Neuroscience Letters xxx (xxxx) 136089

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Research article

Physical exercise increases the production of tyrosine hydroxylase and CDNF in the spinal cord of a Parkinson's disease mouse model

Wagner Antonio Barbosa da Silva [a, c], Karla Ferreira Oliveira [a, c], Louise Caroline Vitorino [b, c], Luciana Ferreira Romão [b], Silvana Allodi [a, b, c], Clynton Lourenço Correa [c, d, *]

[a] Programa de Pós-Graduação em Ciências Biológicas – Fisiologia, Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Av. Carlos Chagas Filho 373, Ilha do Fundão, 21941-902 Rio de Janeiro, RJ, Brazil

[b] Programa de Pós-Graduação em Ciências Morfológicas, Instituto de Ciências Biomédicas, Universidade Federal do Rio de Janeiro, Av. Carlos Chagas Filho 373, Ilha do Fundão, 21941-902 Rio de Janeiro, RJ, Brazil

[c] Laboratório de Neurobiologia Comparativa e do Desenvolvimento, Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Av. Carlos Chagas Filho 373, Ilha do Fundão, 21941-902 Rio de Janeiro, RJ, Brazil

[d] Faculdade de Fisioterapia, Universidade Federal do Rio de Janeiro. R. Rodolpho Paulo Rocco 255 - HUCFF, Ilha do Fundão, 21941-590 Rio de Janeiro, RJ, Brazil

ARTICLE INFO

Keywords:
Parkinson's disease
Cerebral dopamine neurotrophic factor
Tyrosine hydroxylase
Exercise
Animal model

ABSTRACT

Previous research has suggested advocates that exercise is a non-pharmacological therapy for Parkinson's disease (PD). However, few studies have investigated the effects of exercise on central nervous system structures other than the nigrostriatal pathway by using PD animal models. This study investigated the effects of exercise on tyrosine hydroxylase (TH)- and cerebral dopamine neurotrophic factor (CDNF)-containing spinal-cord neurons. Male Swiss mice were divided into 4 groups: sedentary control (SEDCONT), exercise control (EXERCONT), sedentary Parkinson (SEDPD), and exercise Parkinson (EXERPD). The PD groups were submitted to a surgical procedure for stereotaxic bilateral injection of 6-hydroxydopamine into the striatum. TH- and CDNF-containing spinal-cord neurons were evaluated in all groups, using immunohistochemistry and western-blotting. TH content in the ventral horn differed notably between the SEDPD and EXERPD groups. CDNF content was highest in the EXERPD group. SEDPD and EXERPD groups differed the most, as shown by immunohistochemistry and western-blotting. The EXERPD group showed the most intense labeling in immunohistochemistry compared to the SEDCONT and EXERCONT groups. Therefore, we showed here that exercise increased the content of both TH and CDNF in the spinal-cord neurons of a bilateral PD mouse model. We may assume that the spinal cord is affected in a PD model, and therefore this central nervous system region deserves more attention from researchers dealing with PD.

1. Introduction

Parkinson's disease (PD) is a neurodegenerative disorder, characterized by selective degeneration of dopaminergic neurons of the *substantia nigra pars compacta* (SNpc). The subsequent loss of dopamine (DA) in the *striatum* leads to resting tremor, rigidity, bradykinesia, and postural instability. Although specific regions of the motor, limbic, and autonomic systems are affected, the lesions also engulf large portions of the peripheral and enteric nervous systems [1]; therefore, it is not correct to consider PD as a condition of the SNpc alone. Additionally, because the spinal cord has dopaminergic neurons with roles in locomotor activities [2], it is correct to assert that the dopaminergic system is not limited to the nigrostriatal pathway [3].

