of rotenone for 4 weeks. Illustrative images of the pattern of bands corresponding to dose-response (H) and time-course (I) of Syntaphilin 70 and 65 kDa protein expression in hippocampal cell cultures and in hippocampus of 12 months-old rats (J) after rotenone exposure. Normalization was performed by beta-actin (43 kDa) signal. Data are shown as percent of control (DMSO) ± SD. **P*<0.05; ***P*<0.01; ****P*<0.001 as compared to control (DMSO), ## *P*<0.01 as compared to 24 hours, according to One-way ANOVA followed by Tukey post-test to cell cultures and Student's *t*-test for aged animals analysis. Experiments were repeated twice, each run was performed in sample triplicates.

in dynactin expression after exposure to 0.1 and 0.3 nM of rotenone, however after exposure to 0.5 nM of the pesticide for 48 hours the expression of dynactin significantly increased (Fig. 5B). Time-response analysis also demonstrated a significant decrease in dynein c1h1 levels after exposure to 0.3 nM for 48 hours (Fig. 5C). Dynactin expression initially increased after 24 hours proceeding to a significant decrease after exposure to 0.3 nM of rotenone for 48 hours (Fig. 5D).

Aged rats showed a significant decrease in dynein c1h1 and increase in dynactin protein levels after rotenone exposure (Fig. 5E and 5F, respectively). Messenger RNA expression analysis demonstrated a down-regulation of about 60% of both molecular motors after exposure to rotenone (Fig. 5G, H).

Figures 5I and 5J show representative images of immunoblot corresponding of dose-response and time-response experiments of rotenone exposure in cell culture. Figure 5K illustrates the dynein c1h1 and dynactin bands pattern in aged rats experiments.

Rotenone decreased syntaphilin expression in locus coeruleus cell culture and increased it in this area of aged rats

The expression of 70 kDa syntaphilin isoform significantly decreased after rotenone exposure at all tested concentrations (Fig. 6A), however the 65 kDa isoform only decreased after exposure to 0.3 and 0.5 nM (Fig. 6B). 70 kDa syntaphilin isoform levels decreased in a time-dependent manner of rotenone exposure (Fig. 6C), while the 65 kDa isoform only decreased after 48 hours of rotenone exposure (Fig. 6D).

