Research Report

Can physical exercise have a protective effect in an animal model of sleep-related movement disorder?

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Abstract

The purpose of the present study was to determine whether physical exercise (PE) has a protective effect in an experimental animal model of sleep-related movement disorder (A11 dopaminergic nuclei lesions with 6-OHDA). Rats were divided into four groups (Control PE-CTRL/PE, SHAM/PE, A11 lesion/NPE, A11 lesion/PE). Two experiments were performed: (1) the rats underwent PE before (2 weeks) and after (4 weeks) the A11 lesion; and (2) the rats underwent PE only after (4 weeks) the A11 lesion. Electrode insertion surgery was performed and sleep analyses were conducted over a period of 24 h (baseline and after PE) and analyzed in 6 blocks of 4 h. The results demonstrated that the A11 lesion produced an increased percentage of wakefulness in the final block of the dark period (3–7 am) and a significant enhancement of the number of limb movements (LM) throughout the day. Four weeks of PE was important for reducing the number of LMs in the A11 lesion group in the rats that performed PE before and after the A11 lesion. However, in the analysis of the protective effect of PE on LM, the results showed that the number of LMs was lower at baseline in the group that had performed 2 weeks of PE prior to the A11 lesion than in the group that had not previously performed PE. In conclusion, these findings consistently demonstrate that non-pharmacological manipulations had a beneficial effect on the symptoms of sleep-related movement disorder.

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Restless legs syndrome (RLS) is a common sleep-related movement disorder that is characterized by uncomfortable sensations in the limbs. These sensations appear or become worse during the evening/night rest period and are relieved by motor activity (Walters, 1995). Most RLS patients present periodic limb movements (PLMs) in sleep (Thorpy, 2005).

Epidemiologic data have increasingly demonstrated the negative effect of RLS on health. In the Sleep Heart Health Study (Ulfberg et al., 2001), subjects with RLS were found to have a twofold increase in the odds ratios, after adjustment for confounders, for having coronary artery disease and cardiovascular disease in comparison with those without RLS. RLS is significantly associated with diminished quality of life (Allen et al., 2005; Berger et al., 2004) depressed mood, and/or social isolation (Allen, 2004; Berger et al., 2004; Ulfberg et al., 2001). RLS and PLM can negatively affect the quality of sleep, increased sleep latency, number of awakenings and reduced sleep efficiency, thereby resulting in daytime sleepiness (Allen et al., 2003).

Treatment of the idiopathic form of RLS is most commonly pharmacologic (Littner et al., 2004). Specifically, dopamine D2 agonists have become the first-line treatment for this condition (Montplaisir et al., 1999). This is due to association of the dopaminergic system in the pathophysiology of this sleep disorder (Trenkwalder and Paulus, 2010).

As a form of non-pharmacologic treatment, the influence of physical exercise on the improvement of RLS symptoms, specifically, PLMs, has also been the subject of clinical research (ASDA, 1992). Studies have demonstrated that acute or chronic physical exercise provides benefits and significantly reduces PLMs in patients with or without spinal cord injury (De Mello et al., 2002; Esteves et al., 2009; Aukerman et al., 2006; Cavagnolli et al., 2013).

However, on the basis of the current hypotheses concerning the pathophysiology of RLS and PLM, some promising attempts have been made to develop procedures for producing an animal model of sleep-related movement disorder. These attempts have involved lesioning (Esteves et al., 2004; Lopes et al., 2012), dietary manipulations (Qu et al., 2007), and pharmacological interventions (Lopes et al., 2012; Luo et al., 2011; Esteves et al., 2013). In particular, lesions in the A11 core result in sleep disturbances in animals. This manipulation is often considered to be an animal model of RLS/PLM (Lopes et al., 2012; Qu et al., 2007). The A11 cell group in the hypothalamus is a major source of descending dopaminergic projections to the spinal cord and the major source of spinal dopamine (DA). It sends projections to the sympathetic preganglionic neurons (SPNs) in the intermediolateral nucleus and directly projections to the suprachiasmatic nuclei (SCNs) (Hokfelt et al., 1979). The neuronal link between the SCNs and the SPNs via A11 neurons suggests that spinal DA actions are under circadian influence. It is believed that some of the diencephalospinal neurons form a sympathetic-excitatory system (Qu et al., 2006).