Although some studies have found that physical exercise has no beneficial effects in rodents and humans associated with recovery of the nigrostriatal dopamine system [4,5], other studies have shown the oppo-

Abbreviations: AEC, 3-amino-9-ethylcarbazole; BDNF, brain derived neurotrophic factor; CDNF, cerebral dopamine neurotrophic factor; DA, dopamine; DAPI, 4′-6-diamidino-2-phenylindole; EXERCONT, exercise control; EXERPD, exercise Parkinson; 6-OHDA, 6-hydroxydopamine; PB, phosphate buffer; PBS, phosphate buffer saline; PD, Parkinson's disease; PF, paraformaldehyde; SEDCONT, sedentary control; SEDPD, sedentary Parkinson; SNpc, substantia nigra pars compacta; TH, tyrosine hydroxylase; TBS, Tris-Buffered Saline

* Corresponding author at: Laboratório de Neurobiologia Comparativa e do Desenvolvimento, Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Av. Carlos Chagas Filho 373, Bloco G2-001, CCS, Ilha do Fundão, 21941-902 Rio de Janeiro, RJ, Brazil.

E-mail address: clyntoncorrea@hucff.ufrj.br (C. Lourenço Correa).

https://doi.org/10.1016/j.neulet.2021.136089
Received 8 March 2021; Received in revised form 5 May 2021; Accepted 23 June 2021
0304-3940/

site: improved motor function and increased lifespan in PD patients [6–13]. In animal studies, exercise enhances functional recovery after nigrostriatal-pathway lesions, as well as recovery of neurobiological markers such as tyrosine hydroxylase (TH) and brain-derived neurotrophic factor (BDNF), among others [14,15]. A recent study showed that a treadmill exercise protocol prevented the loss of dopaminergic neurons associated with PD, by maintaining neurotrophins, including cerebral dopamine neurotrophic factor (CDNF), in the *striatum* [16].

Due to the neural motor control, spinal-cord stimulation has been proposed as a therapeutic approach in PD to improve motor function [17]. This therapeutic approach is adopted because PD triggers motor impairments, and the spinal cord is not only the link between the brain and the peripheral nervous system (PNS) but also part of the dopaminergic system [3]. Therefore, considering that most studies in this area focus on the *striatum* and SNpc, we wanted to observe the effects of exercise on the spinal cord in a PD mouse model. We investigated the effect of treadmill exercise on motor behavior and expression of CDNF in hierarchically lower-level controller parts of the CNS. We evaluated the content of this protein, together with TH, in the cervical spinal cord of animals bilaterally injected or not (controls) with 6-hydroxydopamine (6-OHDA) into the *striatum*. To the best of our knowledge, this is the first study to analyze the effects of physical exercise on the CDNF content in the spinal cord of a mouse model of bilateral PD. Therefore, this study aims to help understand how physical exercise may act as a neuroprotective agent for spinal-cord neurons affected in PD.

2. Materials and methods

2.1. Animals

In all experiments combined, we used 32 male Swiss mice, 8 weeks old and weighing 30–35 g at the beginning of the experiment. The animals were divided into 4 groups: sedentary control (SEDCONT; n = 8), exercise control (EXERCONT; n = 8), sedentary Parkinson (SEDPD; n = 8), and exercise Parkinson (EXERPD; n = 8). We followed the experimental design schematized in Supplementary material 1. The first step was the surgical procedure for stereotaxic injection of 6-OHDA (SEDPD and EXERPD) or saline (SEDCONT and EXERCONT). This was considered **day 0**. Because Rentsch et al. [18] observed significant and progressive dopaminergic and total neuron loss in the SNpc 4 weeks after injection of 6-OHDA into the medial forebrain bundle, here, 30 days after the 6-OHDA injection, the mice underwent a 30-day training period on a treadmill.

All experiments in this study were conducted according to the National Council for Animal Environmental Committee and as approved by the Ethics Commission on Research Animals. The mice used in the study were housed at 25 °C in a 12/12 h light/dark cycle, with continuous water and food supply.