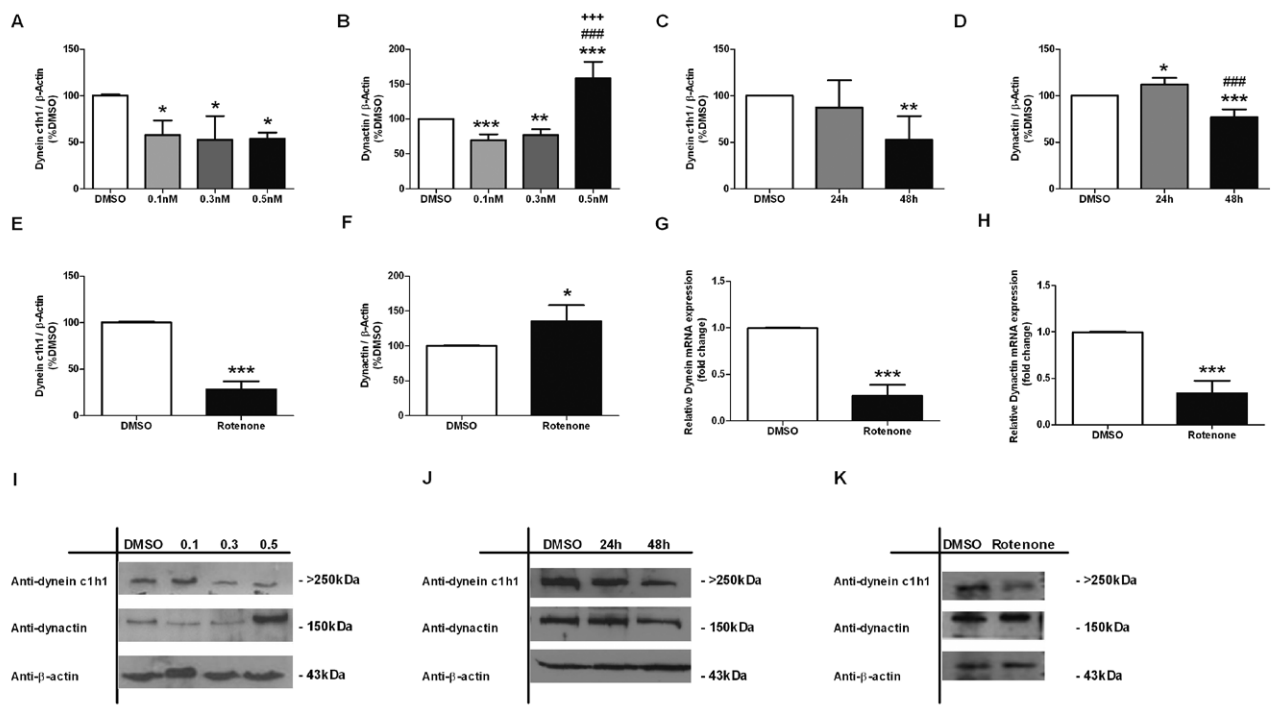

Fig. 5. Locus coeruleus dynein c1h1 and dynactin expression in cell culture and aged rats exposed to rotenone. Dose-response (A and B) and time-course (C and D) of dynein c1h1 (A, C) and dynactin (B, D) protein expression in locus coeruleus cell cultures exposed to 0.1, 0.3 or 0.5 nM of rotenone for 48 hours (dose-response); or 0.3 nM for 24 or 48 hours (time-course). Dynein c1h1 and dynactin protein levels (E, F) and mRNA expression (G, H) in locus coeruleus of 12 months-old rats following exposure to 1 mg/kg/day of rotenone for 4 weeks. Illustrative images of the pattern of bands corresponding to dose-response (I) and time-course (J) of dynein c1h1 and dynactin protein expression in locus coeruleus cell cultures and in locus coeruleus of 12 months-old rats (K) after rotenone exposure. Normalization was performed by beta-actin (43 kDa) signal. Data are shown as percent of control (DMSO) ± SD. * P<0.05; ** P<0.01; *** P<0.001 as compared to DMSO, ### P<0.001 as compared to 0.1 nM (dose-response) or 24 hours (time course), +++ P<0.001 as compared to 0.3 nM (dose-response), according to one-way ANOVA followed by Tukey post-test to cell cultures and Student's t-test for aged animals analysis. Experiments were repeated twice, each run was performed in sample triplicates.