However, the potential influence of non-pharmacological (physical exercise) treatment in this animal model is seldom studied (Esteves et al., 2013).

The purpose of the present study was to determine whether physical exercise has a protective effect in an experimental animal model of sleep-related movement disorder (A11 dopaminergic nuclei lesion with 6-OHDA).

### 2. Results

#### 2.1. Physical exercise before and after A11 lesion

Changes from baseline and after physical exercise in sleep patterns that were assessed by recording sleep in light and dark periods (12 h) are presented in Table 1. In the light period (12 h), a repeated-measures ANOVA revealed a significant difference in the group factor for total sleep time (TST) ($F_{(3,20)} = 4.937, p = 0.01$), slow wave sleep (SWS) ($F_{(3,20)} = 26.402, p < 0.001$), paradoxical sleep (PS) ($F_{(3,20)} = 6.180, p < 0.01$) and wakefulness (WK) ($F_{(3,20)} = 10.655, p < 0.01$) and in the time factor for PS ($F_{(1,20)} = 4.682, p = 0.04$). The main results showed that the group CTRL-PE showed an increase in TST, SE and WK ARS in relation to A11 Lesion NPE and PE groups. For SWS, the A11 lesion NPE showed a significant decrease in comparison with all other groups after PE. For LMs, the CTRL-PE, SHAM-PE and A11 lesion-PE groups showed a statistically significant decrease from the A11 lesion-NPE in baseline and after physical exercise (Kruskal–Wallis, $p < 0.001$).

In the dark period (12 h), a repeated-measures ANOVA showed a significant difference among groups and an interaction with arousal (ARS) ($F_{(3,20)} = 17.718, p < 0.001; F_{(3,20)} = 3.309, p = 0.04$). The A11 lesion-NPE group showed a significant increase in comparison with the other groups in the ARS after four weeks of exercise.

For LMs, the CTRL-PE and SHAM-PE showed a statistically significant difference from the A11 lesion-NPE and A11 lesion-PE in baseline. After exercise, the CTRL-PE, SHAM-PE and A11 lesion-PE groups showed a statistically significant decrease in LMs from the A11 lesion-NPE ($p < 0.001$).

A repeated-measures ANOVA showed a statistically significant difference for group ($F_{(3,20)} = 4.788, p = 0.01$), time ($F_{(5,100)} = 7.372, p = 0.01$) and interaction ($F_{(15,100)} = 2.145, p = 0.01$) in the percentage (%) of WK in the dark period at both baseline and following exercise that was divided into blocks (7–11 pm, 11 pm to 3 am, 3–7 am). The A11 lesion NPE and PE groups showed an increased %WK in the 3–7 am block in comparison with the CTRL-PE and SHAM-PE groups at baseline. After physical exercise, the CTRL-PE, SHAM-PE and A11 lesion-PE groups showed a significant reduction in comparison with the A11 lesion-NPE (baseline and after physical exercise) group and the A11 lesion-NPE (after physical exercise) group. The results are shown in Fig. 1.

#### 2.2. Physical exercise after A11 lesion

Changes from baseline and after physical exercise in sleep patterns, as assessed by recording sleep in the light and dark period (12 h), are presented in Table 2. During the light period (12 h), a repeated-measures ANOVA showed a significant difference in the group factor for TST ($F_{(3,20)} = 8.077, p < 0.01$)
Table 1 – Sleep pattern during light and dark periods (12 h) in the four groups of rats (A11 Lesion NPE, CTRL-PE, SHAM-PE, A11 Lesion PE). Baseline values and values after physical exercise in animals that performed physical exercise before and after the A11 lesion.