2.2. Surgical procedure

For the application of 6-OHDA, prior to surgery the mice were medicated with diazepam and then anesthetized with an intraperitoneal injection of ketamine and xylazine diluted in saline. The anesthetized mice were positioned in the stereotaxic apparatus (Model 940 small-animal stereotaxic instrument with digital display console, David Kopf Instruments, Tujunga, CA, USA). Animals of the SEDPD and EXERPD groups received bilateral stereotaxic injections of 6-OHDA hydrochloride (Sigma®): 5 μg of 6-OHDA with 0.03% ascorbic acid in a total volume of 2 μL of saline were injected into each hemisphere, at a flow rate of 0.1 μL/min. Animals of the SEDCONT and EXERCONT groups received only 2 μL of saline. The following coordinates were used to inject the solutions into the *striatum*: (1) anteroposterior +0.5 mm, (2) lateral +2 mm and −2 mm, and (3) vertical −3 mm [19].

2.3. Treadmill training

The EXERCONT (n = 8) and EXERPD groups (n = 8) followed a protocol consisting of treadmill training (Insight®, São Paulo, Brazil) twice a week, 50 min/day at a speed of 9 m/min, without inclination or electrical stimulation, for a period of 4 weeks, totaling 8 training sessions.

2.4. Tissue collection

Of the total number of animals (32), 20 animals were anesthetized, infused by transcardiac perfusion with saline, and fixed with 4% paraformaldehyde (4% PF) in phosphate buffer (PB) 0.1 M pH 7.4. The brain and cervical spinal cord were removed and the dissected tissues were immersed in 4% PF overnight, and placed in solutions with increasing concentrations of sucrose in PB for tissue cryoprotection.

2.5. Immunohistochemistry

Slides containing sections (20 μm thick) of the spinal cord and *striatum* (5 animals per group, 4 groups) were prepared for immunoperoxidase in order to evaluate whether the effect of 6-OHDA on TH-producing cells, using a protocol described in the Supplementary data (Immunoperoxidase method).

Other slides with sections 20 μm thick containing the spinal cords of 3 animals per group (from the above 5 animals per group, 4 groups) were washed with 0.3% PBS-Triton X-100 and blocked with 10% NGS in PBS for 1 h at room temperature. Next, the material was incubated with: rabbit anti-tyrosine hydroxylase (1:600, Merck/Millipore, code: AB152), and mouse anti-CDNF (1:100, R&D Systems, code: AF5187) diluted in 0.3% PBS Triton X-100/1% BSA overnight at 4 °C. The next day, after washing in 0.3% PBS-Triton X-100, the sections were incubated with the secondary antibodies diluted in 0.3% PBS-Triton X-100/1% BSA for 1 h, at room temperature: Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:500, Abcam, code: AB150077) and Alexa Fluor 546-conjugated goat anti-mouse IgG (1:500, Invitrogen, code: A-11010). After washing, the sections were immersed in 4′-6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Inc., Burlingame, CA, USA), washed with saline, and mounted with Fluoromount™ (Sigma-Aldrich). The sections were inspected and photographed under a Zeiss Apotome® Imager M2 microscope (Carl Zeiss Microscopy® GmbH, 07745 Jena, Germany), using the program Zen Blue edition 2012. The optical density corresponding to the immunolabeling of the acquired images from the ventral horn of the spinal cord was obtained using ImageJ software (ImageJ, U.S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij), as previously described [20].

2.6. Western-blotting

Western-blotting procedures followed a previous study [21]. Cervical spinal-cord samples of 3 animals per group (4 groups) were used for all the experiments. The samples were isolated and dissected and the material was kept in cryotubes containing a cocktail of protease inhibitors, phosphate buffer, phenylmethylsulfonyl fluoride, Laemmli buffer, and beta-mercaptoethanol. The samples were then processed to separate the protein of interest by polyacrylamide running-gel electrophoresis.