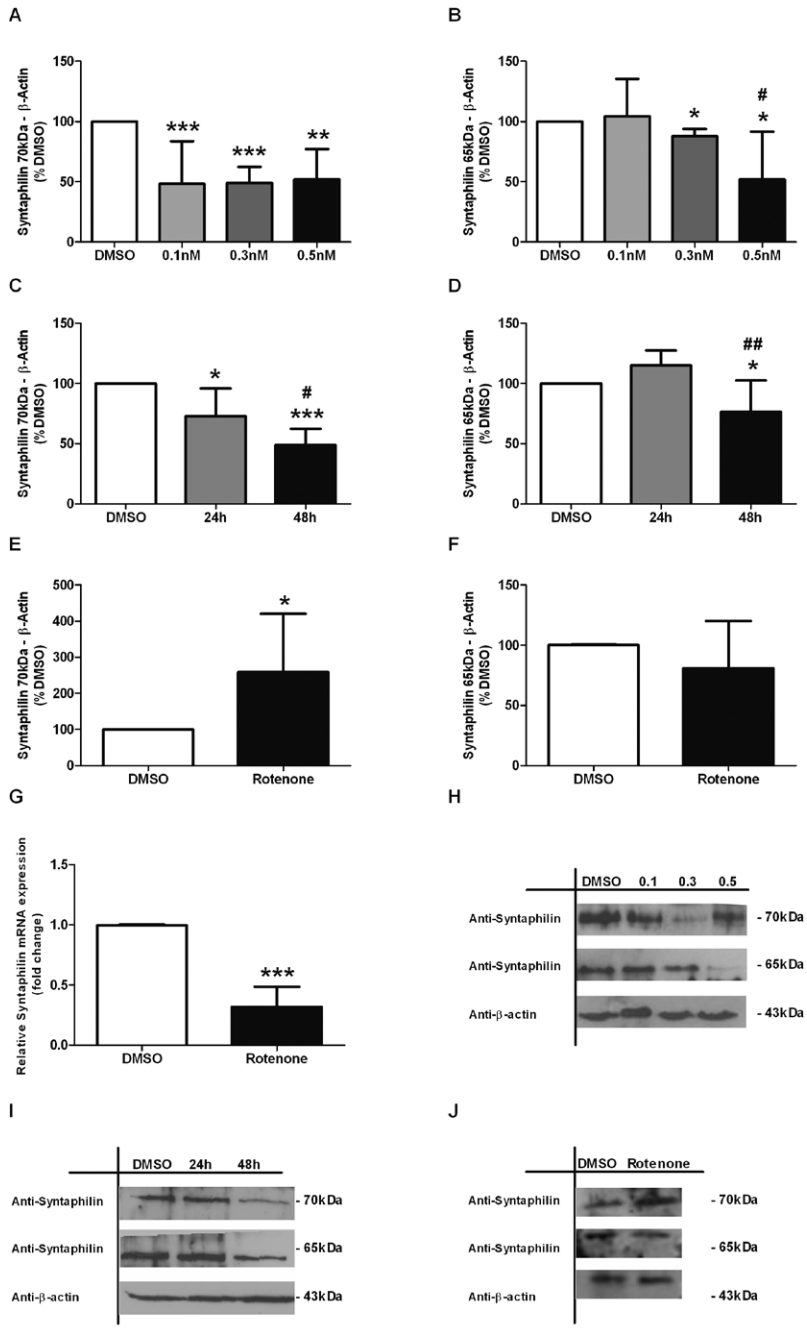

Fig. 6. Locus coeruleus syntaphilin expression in cell culture and aged rats exposed to rotenone. Dose-response (A and B) and time-course (C and D) of syntaphilin isoforms of 70 and 65 kDa expression in locus coeruleus cell cultures exposed to 0.1, 0.3 or 0.5 nM for 48 hours (dose-response); or 0.3 nM for 24 or 48 hours (time-course). Syntaphilin isoforms of 70 (E) and 65 kDa (F) protein levels and mRNA expression (G) in locus coeruleus of 12 months-old rats following exposure to 1 mg/kg/day of rotenone for 4 weeks. Illustrative images of the pattern of bands corresponding to dose-response (H) and time-course (I) of Syntaphilin 70 and 65 kDa protein expression in locus coeruleus cell cultures and in locus coeruleus of 12 months-old rats (J) after rotenone exposure. Normalization was performed by beta-actin (43 kDa) signal. Data are shown as percent of control (DMSO) ± SD. **P*<0.05; ***P*<0.01; ****P*<0.001 as compared to control (DMSO), #*P*<0.05, ##*P*<0.01 as compared to 0.1 nM (dose-dependent) or 24 hours (time-course), according to One-way ANOVA followed by Tukey post-test to cell cultures and Student's *t*-test for aged animals analysis. Experiments were repeated twice, each run was performed in sample triplicates.

Syntaphilin of 70 kDa increased in the locus coeruleus of aged rats exposed to 1 mg/kg/day of rotenone (Fig. 6E) with no significant alteration in 65 kDa isoform (Fig. 6F). The increase in syntaphilin protein expression was accompanied by a significant down-regulation of its mRNA after rotenone exposure (Fig. 6G).