<table>
<thead>
<tr>
<th>Sleep Pattern</th>
<th>Period</th>
<th>Baseline</th>
<th>After exercise (4 weeks)</th>
<th>ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>TST (min)</td>
<td>Light</td>
<td>415.7 ± 59.8</td>
<td>430.8 ± 86.1</td>
<td>462.1 ± 19.5</td>
</tr>
<tr>
<td></td>
<td>Dark</td>
<td>275.2 ± 52.8</td>
<td>251.5 ± 32.2</td>
<td>288.4 ± 23.2</td>
</tr>
<tr>
<td>SE (%)</td>
<td>Light</td>
<td>58.1 ± 8.1</td>
<td>64.9 ± 6.6</td>
<td>65.5 ± 2.6</td>
</tr>
<tr>
<td></td>
<td>Dark</td>
<td>38.3 ± 7.3</td>
<td>34.5 ± 4.4</td>
<td>39.2 ± 3.2</td>
</tr>
<tr>
<td>ARS (ev/h)</td>
<td>Light</td>
<td>38.3 ± 20.3</td>
<td>36.0 ± 7.0</td>
<td>27.1 ± 7.0</td>
</tr>
<tr>
<td></td>
<td>Dark</td>
<td>43.1 ± 18.4</td>
<td>36.8 ± 22.0</td>
<td>23.0 ± 9.7</td>
</tr>
<tr>
<td>SWS (%)</td>
<td>Light</td>
<td>39.9 ± 4.0</td>
<td>58.2 ± 6.7</td>
<td>54.2 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>Dark</td>
<td>35.1 ± 7.0</td>
<td>31.4 ± 5.8</td>
<td>35.0 ± 2.9</td>
</tr>
<tr>
<td>PS (%)</td>
<td>Light</td>
<td>18.5 ± 7.8</td>
<td>8.3 ± 5.3</td>
<td>13.1 ± 4.2</td>
</tr>
<tr>
<td></td>
<td>Dark</td>
<td>5.3 ± 1.4</td>
<td>4.2 ± 1.4</td>
<td>6.8 ± 1.0</td>
</tr>
<tr>
<td>WK (%)</td>
<td>Light</td>
<td>41.4 ± 8.3</td>
<td>33.4 ± 6.1</td>
<td>32.5 ± 4.0</td>
</tr>
<tr>
<td></td>
<td>Dark</td>
<td>59.4 ± 7.3</td>
<td>63.9 ± 4.8</td>
<td>58.1 ± 3.6</td>
</tr>
<tr>
<td>LM (ev)</td>
<td>Light</td>
<td>86.1 ± 12.2</td>
<td>0.6 ± 0.8</td>
<td>1.0 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>Dark</td>
<td>19.2 ± 9.2</td>
<td>2.3 ± 3.6</td>
<td>0.2 ± 0.3</td>
</tr>
</tbody>
</table>

TST: total sleep time; SE: sleep efficiency; ARS (ev/h): arousal (events/h); SWS: slow-wave sleep; PS: paradoxical sleep; WK: wakefulness; LM (ev): limb movement (events). Comparisons among the four groups of rats for the time factor (*), group factor (#), and interaction (+). Light period: comparison among groups (post-hoc test) for time factor (a differ b and c differ d) and the group factor (e differ f and g differ h). Dark period: comparison among groups (post-hoc test) for the time factor (a differ b and c differ d) and the group factor (e differ f). Repeated-measures ANOVA followed by the Duncan post-hoc test, \( p<0.05 \). A Kruskal–Wallis test (\( p<0.05 \)) was used for the LM variable. * Differs significantly from the A11 Lesion NPE group.
and PS ($F_{(1,20)}=4.533, p=0.01$) and in the time factor for TST ($F_{(1,20)}=7.807, p=0.01$), ARS ($F_{(1,20)}=12.592, p<0.01$) and SWS ($F_{(1,20)}=4.673, p=0.04$). All groups after four weeks of exercise showed a reduction in ARS in comparison with the A11 lesion-PE group at baseline. However, the A11 lesion-NPE group after exercise showed a decrease in SWS in comparison with all other groups.