The separated proteins were transferred to a nitrocellulose membrane (Bio-Rad), blocked, and then incubated overnight with: rabbit anti-tyrosine hydroxylase (62 KDa, 1:1000, Merck/Millipore, code: AB152), mouse anti-CDNF (17–19 KDa, 1:1000, R&D Systems, code: AF5187) or mouse anti-actin (42 KDa, 1:1000, Sigma, code A2228). Then, the membranes were washed with Tris-Buffered Saline (TBS) Tween and incubated with TBS-diluted secondary antibodies (ExtrA-

vidin Peroxidase Staining Kit (Sigma, code: E8386), anti-rabbit (1:100) or ExtrAvidin Peroxidase Staining Kit, anti-mouse (1:100), followed by detection using ImageQuant LAS 4000® (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The membranes were quantified in Scion for Windows 4.0.2 (Scion Corporation, Frederick, MD, USA, https://scion-image.software.informer.com) and all quantitative data were normalized by their respective loading controls, using the actin protein as the control.

2.7. Statistical analyses

The violation of normality hypotheses was assessed using the Shapiro-Wilk test. When the distribution showed normal data, the parametric inferential statistic was applied. In addition, the unpaired Student's *t*-test (*striatum* and *substantia nigra*) and the two-way ANOVA followed by Tukey's post-hoc tests were performed for the microscopy and western-blotting analyses. Statistical significance was set at $p < 0.05$ for all analyses. Analyses were performed using a commercially available statistical package (IBM SPSS Inc. Released 2008. SPSS Statistics for Windows, Version 17.0. SPSS Inc., Chicago, IL, USA, https://ibm.com/analytics/spss-statistics-software). Graphs were made using GraphPad Prism for Windows, version 7.00 for Windows (GraphPad Software, La Jolla, CA, USA, https://graphpad.com).

3. Results

3.1. 6-OHDA-caused degeneration of the striatum was partially prevented by exercise

To determine whether the injections of 6-OHDA depleted dopaminergic neurons in the *striatum* and in the *substantia nigra*, we conducted immunoperoxidase reactions for TH in all groups. The results are shown in Supplementary material 2.

3.2. 6-OHDA striatal degeneration reduced the number of cervical spinal cord TH+ cells

Immunofluorescence for TH in the ventral horn of the cervical spinal cord showed differences in labeling among the experimental groups (Fig. 1). The difference between the PD groups (SEDPD 18.51 ± 1.16; EXERPD 35.81 ± 4.75, $p < 0.001$) was especially notable (Fig. 1c, d, f). Western-blotting revealed differences among all groups (Fig. 1g, h) and the results are in accordance with the immunofluorescence regarding the SEDPD and EXERPD groups (SEDPD 0.957 ± 0.006; EXERPD 1.153 ± 0.012, $p < 0.001$).

