Effects of mild running on substantia nigra during early neurodegeneration

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Abstract
Moderate physical exercise acts at molecular and behavioural levels, such as interfering in neuroplasticity, cell death, neurogenesis, cognition and motor functions. Therefore, the aim of this study is to analyse the cellular effects of moderate treadmill running upon substantia nigra during early neurodegeneration. Aged male Lewis rats (9-month-old) were exposed to rotenone 1mg/kg/day (8 weeks) and 6 weeks of moderate treadmill running, beginning 4 weeks after rotenone exposure. Substantia nigra was extracted and submitted to proteasome and antioxidant enzymes activities, hydrogen peroxide levels and Western blot to evaluate tyrosine hydroxylase (TH), alpha-synuclein, Tom-20, PINK1, TrkB, SLP1, CRMP-2, Rab-27b, LC3II and Beclin-1 level. It was demonstrated that moderate treadmill running, practiced during early neurodegeneration, prevented the increase of alpha-synuclein and maintained the levels of TH unaltered in substantia nigra of aged rats. Physical exercise also stimulated autophagy and prevented impairment of mitophagy, but decreased proteasome activity in rotenone-exposed aged rats. Physical activity also prevented H2O2 increase during early neurodegeneration, although the involved mechanism remains to be elucidated. TrkB levels and its anterograde trafficking seem not to be influenced by moderate treadmill running. In conclusion, moderate physical training could prevent early neurodegeneration in substantia nigra through the improvement of autophagy and mitophagy.

Introduction
Physical exercise has been postulated as an intervention that may attenuate progression of system aging. Physical training, at moderate levels, also protects brain against aging effects, restoring structural and functional impairments. The benefits of physical exercise has been attributed to changes at the cellular level in brain, such as enhanced angiogenesis (Al-Jarrah, Jamous, Al Zailaey, & Bweir, 2011); anti-inflammatory responses (Cadet et al., 2003; Wu et al., 2011a); mitochondrial function (Lau, Patki, Das-Panja, Le, & Ahmad, 2011); neurogenesis (Real et al., 2013; Tajiri et al., 2010); and protection against loss of tyrosine hydroxylase (TH) positive cells (Tuon et al., 2012). All these effects may translate to attenuation of age-related diseases, including Parkinson’s disease (PD) (Speelman et al., 2011).

PD is the second most common age-related neurodegenerative disease, characterized by motor and non-motor symptoms, which decrease quality of life and increase mortality. PD is characterized by progressive degeneration of dopaminergic neurons of the substantia nigra pars compacta as well as the presence of intracellular insoluble inclusions, called Lewy Bodies, consisting primarily of aggregated α-synuclein protein. Although the cause of PD is unknown, aging is considered the major risk factor; nevertheless, genetic and environmental factors contribute to neurodegeneration (Collier, Kanaan, & Kordower, 2011; Dawson & Dawson, 2003).

Motor symptoms appear after the loss of 50–70% of dopaminergic neurons (TH-positive neurons) in substantia nigra making diagnosis difficult in early stages PD. In the last decade, studies have reported alterations at the cellular level, such as changes in proteostasis (Bourdenx et al., 2016; Chang et al., 2016; Mazzulli, Zunke, Isacson, Studer, & Krainc, 2016); mitochondrial function (Gautier, Corti, & Brice, 2014; Pickrell, Pintro, Hida, & Moraes, 2011); oxidative stress (Kim, Kim, Rhie, & Yoon, 2015; Singal, Akaravichien, Kukongvirkul, & Sattayasai, 2015); disruption of axonal transport (Goldstein, 2012; Millemamps & Julien, 2013); and neurotrophic factors pathways (Berghauzen-Maciejewska et al., 2015; Mariani et al., 2015; van der Kolk et al., 2015), that are closely related to early stages of PD.