Illustrative immunoblot stained with anti-syntaphilin of dose-response and time-response experiments are show in Figure 6H and 6I, Figure 6J illustrate a representative image of immunoblot of aged rat experiments.

Rotenone exposure promoted a dual effect in dynein c1h1 and dynactin expression in substantia nigra cell culture and increased both proteins in this nucleus of aged rats

Cultured cells of substantia nigra exhibited significant decrease of dynein c1h1 levels after exposure to 0.3 nM of rotenone as compared to DMSO-treated cells, while 0.5 nM of rotenone significantly increased dynein c1h1 expression as compared to 0.1 and 0.3 nM (Fig. 7A). Dynactin protein expression decreased with 0.1 nM and increased after 0.3 nM of rotenone as compared to DMSO-exposed cells (Fig. 7B). Time-course of mitochondria retrograde proteins responded accordingly to dose-response study, where it was observed a decrease in dynein c1h1 and an increase in dynactin after 0.3 nM of rotenone for 48 hours (Fig. 7C and 7D, respectively).

Rotenone decreased significantly dynein c1h1 and increased dynactin protein levels (Fig. 7E and 7F, respectively) in substantia nigra of aged animals. Messenger RNA of both proteins increased after 1 mg/kg/day of rotenone (Fig. 7G, H).

Representative immunoblots images of dose-response and time-response experiments are show in