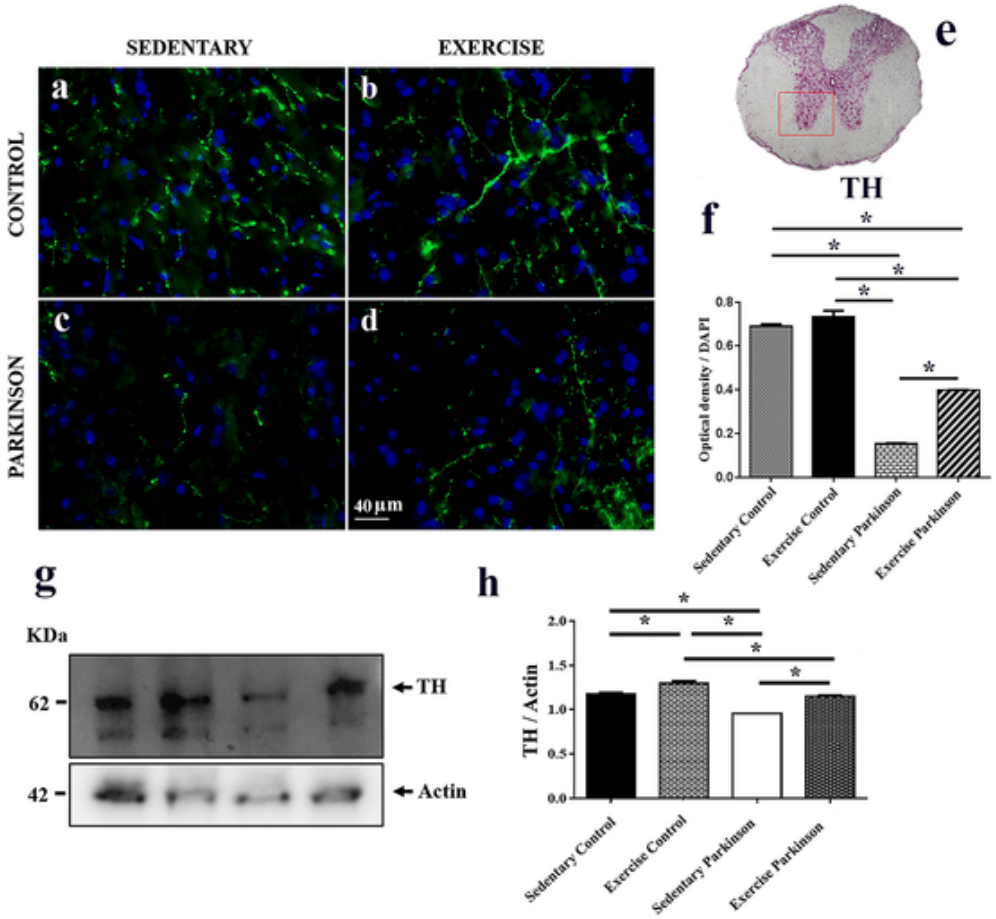

Fig. 1. Tyrosine hydroxylase (TH) immunostaining in the cervical spinal cord for sedentary (control [a] and Parkinson [c]), and exercise (control [b] and Parkinson [d]) groups. g: Western-blotting of cervical spinal-cord protein content. Arrows indicate the molecular weight of TH and actin. Two-way ANOVA revealed significant effects of the factor 6-OHDA lesion (immunostaining (f) [$F_{(3.8)} = 526.754$; $p < 0.001$]), (western-blotting (h) [$F_{(3.8)} = 406.593$; $p < 0.001$]), with a significant 6-OHDA lesion *vs* exercise interaction (immunostaining (f) [$F_{(3.8)} = 6.778$; $p < 0.001$]), (western-blotting (h) [$F_{(3.8)} = 5.032$; $p < 0.001$]), confirming that exercise prevented TH loss in parkinsonian mice. e: Cross section of spinal cord; rectangle shows the ventral horn, where quantifications were made in all groups (Nissl staining). * $p < 0.001$.