Animal models are reliable to understand the mechanisms of neurodegeneration (Jagmag, Tripathi, Shukla, Maiti, & Khurana, 2015). In this way, rotenone, a high affinity specific inhibitor of mitochondrial NADH dehydrogenase within complex I of the respiratory chain, have been used as a model of PD (Betarbet et al., 2000; Cannon et al., 2009), since it is able to promote α-synuclein aggregation in vitro (Chaves, Melo, Martins, & Ferrari, 2010; Radad, Gille, & Rausch, 2008; Ullumich & Humpel, 2009) and in vivo (Almeida, Silva, D’Unhao, & Ferrari, 2016; Hoglinger et al.,...
Additionally, rotenone exposure leads to impairment of proteasome activity, oxidative/nitrosative stress, dysfunction of cytoskeleton; reduction of axonal transport and autophagic flux (Almeida, Chaves, et al., 2016; Chaves et al., 2016; Chaves, Melo, D’Unhao, Farizatto, & Ferrari, 2013; Henchcliffe & Beal, 2008), prior to formation of protein aggregates.

Considering that the effects of physical exercise in early stages of protein aggregation remains poorly understood, the objective of the present study is to evaluate the effects of physical exercise on oxidative stress, proteostasis, mitophagy and TrkB receptors levels and trafficking in the substantia nigra of aged rats during early neurodegeneration promoted by rotenone exposure.

Methods

All procedures were performed in accordance with the International Guideline for Animal Experimentation care and use (Demers et al., 2006), as well as respecting the Brazilian federal law 11,794/08 for animal welfare and approved by the institutional ethics committee (CEUA IB 451/11) of the Institute of Biosciences of the University of Sao Paulo.

Animals

Twenty-six aged male Lewis rats (9 months old), supplied by the central animal facility of the Institute of Biosciences of the University of Sao Paulo were used in this study. The animals were housed in groups of 3–4 animals per conventional cage, maintained at 23°C ± 2, in an inverted 12h light/12h dark cycle (lights off at 7 am), with free access to food and water.

Rotenone exposure

Rats were anesthetized with ketamine (1.25ml/kg) and xylazine (0.5ml/kg) and had osmotic minipumps (4ML2, Alzet, USA) implanted subcutaneously on their back, containing dimethyl sulfoxide (DMSO, Sigma, USA) and polyethylene glycol (PEG, Sigma, USA) (DMSO:PEG – 1:1) as Control Group (DMSO) or Rotenone (Sigma, USA) dissolved in DMSO:PEG in equal volumes which were delivered at the rate of 1mg/kg/day during 4 weeks. After 4 weeks same procedure was repeated to ensure the exposure to rotenone for 8 weeks.

Physical exercise training

After 1 week of minipumps implantation all Lewis rats were familiarized to treadmill running 3 times per week, 10–30 minutes per day, 0.3km.h⁻¹, during 3 weeks. Rats were then preselected for their ability to run in a treadmill and allocated to physical exercise training group (exe, 50–60% of maximal exercise capacity, 5 days/week, 40 minutes/day, during six weeks) or kept sedentary (sed). Preselection of rats avoid the possible stress caused by forced training, as well as decrease the losses during training, since no electric shock was employed to motivate rats to run. Willingness to run per se do not influence the results, however the stimulation to keep running when rats are not prone to run is stressful (Greenwood et al., 2013), and this negatively influence neurodegeneration.

Rats that did not run or stopped running (total of 6 rats) during protocol were excluded from the analysis. By the end of the exercise protocol we had 20 rats (n = 5 for each experimental group, i.e. DMSO sedentary, DMSO trained, Rotenone sedentary, Rotenone trained). Sedentary rats were exposed to the switched off treadmill during the same period as the trained rats did. Exercise protocol started after 4 weeks of rotenone exposure and lasted 6 weeks, during the second round of rotenone delivery and 2 additional weeks. Figure 1 summarizes the training protocol and experimental groups.

Maximal exercise capacity was determined by the progressive maximal test starting at 0.3km.h⁻¹, with increments of 0.3km.h⁻¹ every 3 min until exhaustion, which was recognized when rats were at semireclining posture, did not recover physical strength after reduction of speed and react slowly to hand stimulation on their back.