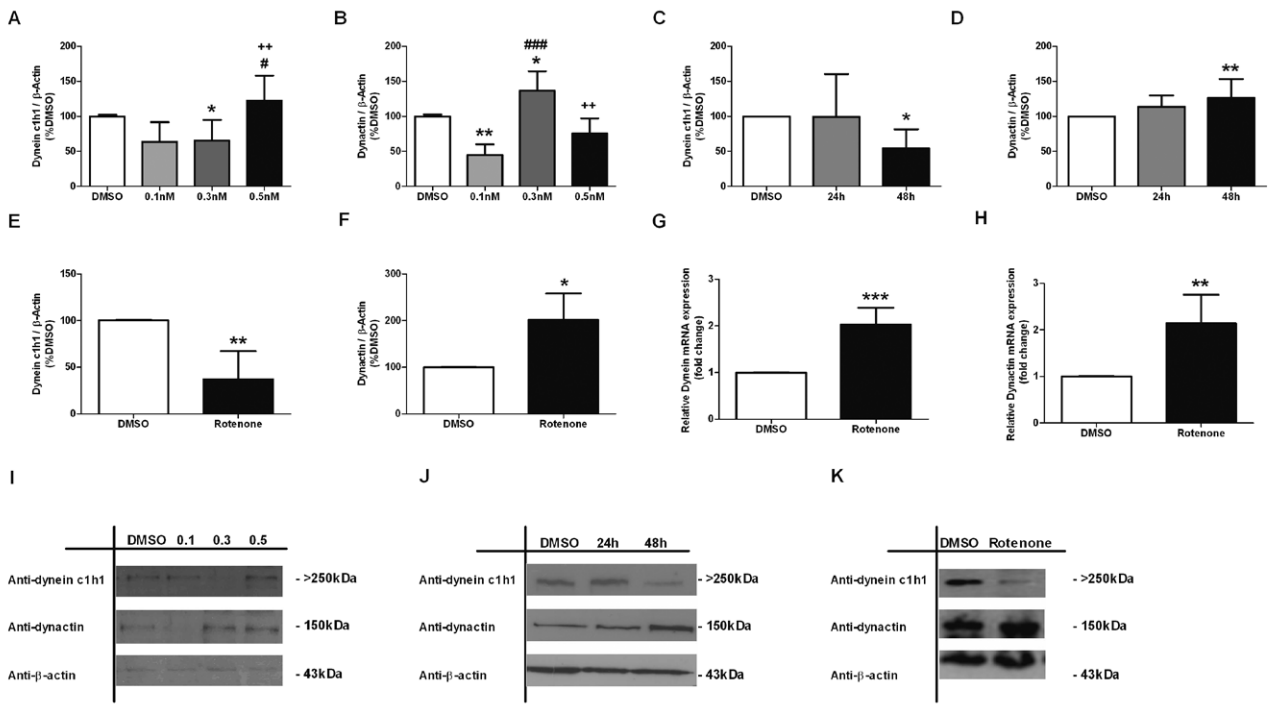

Fig. 7. Substantia nigra dynein c1h1 and dynactin expression in cell culture and aged rats exposed to rotenone. Dose-response (A and B) and time-course (C and D) of dynein c1h1 (A, C) and dynactin (B, D) protein expression in substantia nigra cell cultures exposed to 0.1, 0.3 or 0.5 nM of rotenone for 48 hours (dose-response); or 0.3 nM for 24 or 48 hours (time-course). Dynein c1h1 and dynactin protein levels (E, F) and mRNA expression (G, H) in substantia nigra of 12 months-old rats following exposure to 1 mg/kg/day of rotenone for 4 weeks. Illustrative images of the pattern of bands corresponding to dose-response (I) and time-course (J) of dynein c1h1 and dynactin protein expression in substantia nigra cell cultures and in substantia nigra of 12 months-old rats (K) after rotenone exposure. Normalization was performed by beta-actin (43 kDa) signal. Data are shown as percent of control (DMSO) ± SD. * $P<0.05$; ** $P<0.01$; *** $P<0.001$ as compared to DMSO, # $P<0.05$; ### $P<0.001$ as compared to 0.1 nM (dose-response), ++ $P<0.01$ as compared to 0.3 nM (dose-response), according to one-way ANOVA followed by Tukey post-test to cell cultures and Student's *t*-test for aged animals analysis. Experiments were repeated twice, each run was performed in sample triplicates.