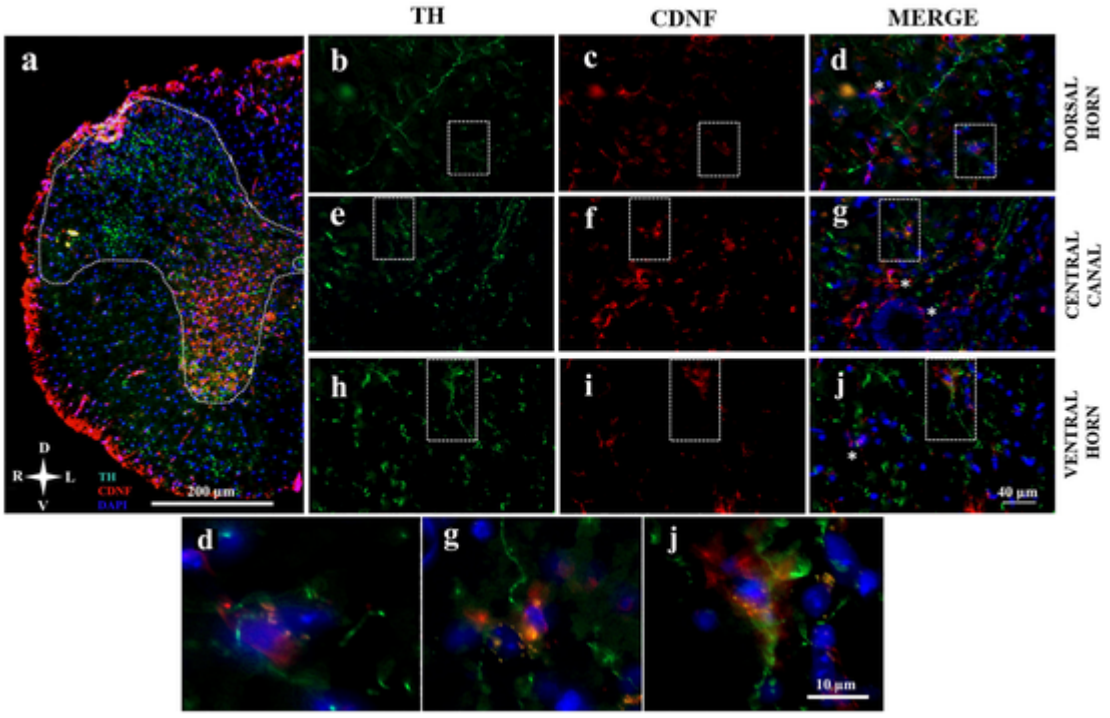

Fig. 2. Tyrosine hydroxylase (TH) and cerebral dopamine neurotrophic factor (CDNF) immunofluorescence in the cervical spinal cord. Labeling is most intense in the ventral horn (a). Labeling for TH (b, e, h) and CDNF (c, f, i) is evident in the ventral horn, central canal, and dorsal horn. Double labeling shows TH/CDNF+ cells (d, g, j).

3.3. CDNF is produced by TH-expressing neurons

Immunofluorescence using antibodies for TH and CDNF indicated that CDNF was produced by neurons that are involved in DA production. The ventral horn of the cervical spinal cord, the location of most of the motor neurons, showed more CDNF+ cells than either the dorsal horn or the region close to the central canal (Fig. 2).

3.4. Effects of exercise on production of CDNF

The effects of exercise on parkinsonian mice are seen in the immunofluorescence results shown in Fig. 3. All groups showed significant differences, SEDCONT *vs* EXERCONT (1.126 ± 0.0193, 2.127 ± 0.0015; $p < 0.001$); SEDCONT *vs* SEDPD (1.126 ± 0.0193, 0.151 ± 0.0036; $p < 0.001$); SEDCONT *vs* EXERPD (1.126 ± 0.0193, 2.696 ± 0.0774; $p < 0.001$); EXERCONT *vs* SEDPD (2.127 ± 0.0015, 0.151 ± 0.0036; $p < 0.001$); EXERCONT *vs* EXERPD (2.127 ± 0.0015, 2.696 ± 0.0774; $p < 0.001$); and SEDPD *vs* EXERPD (0.151 ± 0.0036, 2.696 ± 0.0774; $p < 0.001$); but the large difference between SEDPD and EXERPD groups is especially important (Fig. 3f). This last group showed the most intense labeling compared to the SEDCONT and EXERCONT groups.

4. Discussion

There is no consensus among studies that use physical exercise as a form of therapy: some studies have shown large decreases [22] in neurotrophic factors, although others have shown the opposite [4,5,7,8,22]. Therefore, here, we investigated the effects of treadmill training on the content of TH+/CDNF+ cells in the spinal cord of bilateral parkinsonism-induced Swiss mice in order to contribute to this issue.

Our approach did not reveal whether the impairment of the spinal cord was the result of anterograde degeneration (from the *striatum* toward cortical areas) or of retrograde degeneration (from the *striatum* to the SNpc). Still, we could observe a clear deficit in the motor system of the animals, shown by the immunohistochemistry for TH. Because the lower motor neurons are located in the ventral horn, the consequence of this degeneration is impairment of the axons projecting from the ventral spinal cord to the PNS [23]. Therefore, it seems reasonable to suppose that a lesion in a CNS structure, in this case the *striatum*, may affect the PNS due to dysfunctions of the lower motor neurons.