Progressive maximal test was repeated every 2 weeks in order to re-adapt the velocity, thus maintaining the intensity of training (Ceroni, Chaar, Bombein, & Michelini, 2009; Ichige et al., 2016; Rodrigues et al., 2007)

With the end of physical exercise animals were euthanized and their soleus and gastrocnemius were removed to access skeletal muscle adaptation to exercise, by citrate synthase activity. Also, substantia nigra was removed and either immediately processed for enzymes activity and H₂O₂ concentration, or stored at −70°C in extraction buffer (400 µl of PBS, pH 7.4, containing 1% NP40, 0.5% sodium deoxycholate, 1%SDS, 1mM EDTA, 1mM EGTA and 1% protease inhibitor cocktail, all reagents from Sigma) for Western Blot assay.

Citrate synthase activity

Citrate synthase activity was measured in gastrocnemius and soleus homogenates based on the previously described method (Sere, 1969). Muscles were homogenized in a buffer containing 0.1 M Tris, pH 8.1, 0.15 M NaCl, 0.1% Triton X-100, 1 mM EDTA and

Figure 1. Experimental design. Nine months old male Lewis rats were exposed to rotenone (1mg/kg/day) or DMSO:PEG as vehicle control (DMSO) delivered subcutaneously through Alzet minipumps. One week after minipumps implantation all rats were familiarized to treadmill running for 3 weeks (3 times per week, 10–30 minutes per day, 0.3km.h⁻¹). Rats were then divided in two groups: sedentary and physical exercise. Exercised rats run on treadmill 5 days/week, 40min., at 50% of their maximal capacity, during 6 weeks. Sedentary groups (DMSO, n = 5; and Rotenone, n = 5) did not practiced physical exercise, they were only exposed to the switched off treadmill. Groups DMSO EXE (n = 5) and ROT EXE (n = 5), receive DMSO or rotenone, respectively, followed by physical training.
0.2 mM protease inhibitor (PMSF). Total protein concentration was accessed through Bradford method. Samples were prepared in duplicates using 100 mM Tris (pH 8.1), 3 mM acetyl-CoA, 1 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) and 5 mM oxaloacetate.

Reaction was quantified by spectrophotometry measuring the indirect formation of the Coa-SH. Citrate synthase catalyzes a reaction that ends up with the formation of CoA conjugated with a thiol group (Coa-SH), which reacts with DTNB to form TNB, a yellow product, measured at 412 nm.

**Proteasome activity**

Proteasome activity was determined according to the previously described method (Silva et al., 2012). Substantia nigra was lysed in RIPA buffer (400 µl of PBS, pH 7.4, containing 1% NP40, 0.5% sodium deoxycholate, 1% SDS, 1 mM EDTA, 1 mM EGTA). Thirty micrograms of total protein was incubated with 125 µM of the fluorogenic substrate s-LLVY-MCA (Calbiochem) in 20 mM Tris/HCl buffer, pH 7.5, at 37ºC. Fluorescence emission was recorded at 440 nm (excitation at 365 nm) for 45 minutes in Gemini XPS Fluorescence Microplate Reader.

**Levels of hydrogen peroxide**

To evaluate the intracellular H2O2 content, a modification of Zhou, Diwu, Panchuk-Voloshina, and Haugland (1997) method was employed. Briefly, 30 µg of total protein extract of brain tissue was incubated with 50 µM of the fluorogenic reagent Amplex red (Molecular probes) and 1.0 U/mL of Horseradish peroxidase diluted in 0.1M PBS, pH 7.0, at 37ºC. Fluorescence emission was recorded at 587 nm (excitation at 563 nm) for 10 minutes in Gemini XPS Fluorescence Microplate Reader. Hydrogen peroxide levels were shown as percentage of control.

**Antioxidant enzymes assays**

Glutathione peroxidase (GPx) activity was analysed by a colorimetric assay (Sigma cat. # CGP1), which is based on the reduction of tert-butyl hydroperoxide by glutathione (GSH), in a reaction catalysed by GPx that generates the oxidized form of glutathione (GSSG). Then, NADPH reduces GSSG in a reaction catalysed by glutathione reductase (GR). The course of these coupled reactions were followed by the decay of NADPH absorbance at 340 nm. Eighty micrograms of total SN protein extracts were loaded in a 96-well microplate containing GPx assay buffer, pH 7.0, NADPH assay reagent, GPx and tert-butyl hydroperoxide.