For LMs, the CTRL-PE and SHAM-PE groups showed a statistically significant decrease from the A11 lesion NPE and PE groups at the baseline. The CTRL-PE, SHAM-PE and A11 lesion-PE groups presented a significant reduction in LMs from the A11 lesion-NPE group after 4 weeks of physical exercise (Kruskal–Wallis, $p<0.001$).

In the dark period (12 h), a repeated-measures ANOVA showed a statistically significant difference in the group and time factors and in interaction for ARS ($F_{(3,20)}=30.668, p<0.001$; $F_{(1,20)}=4.989, p<0.001$; $F_{(3,20)}=4.623, p=0.01$) and in the time factor for TST ($F_{(1,20)}=6.288, p=.02$). The main results showed that the group A11 lesion NPE after 4 weeks of physical exercise showed an increase in ARS in relation to all groups both at the baseline and after physical exercise.

For LM, the CTRL-PE and SHAM-PE groups showed a statistically significant reduction from the A11 lesion-NPE and PE groups at the baseline. After physical exercise, the CTRL-PE, SHAM-PE and A11 lesion-PE groups showed a statistically significant reduction in LMs from the A11 lesion-NPE group (Kruskal–Wallis, $p<0.001$).

A repeated-measures ANOVA showed a statistically significant difference in the group factor ($F_{(3,20)}=5.277, p<0.01$), time ($F_{(6,100)}=13.815, p<0.01$) factor and interaction ($F_{(15,100)}=2.836, p<0.01$) in the %WK during the dark period at baseline and following exercise that was divided into blocks (7–11 pm, 11 pm to 3 am, 3–7 am). The A11 lesion NPE and PE groups showed an increased %WK in the 3–7 am block in comparison with the CTRL-PE and SHAM-PE groups in the baseline values. After physical exercise, the CTRL-PE and A11 lesion-PE groups showed a significant reduction in comparison with the baseline values for the A11 lesion-NPE (baseline and after physical exercise) and PE (baseline) groups (baseline). The results are shown in Figs. 2 and 3.

3. Discussion

The present study investigated the influence of a non-pharmacological treatment of sleep-related movement disorder in an experimental animal model. The rats performed physical activity for four weeks. The protective effect of physical exercise in rats that received an A11 lesioned with 6-OHDA was also evaluated.

Our study demonstrated changes in sleep patterns for SE, SWS and SP in all groups. Four weeks of physical exercise substantially reduced the number of LMs in the A11 lesion group in rats that had performed physical exercise. Moreover, the physical exercise before the A11 lesion decreased the prevalence of LMs in rats in the A11 lesion-PE group at the PSG baseline.

A separate analysis by blocks of %wakefulness showed that there was an improvement in the last block (3–7 am) before and after the A11 lesion. Yet, other changes in sleep pattern related to the SWS (increase) and PS (decrease) were also demonstrated. Possible changes in sleep patterns as a result of exercise are discussed in a meta-analysis that performed by Youngstedt et al. (1997), who found that exercise increases the latency to REM sleep and/or decreases the time of this sleep stage by about six minutes and 11.6 min, respectively. These changes can be used as a stress index that is induced by exercise (Driver and Taylor, 2000).

Exercise can alter some neurotransmitters and neurotrophin expression (Meeusen and De Meirleir, 1995; Vučković et al., 2010). Additionally, changes in sleep patterns resulting
### Table 2 – Sleep pattern during light and dark periods (12 h) in the four groups of rats (A11 Lesion-NPE, CTRL-PE, SHAM-PE, A11 Lesion-PE). Baseline values and values after physical exercise in animals that performed physical exercise only after the A11 lesion.