Because the EXERPD and EXERCONT groups showed increased contents of TH and CDNF, the findings confirm the positive effects on the production of a protective neurotrophin, CDNF, by the remaining TH+ cells, influenced by exercise [16], specifically in the spinal cord. These findings indicate that the training protocol used here had a neuroprotective effect against 6-OHDA-induced PD, through the increase in the enzyme responsible for the production of DA and the neurotrophin CDNF. Since we observed double labeling of TH and CDNF, we assume that neurons were responsible for producing CDNF.

As shown in a rat model of PD induced by 6-OHDA, CDNF prevents the degeneration of dopaminergic neurons [24]. Also, parkinsonian rats following a program of progressive exercise training on treadmills showed increased concentrations of CDNF in the *striatum* [16]. Because we observed intense labeling for CDNF and TH in the ventral horn, the region that contains lower motor neurons, and one of the regions of the CNS committed to motor functions, we suggest that lower motor neurons are responsive to external stimuli such as the treadmill training. This is noteworthy, since these neurons were affected by a supraspinal lesion (6-OHDA injected into the *striatum*) and exercise was capable of influencing the content of CDNF. Because the spinal cord connects the CNS to the PNS, these results show that neurons from spinal cord were influenced by physical exercise.

PD is not only a movement disorder arising from a malfunction of certain encephalic areas, but also a complex disease involving the entire nervous system. The spinal cord is frequently involved in PD, and further studies are needed to correlate symptoms with the site of the lesions [25]. A previous study reported that TH+ cells were concentrated in the dorsal horn, while the ventral horn was practically devoid of TH+ cells [26]. However, [27] reported that the DA immunoreactivity