GR activity was determined by a colorimetric (Sigma cat. #GRSA) that is based on the reduction of GSSG by NADPH coupled with the reduction of DTNB by GSH, generating the product TNB, which is followed by visible absorbance. Eighty micrograms of protein extracts were incubated with 2 mM GSSG (100 µl), assay buffer (20 µl), 3 mM DTNB (50 µl) and 2 mM NADPH (10 µl) in a 96-well plate. The reduction GSSG by NADPH catalysed by GR was verified by spectrophotometry analysis for five minutes, the first reaction was measured by the decrease of NADPH absorbance at 340 nm for NADPH and the second reaction measured by the increase of TNB absorbance at 412 nm for TNB. Superoxide dismutase (SOD) activity was determined by a colorimetric assay (Cayman, cat # 706,002) that utilizes a tetrazolium salt for detection of superoxide radicals that can be generated by leakage of electrons in the mitochondrial respiratory chain. Superoxide radicals can reduce a tetrazolium salt into formazan products that exhibited absorbance in the visible spectrum. Therefore, all three isoforms of SOD (Cu/Zn, Mn, and FeSOD) can inhibit the reaction of superoxide radicals with this tetrazolium salt. Ten micrograms of protein extracts (1µg/µl) were added to 200µl of the radical detector per well. Reaction started with 20 µl of diluted xanthine oxidase to all the wells. Plate was protected from light and incubated in a shaker for 30 minutes at room temperature. Absorbance was measured at 440 nm.

**Western blot**

Substantia nigra samples were fractionated by SDS-PAGE (30 µg of protein/lane) using a 12% tris-HCl gel at 100 V for 1h. Proteins were transferred to nitrocellulose membrane in transfer buffer (25 mM Tris, 190 mM glycine, 10% methanol) for 1 hour at 100 V at 4ºC. Membranes were blocked for 1 hour at room temperature in Tris-Buffered saline containing Tween 20 (TBS-T; 50 mM Tris, pH 8.0, 133 mM NaCl, 0.2% Tween 20) with 5% non-fat dry milk or 5% BSA (Sigma).

Membranes were incubated with primary antibodies against TH (h-196, sc-14,007, Santa Cruz, 1/500), alpha-synuclein (C-20-R, sc-7011-R, Santa Cruz, 1/500), Tom-20 (sc-11,415, Santa Cruz, 1/500), PINK1 (ab23707, Abcam, 1/1000), TrkB (H-181, sc-8316, Santa Cruz, 1/1000); SLP1 (sc-136,480, Santa Cruz, 1/2000); CRMP-2 (C2993, Sigma, 1/7000); Rab-27b (R-4655, Sigma, 1/1000); LC3II (3868, Cell Signalling Technology, 1:1000) or Beclin-1 (3738, Cell Signalling Technology, 1:1000); in 3% non-fat dry milk in TBS-T, overnight at 4°C, followed by horseradish peroxidase-conjugated anti-mouse (1/6000, Amersham) or anti-rabbit (1/10,000, Amersham). Secondary antibodies incubations were performed at room temperature for 1 hour.

Development was done after 5-minute incubation with enhanced chemiluminescence reagent (Millipore) and exposure to chemiluminescence sensitive films (Hyperfilm ECL, Amersham Biosciences). After development, blots were incubated with anti-beta-actin antibody (sc-47,778, Santa Cruz, 1/1000) during 1h at room temperature, followed by horseradish peroxidase-conjugated anti-mouse (1/6000, Amersham), incubated for 1 hour also at room temperature and were 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 values.

**Statistical analysis**

Results were analysed either by Student’s T-test (citrate synthase activity), repeated measures ANOVA (body mass), or two-way ANOVA followed by Bonferroni post-hoc test accessed through GraphPad Prism (GraphPad Software Inc., version 5.00, CA). Data are expressed as percent of control (DMSO) ± standard deviation.
Results

Effects of physical activity on body weight, physical capacity and activity of citrate synthase

Body mass decreased about 10% at the end of experimental protocol as compared with the initial situation for rats from Sedentary Rotenone and DMSO Exercise groups (Figure 2(a)), exercised rats that received rotenone lost 15% of the initial body mass (Figure 2(a)). Physical activity did not interfere with body weight since there was no significant change in this parameter after the beginning of training (Figure 2(a), 4th week). The lower body mass of control rats at the beginning of the experimental protocol is not statistically different from the other groups. Improvement in physical activity was confirmed by the test at the end of training confirming that exercised animals run during 50% more time than sedentary rats (Figure 2(b)), rotenone exposure did not affect rats performance during training (Figure 2(b)).