<table>
<thead>
<tr>
<th>Sleep pattern</th>
<th>Period</th>
<th>Baseline</th>
<th>After exercise</th>
<th>ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TST (min)</strong></td>
<td>Light</td>
<td>415.7 ± 59.8</td>
<td>380.7 ± 87.5</td>
<td>322.8 ± 97.9*</td>
</tr>
<tr>
<td></td>
<td>Dark</td>
<td>275.2 ± 52.8a</td>
<td>265.8 ± 37.0a</td>
<td>260.7 ± 17.0a</td>
</tr>
<tr>
<td><strong>SE (%)</strong></td>
<td>Light</td>
<td>58.1 ± 8.1</td>
<td>61.5 ± 10.6</td>
<td>58.3 ± 5.2</td>
</tr>
<tr>
<td></td>
<td>Dark</td>
<td>38.3 ± 7.3</td>
<td>42.0 ± 11.0</td>
<td>35.3 ± 2.6</td>
</tr>
<tr>
<td><strong>ARS (ev/h)</strong></td>
<td>Light</td>
<td>37.8 ± 20.4</td>
<td>47.1621.7c</td>
<td>34.0 ± 7.1</td>
</tr>
<tr>
<td></td>
<td>Dark</td>
<td>43.1 ± 18.4**</td>
<td>33.6 ± 6.7a</td>
<td>23.6 ± 5.6a</td>
</tr>
<tr>
<td><strong>SWS (%)</strong></td>
<td>Light</td>
<td>39.9 ± 4.0**</td>
<td>49.8 ± 7.3ab</td>
<td>48.3 ± 4.9b</td>
</tr>
<tr>
<td></td>
<td>Dark</td>
<td>35.1 ± 7.0</td>
<td>36.7 ± 8.2</td>
<td>31.8 ± 1.7</td>
</tr>
<tr>
<td><strong>PS (%)</strong></td>
<td>Light</td>
<td>18.5 ± 7.8a</td>
<td>12.7 ± 2.9</td>
<td>11.6 ± 1.2f</td>
</tr>
<tr>
<td></td>
<td>Dark</td>
<td>5.3 ± 1.4</td>
<td>6.1 ± 2.6</td>
<td>5.2 ± 0.8</td>
</tr>
<tr>
<td><strong>WK (%)</strong></td>
<td>Light</td>
<td>41.4 ± 8.3</td>
<td>37.439.7</td>
<td>40.1 ± 4.4</td>
</tr>
<tr>
<td></td>
<td>Dark</td>
<td>59.4 ± 7.3</td>
<td>57.1 ± 10.8</td>
<td>62.9 ± 2.2</td>
</tr>
<tr>
<td><strong>LM (ev)</strong></td>
<td>Light</td>
<td>86.1 ± 12.2</td>
<td>1.6 ± 1.3**</td>
<td>1.33 ± 1.0**</td>
</tr>
<tr>
<td></td>
<td>Dark</td>
<td>19.2 ± 9.2</td>
<td>1.6 ± 1.8**</td>
<td>0.3 ± 0.5**</td>
</tr>
</tbody>
</table>

TST: total sleep time; SE: sleep efficiency; ARS (ev/h): arousal (events/h); SWS: slow-wave sleep; PS: paradoxical sleep; WK: wakefulness; LM (ev): limb movement (events). ANOVA columns: comparisons among groups for the time factor (*), group factor (#) and interaction (+). Light period: Comparison among groups (post-hoc test) for the time factor (a differ b and c differ d) and the group factor (e differ f). Dark period: Comparison among groups (post-hoc test) for the time factor (a differ band c differ d) and the group factor (e differ f and g differ h). Repeated-measures ANOVA followed by the Duncan post-hoc test, p < 0.05. Kruskal-Wallis test (p < 0.05) used for LM and PLM variables. * Differs significantly from A11 Lesion NPE group; # differs significantly from A11 Lesion group PE.
from physical exercise are most often linked to its relation-
ship with the release of neurotransmitters (Cotman and
Berchtold, 2002; Vučković et al., 2010; Esteves et al., 2009).
Esteves et al. (2009) showed a negative correlation between
the release of plasma levels of beta-endorphins and reduc-
tions in leg movements during sleep in volunteers following
sessions of acute physical exercise.

Moreover, physical exercise is beneficial for treating cogni-
tive deficits (Zagaar et al., 2013; Antunes et al., 2015), in
muscular or neuromuscular systems (Bazzucchi et al., 2015),
non-insulin-dependent diabetes mellitus (Pandey et al., 2015),
prevention of mild or moderate systemic arterial hypertension
(Lin et al., 2015), and in cancer patients (van Putten et al., 2015).