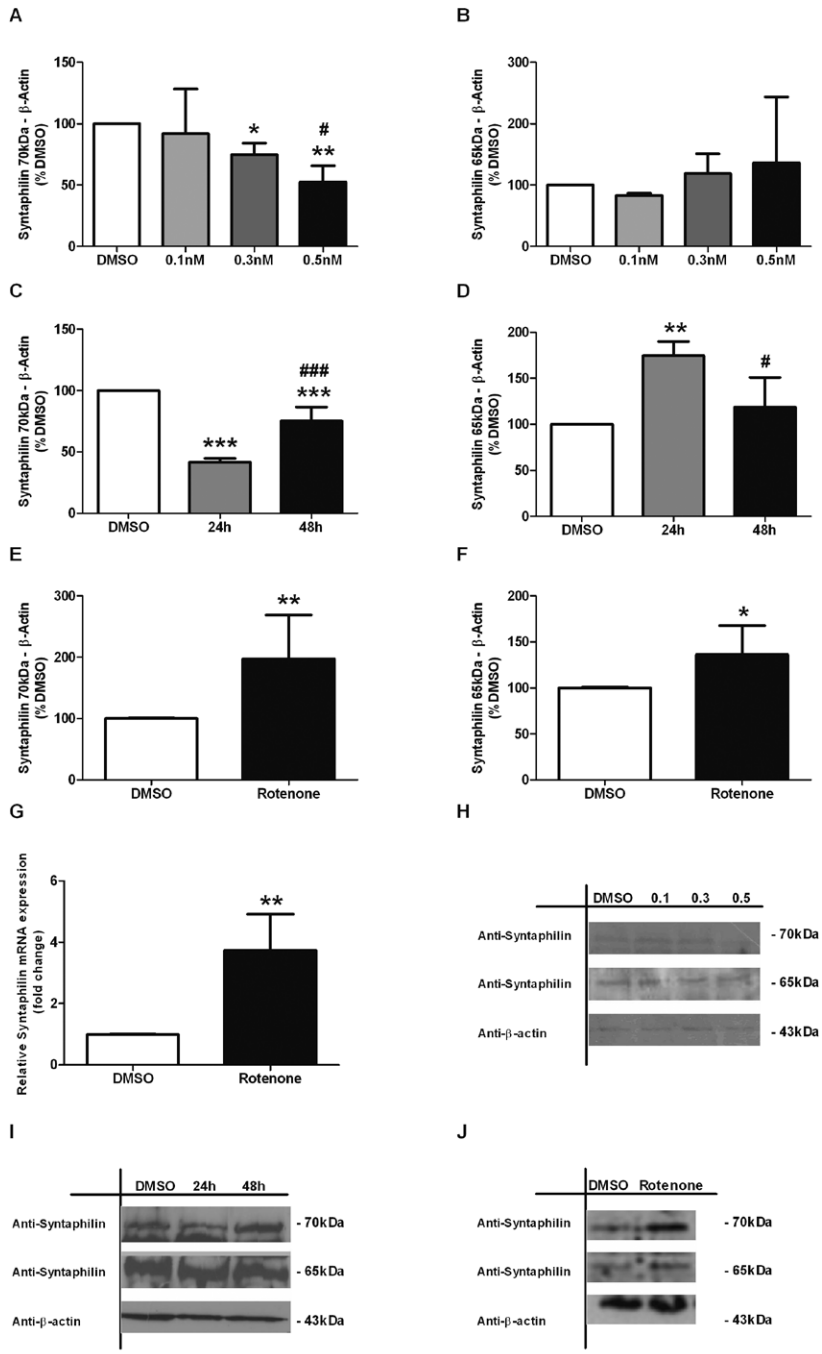

Fig. 8. Substantia nigra syntaphilin expression in cell culture and aged rats exposed to rotenone. Dose-response (A and B) and time-course (C and D) of syntaphilin isoforms of 70 and 65 kDa expression in substantia nigra cell cultures exposed to 0.1, 0.3 or 0.5 nM for 48 hours (dose-response); or 0.3 nM for 24 or 48 hours (time-course). Syntaphilin isoforms of 70 (E) and 65 kDa (F) protein levels and mRNA expression (G) in substantia nigra of 12 months-old rats following exposure to 1 mg/kg/day of rotenone for 4 weeks. Illustrative images of the pattern of bands corresponding to dose-response (H) and time-course (I) of Syntaphilin 70 and 65 kDa protein expression in substantia nigra cell cultures and in substantia nigra of 12 months-old rats (J) after rotenone exposure. Normalization was performed by beta-actin (43 kDa) signal. Data are shown as percent of control (DMSO) ± SD. **P*<0.05; ***P*<0.01; ****P*<0.001 as compared to control (DMSO), # *P*<0.05, ### *P*<0.001 as compared to 0.1 nM (dose-dependent) or 24 hours (time-course), according to one-way ANOVA followed by Tukey post-test to cell cultures and Student's *t*-test for aged animals analysis. Experiments were repeated twice, each run was performed in sample triplicates.

Figure 7I and 7J, Figure 7K illustrate aged rat experiment immunoblot.

Syntaphilin isoforms are differently regulated in substantia nigra cell culture and are increased in this nucleus of aged rats

Dose-response analysis of rotenone-exposed cells demonstrated a decrease in 70 kDa syntaphilin after 0.3 and 0.5 nM (Fig. 8A), however the isoform of 65 kDa did not change (Fig. 8B). The time of rotenone exposure differently influenced syntaphilin isoforms expression, the 70 kDa isoform decreased after 24 and 48 hours of rotenone exposure (Fig. 8C), however protein expression of the 65 kDa syntaphilin isoform increased after 24 hours of rotenone exposure as compared to DMSO control cells, returning to basal levels after 48 hours (Fig. 8D).

Both isoforms of syntaphilin increased in the substantia nigra of aged rats exposed to 1 mg/kg/day of rotenone for 4 weeks (Fig. 8E, F), concomitantly syntaphilin mRNA also increased after rotenone exposure (Fig. 8G).