Effectiveness of aerobic physical activity was confirmed in trained animals since they presented increased activity of citrate synthase in gastrocnemius and soleus muscles, indicating oxidative adaption to exercise training (Figure 2(c)).

Moderate physical training attenuates α-synuclein levels generated by rotenone exposure

Rotenone increased the levels of α-synuclein by 50% (Figure 3(a)), as exercise alone also did. However, moderate treadmill running prevented the increase of α-synuclein levels in substantia nigra after rotenone exposure (Figure 3(a)).

Tyrosine hydroxylase levels did not change after rotenone exposure or physical training (Figure 3(b)), however there was an increase in TH levels in rotenone-exposed rats after treadmill (Figure 3(b)).

Moderate physical training increased autophagy during neurodegeneration and decreased proteasome activity

Autophagy was measured comparing the levels of beclin-1 and LC3II. It was demonstrated that rotenone promoted a decrease in autophagy illustrated by the absence of LC3II degradation (LC3II accumulation, 90%) even in the scenario where beclin-1, which initiates autophagy, is 30% upregulated (Figure 4(a), 4(b)). Exercise training stimulated an increase in beclin-1 levels and degradation of LC3II in substantia nigra during neurodegeneration (Figure 4(a), 4(b)), demonstrating an increase in autophagy promoted by exercise training only in rats exposed to rotenone.

Proteasome chymotrypsin-like activity was not changed in early neurodegeneration while exercise induced loss of proteasomal activity. In animals treated with rotenone and submitted to exercise, the loss of proteasomal activity was significantly higher (Figure 4(c)).

Moderate physical training during early neurodegeneration prevents impairment of mitophagy

TOM-20, a subunit of the mitochondrial outer membrane translocase, levels significantly increased 40% in substantia nigra of the aged Lewis rats exposed to rotenone, indicating...
accumulation of mitochondria, whereas physical training during early neurodegeneration prevented that increase (Figure 5 (a)). PINK1 levels showed a tendency of decrease in substantia nigra of aged rats exposed to rotenone (p = 0.08), which was not present in exercised rats (Figure 5(b)), illustrating a possible improvement in mitophagy after physical activity.

**Moderate physical training decreases hydrogen peroxide during neurodegeneration**

Hydrogen peroxide levels increased 16% after low rotenone exposure; however, moderate physical exercise practiced during early neurodegeneration prevented increased H$_2$O$_2$ production (Figure 6(a)).

Activity of the antioxidant enzyme GPx in substantia nigra was evaluated, as this enzyme is one of the most reactive enzymes for H$_2$O$_2$ removal. The other antioxidant enzyme evaluated was SOD responsible for superoxide decomposition and H$_2$O$_2$ production. It was observed no change either in SOD activity (Figure 6(b)) or in GPx activity (Figure 6(c)). There was a discrete loss in GR activity in animals treated with decreased after rotenone or submitted to exercise when compared to Control animals. However, GR activity was recovered when rotenone-treated animals were submitted to exercise (Figure 6(d)).
Moderate physical exercise does not interfere with trkB levels and proteins involved in its anterograde traffic in substantia nigra during neurodegeneration

Rotenone decreased TrkB receptor levels by 45%, whereas moderate physical training did not change this decrease during early neurodegeneration (Figure 7(a)).

Specific protein complex of TrkB anterograde trafficking (CRMP-2, Rab27B and SLP-1) increased before protein aggregation (42% for CRMP-2, 118% for Rab27B and 96% for SLP-1), in the same way physical activity increased these proteins levels independently of rotenone exposure (Figure 7(b), 7(c) and 7(d)).