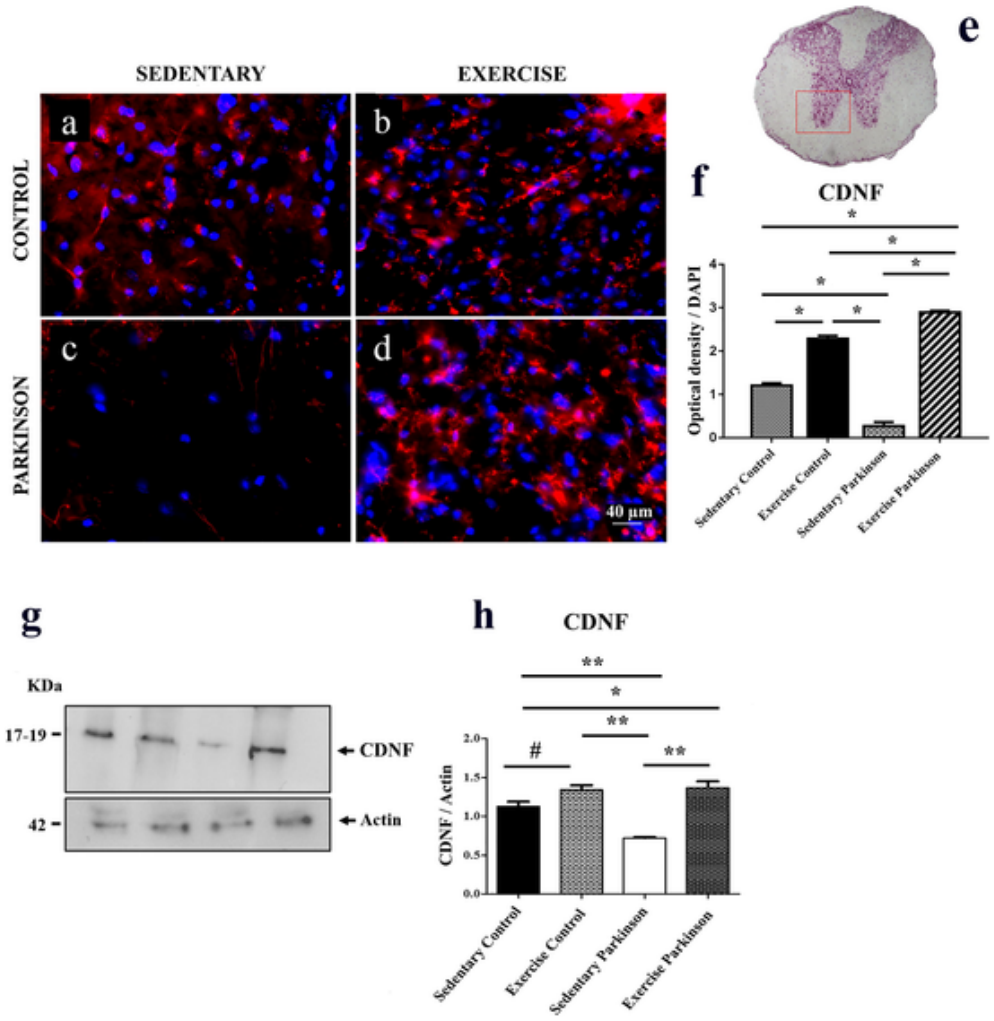

Fig. 3. Cerebral dopamine neurotrophic factor (CDNF) immunofluorescence in the cervical spinal cord for sedentary (control [a] and Parkinson [c]), and exercise (control [b] and Parkinson [d]) groups. g: Western-blotting of cervical spinal-cord protein content. Arrows indicate the molecular weight of CDNF and actin. Two-way ANOVA revealed significant effects of the factor 6-OHDA lesion (immunostaining (f) [$F_{(3.8)} = 23.690$; $p < 0.001$]), (western-blotting (h) [$F_{(3.8)} = 74.399$; $p < 0.001$]), with a significant 6-OHDA lesion *vs* exercise interaction (immunostaining (f) [$F_{(3.8)} = 3.781$; $p < 0.001$]), (western-blotting (g, h) [$F_{(3.8)} = 16.195$; $p < 0.001$]), confirming that exercise prevented loss of CDNF in parkinsonian mice. e: Cross section of spinal cord; rectangle shows the ventral horn, where quantifications were made in all groups (Nissl staining). f: $*p < 0.001$, h: $\#p = 0.032$, $*p = 0.006$, $**p < 0.001$.

in the ventral horn has a very different appearance from the dorsal horn. This difference was attributed mostly to the type of innervation of the motoneuronal cell groups. These authors stated that DA fibers and terminals are located throughout the spinal gray matter. Consequently, it is to be expected that TH+ cells located in the spinal cord would influence both sensory and motor systems as well as the autonomic nervous system.

In view of the presence of TH+ cells in the motoneuronal cell groups, the question arises of whether the disturbed motor performance in PD is partly attributable to changes in TH+ cells at the spinal level [26]. It has been shown that a unilateral lesion of the *substantia nigra,* produced by injection of 6-OHDA, significantly reduced the DA content in the *striatum* and spinal cord on the side of the injection [28]. There is good evidence favoring the idea that the descending ventral dopaminergic innervation of the spinal cord is involved in the control of motor functions. It has been further demonstrated that the three neuronal elements involved in somatic motor activity, i.e., α and γ motoneurons, as well as interneurons, are affected by DA [29]. It is known that neurobiological effects of exercise on spinal cord have several metabolic and neurochemical pathways through which physical activity and exercise might influence the CNS [30]. For example, spinal motor learning that is specific to a practiced task can occur within the neuronal networks in the spinal cord in mice, rats, cats, and humans [31].

The functions performed by CDNF are not completely elucidated [32,33]. However, in this study we showed that treadmill training following a protocol of 9 m/min for four weeks may have beneficial effects on spinal-cord neurons. We cannot state whether CDNF participates in the survival of these neurons, although exercise clearly had some influence on CDNF production, as occurs with other neurotrophic factors [15,22]. To conclude, we can affirm that the spinal cord is affected in a PD model, and therefore this CNS region deserves more attention from researchers. Studies of other CNS structures affected by parkinsonism may help to understand how the motor function is impaired, in order to search for effective treatments.

Acknowledgments

The authors thank Sergio Luís de Carvalho for technical support.

Funding information

This study was supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Conselho Nacional de Desenvolvi-

mento Científico e Tecnológico (CNPq), and Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neulet.2021.136089.

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