Figures 8H and 8I show illustrative images of the pattern of bands of anti-syntaphilin in dose-response and time-course experiments in hippocampal cell cultures and aged rats (Fig. 8J).

DISCUSSION

The present study described for the first time the expression of dynein c1h1, dynactin and syntaphilin in cell cultures and aged rats after exposure to low doses of rotenone, correlating this with cellular events triggered by rotenone exposure prior to neurodegeneration.

Overall the results presented herein indicate that low concentrations of rotenone, which cannot induce cell death in cells cultures, decrease dynein c1h1 protein levels in cultured cells and brain areas involved in neurodegeneration of aged rats. On the other hand the adaptor protein dynactin is decreased after 0.1 and 0.3 nM of rotenone, and increased at the concentration of 0.5 nM, when the first aggregates appear in cell culture (Chaves et al. 2010). Aged rats present increased dynactin expression in analyzed brain nuclei. Regarding syntaphilin, the response to rotenone is opposite between cell cultures and brain areas from aged animals since its expression decreased *in vitro* and increased *in vivo* following rotenone exposure.

The present data interestingly corroborates several previous studies suggesting that alteration in intracellular trafficking, including mitochondrial transport, occurs early in neurodegeneration (Williamson and Cleveland 1999, Ross and Poirier 2005, Martin 2007, Bilsland et al. 2010).

Mitochondrial respiratory chain impairment and oxidative stress has been identified *in vivo*, *in vitro* and in *post mortem* brains of Parkinson's (reviewed by Abou-Sleiman et al. 2006), Huntington's (Lodi et al. 2000) and Alzheimer's disease patients (Mancuso et al. 2008). Moreover, mitochondrial dysfunction and oxidative stress are also related to the natural ageing process (reviewed by Bowling and Beal 1995, Weber and Reichert 2010, Leeuwenburgh et al. 2011).

The experimental model using rotenone exposure mimics mitochondrial defects related to neurodegenerative diseases (Rizzardini et al. 2006, Hollerhage et al. 2009), making it a valuable tool to understand the pathophysiology of ageing and neurodegeneration.

Rotenone has the ability of altering mitochondria fusion/fission dynamics, traffic direction and velocity (Arnold et al. 2011), impairment of mitochondrial respiratory chain complex I induces oxidative/nitrative stress and proteasome inhibition (Chou et al. 2010), dysfunction in microtubule dynamics (Choi et al. 2011), and promotes protein aggregation (Chaves et al. 2010).

Exposure to rotenone reproduces several alterations in cell homeostasis similar to what occur in ageing and neurodegenerative diseases, such as protein inclusions and deficient axonal transport. Our group recently illustrated rotenone capability to alter protein and messenger RNA expression levels of KIF1B and KIF5 and disrupt mitochondria total mobility in the same brain areas and culture system described here, without disrupt mitochondrial membrane potential (Melo et al. 2012). Suggesting that alterations in expression of proteins related to mitochondrial transport could triggers alterations in mitochondria mobility.

However this is still a matter of investigation whether rotenone directly modulates protein expression related to mitochondrial anchoring and retrograde transport or the results are secondary to rotenone effects on mitochondria.

This is the first study reporting that exposure to 0.5 nM of rotenone, the higher concentration used in the study, did not trigger cell death in cell cultures demonstrating that the results presented herein are independent of apoptotic processes. Several studies have

reported the rotenone capability to induce cell death in dopaminergic (Ren et al. 2005, Radad et al. 2008) and non-dopaminergic neurons (Ren and Feng 2007, Ullrich and Humpel 2009, Chaves et al. 2010), however only at concentrations higher than 1nM, suggesting an effect dependent of dose.

Dynein c1h1 protein expression decreased in cell cultures and in aged rats brain areas after rotenone exposure, which might impact retrograde axonal transport. Potentially the reduced expression levels of dynein c1h1 could decrease dynein-dependent transport affecting exosome release (Kimura et al. 2009), autophagic clearance (Ravikumar et al. 2005) and mitochondrial retrograde transport.