Discussion

It was demonstrated, for the first time, that moderate treadmill running, practiced during early neurodegeneration, prevented the increase of α-synuclein and promoted the increase of TH levels in substantia nigra of aged rats. Physical exercise also stimulated autophagy, namely mitophagy, but decrease proteasome activity. Physical activity also prevented H2O2 increase during early neurodegeneration, although the mechanism involved remains to be elucidated. One possibility might be increased mitogenesis upon increased physical activity. TrkB levels and anterograde trafficking seems not to be influenced by moderate treadmill running. Overall results indicate that moderate treadmill running can delay neurodegeneration of substantia nigra.

Training protocol did not interfere with body mass, while trained groups increased aerobic capacity and citrate synthase activity, as expected, even for old animals (Sanchez, Bastien, & Monod, 1983). Also, the physical capacity was decreased in sedentary groups around 40–50% compared with initial condition. It was expected that older rats present lower performance at the maximal capacity test, at the end of the protocol rats were 10 weeks older (they were 10 months old), thus it could be the reason for the decline in strength, since studies in young rats already showed that physical inactivity reduces the performance (25–35%) after 13 weeks (Ceroni et al., 2009; Jordao, Ladd, Coppi, Chopard, & Michelini, 2011), while in old rats the exercise capacity decreased significantly with age and sedentarism.

Rotenone exposure increased α-synuclein levels, which was not supposed to occur with only 1 month of 1mg/kg/day of rotenone treatment (Almeida et al., 2016). However, neurodegeneration was not yet installed as TH levels remained unchanged. Although α-synuclein was up-regulated by rotenone, TH-positive neurons were preserved demonstrating that the observed results occur prior neuron death, in early neurodegeneration. Interestingly, TH-positive neurons increase after physical activity in rotenone trained rats.

Alpha synuclein is a protein involved in synaptic transmission, the increased expression of this protein after exercise training may be related to the synaptic plasticity, while the increased expression of α-synuclein after rotenone may be associated to decrease of its degradation and/or aggregation as occur in early neurodegeneration. This hypothesis is strengthened by the evidence that exercise training prevented the increase of α-synuclein promoted by rotenone, which indicate that the increase of α-synuclein in the two groups is driven by different and antagonistic pathways. The mechanisms underlying increase of α-synuclein in both situations remains to be further evaluated. This is evidence that physical activity applied during early neurodegeneration has a possible protective response, as occurs in other PD animal models (Shin, Jeong, An, Lee, & Sung, 2016).

Physical exercise also interferes with autophagy during early neurodegeneration. It was demonstrated a decrease in autophagy flux by the accumulation of LC3II, which was prevented by mild treadmill running during neurodegeneration. The increase in autophagy induction, promoted by increase in beclin-1, as well as degradation of LC3II (Klionsky et al., 2016), may correlate with the observed decrease of α-synuclein in the substantia nigra of aged rats exposed to rotenone and moderate running.

In the context of autophagy, mitophagy was also evaluated by the expression of PINK1 and TOM-20. The increase in TOM-20 during early neurodegeneration is associated with decrease of PINK1 and possibly accumulation of mitochondria (Nardin, Schrepfer, & Ziviani, 2016) restoration of their levels by exercise might ameliorate mitochondria dynamics.

Mitophagy dysfunction during early neurodegeneration is associated with increased oxidative stress, since mitochondria are the primary source of reactive oxygen species (Palikaras & Tavernarakis, 2012). In the present study, an increased production of H2O2 was observed, which correlated with mitochondria accumulation. Moderate physical activity prevented the...
increase of H$_2$O$_2$ and did not change the activity of antioxidante enzymes. Decrease of GR activity may be result of unchanged of GSH activity, decreasing the substrate GSSH for GR. This strengthens the hypothesis that H$_2$O$_2$ was mainly produced by dysfunctional mitochondria, which was re-established by moderate physical activity.

The present data corroborate previous studies that associate decrease in TrkB receptors during neurodegeneration, which is associated with the possible feedback effect of increase in motor proteins of these receptors. However, results presented herein demonstrated that proteins associated to BDNF system, such as TrkB receptor and proteins related to antioxidating trafficking of TrkB receptor, seem not to be involved with the cellular protection of moderate physical activity during early neurodegeneration, as well established for late PD animal models where neuron loss is present (Real et al., 2013; Wu et al., 2011b).

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Disclosure statement
No potential conflict of interest was reported by the authors.

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References


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