Dynactin expression initially decreased after the lower doses of rotenone and subsequently increased with the elevation of rotenone concentration. Considering that dynein and dynactin are connected for mitochondrial trafficking, the increase in expression of dynactin may compensate the decrease in dynein expression. Kimura and colleagues (2009) reported a similar response in dynactin expression after dynein depletion.

The imparment of retrograde mitochondrial transport can impair the proper function of the organelle affecting also its biogenesis, which is critically involved in the formation of synapses and dendritic spines, as well as in apoptotic process and neurodegenerative diseases (Van Laar and Berman 2009).

The dual syntaphilin response to rotenone exposure *in vitro* and *in vivo* remains to be elucidated, however the heterogeneity of response illustrates that rotenone exposure can alter syntaphilin expression and possibly mitochondrial anchoring in different ways depending upon organism and procedures of drug administration.

The decrease of syntaphilin expression levels accompanied with dynein expression decrease can illustrate a depression of mitochondrial anchoring and retrograde transport in cell culture possibly causing local energy depletion and toxic changes in Ca^{2+} buffering, synaptic dysfunction and loss, triggering nervous cell death.

In view of the present data where the protein levels of dynein c1h1, dynactin and syntaphilin decreased after exposure to 0.1, 0.3 and 0.5 nM of rotenone in cell cultures, it may be concluded that the capacity of rotenone to down-regulate the level of these proteins is not only influenced by neurodegeneration process, but rather any effect of rotenone over motor and anchor proteins.

Furthermore, animals exposed to 1 mg/kg/day of rotenone which is lower than used to triggers protein aggregation, also illustrated decrease in dynein expression and increase in dynactin and syntaphilin expression illustrating the possible effects of rotenone upon motor proteins expression also *in vivo*.

One possible mechanism by which rotenone can alter protein expression is inducing reactive oxygen (ROS) and nitrogen (RNS) species (Chou et al. 2010), therefore increasing cellular stress (Choi et al. 2011).

Massaad and colleagues (2010) reported that increase in ROS is accompanied by decrease of axonal transport which can be alleviated by the overexpression of superoxide dismutase 2 enzyme, illustrating that ROS generation may alter intracellular transport, possibly acting upon hyperphosphorylation of tau protein and microtubule destabilization. Rotenone is able to increase ROS and also directly depolymerize microtubules (Choi et al. 2011).

In addition to rotenone, 1-methyl-4-phenylpyridinium (MPTP) also can induce ROS (Smith and Bennett 1997), change microtubule dynamics (Cartelli et al. 2010), and mitochondrial transport (Morfini et al. 2007, Borland et al. 2008) illustrating that increased ROS and microtubule dysfunction participate of a common pathway which possibly modulate protein expression related to mitochondrial anchoring and retrograde transport, culminating in neurodegeneration.

Chou and others (2010) reported that microtubule depolymerizing toxins and rotenone can impair proteasome activity, which may alter cell homeostasis culminating in neurodegenerative disorders such as Parkinson's disease (Xie et al. 2010). Rotenone exposure at low concentrations also increases the levels of hyperphosphorylated tau (Chaves et al. 2010) collaborating to microtubules destabilization. Aberrant patterns of protein phosphorylation and tau hyperphosphorylation represent the major hallmarks of tauopathies and Alzheimer´s disease. Therefore the mechanism of how rotenone exposure alters motor protein expression remains elusive.

CONCLUSION

The present study suggests that changes in protein expression related to mitochondrial retrograde trans-

port and anchoring occurs before neurodegeneration triggered by rotenone exposure, which suggests that changes in protein phosphorylation, oxidation modifications, axonal transport impairment, proteolysis cleavage and impairment of degradation pathways could occur in the absence of neurodegeneration and could be primary factors to trigger neurodegenerative mechanisms, such as protein inclusions.

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