In human subjects, Aukerman et al. (2006) demonstrated
that a 12-week conditioning program of 3-days-per-week
aerobic and lower-body resistance training was effective in
improving the symptoms of RLS. Esteves et al. (2009) have
demonstrated that both acute intensive exercise (only one
session) and chronic exercise (72 sessions, 50 min) were
effective in reducing PLM and that volunteers showed a
negative correlation between the release of plasma levels of
ß-endorphins and reductions in leg movements during sleep.
Changes in the central nervous system (CSN) that were due to β-endorphin release in the system through physical exercise were enough to alter neurotransmission and receptors that are involved in its regulation (Smith and Lyle, 2006). This release of beta-endorphin, which is based upon the amount and intensity of exercise, affects the dopaminergic system (Schwarz and Kindermann, 1992).

Of further note is a study by De Mello et al. (2004). Those authors conducted a crossover trial in 13 patients who showed PLM subsequent to spinal cord injury. The participants received 200 mg L-DOPA and 50 mg benzerazine for 30 days or performed exercise on an ergometer three times a week for 45 days. Both treatments resulted in significant reductions in the PLM index, from 35.1 to 19.9 for the L-DOPA group and from 35.1 to 18.5 for the exercise group.

However, the results of our current analysis of the protective effect of physical exercise on LMs showed that LMs were lower at baseline for rats that performed physical exercise for two weeks prior to the A11 lesion. O’Dell et al. (2007) have demonstrated in Sprague-Dawley rats that exercise prior to 6-OHDA infusion caused a functionally meaningful down-regulation of striatal DAT. Thus, it might be expected that nigrostriatal terminals in exercised rats would less efficiently accumulate 6-OHDA. Kleim et al. (2003) have shown that motor enrichment may prime the brain to respond more adaptively to injury, in part by up-regulating trophic factors, such as GDNF, FGF-2, or BDNF. Discontinuation of exercise in advance brain injury may cause levels of trophic factor expression to plummet below baseline, which may leave the brain more vulnerable to degeneration.

Esteves et al. (2013) have previously demonstrated that the SHR strain is a promising model for PLM-RLS in association with Attention Deficit Hyperactivity Disorder (ADHD). Both pharmacological (pramipexole) and non-pharmacological (physical exercise) manipulations were performed to evaluate the validity of this animal model. Moreover, Lopes et al. (2012) have shown in rats with 6-OHDA-induced A11 lesions that the percentage of wakefulness increased during the last block of the dark period. Furthermore, these alterations were reversed by the acute administration of the dopaminergic agonist pramipexole.

However, a possible limitation of our study was that rats spent a week without physical exercise after the A11 lesion and recovery from the electrode implantation surgery. Furthermore, as a necessity for the adequacy of research in animal models (i.e., Refine, Reduce and Replace), the number of rats that used in the experiment was reduced. In addition, we chose to use the CTRL/PE group for our control group because the object of the study was the effect of physical exercise effect on A11 injury. In this context it is an important caution when discussing the results of this study, because we cannot affirm that results are due to interventions or they would appear by chance in regular naive animals. In a study with similar experimental protocol, Goes et al. (2014) demonstrated that a 4-week swimming training (ST) was effective in attenuating the impairments resulting from 6-OHDA exposure. The experiments were performed using male C57B/6J mice randomly assigned into four groups: (1) vehicle/sedentary; (2) 6-OHDA/sedentary; (3) vehicle/exercise and (4) 6-OHDA/exercise. In this experimental design, exercise groups were submitted to ST for 4 weeks with a progressive increase time and constant intensity. Sedentary groups were maintained in physical inactivity. Thus, the results demonstrated by Goes et al. (2014) in the vehicle/sedentary group could support a similar response in the speculations of our results. Nonetheless, they used different strains and physical exercise of our experiment.

In summary, the results of the current study verify the results of a previous study (Lopes et al., 2012) that demonstrated increases in locomotor activity in rats with 6-OHDA-induced A11 lesions only at the circadian time point corresponding to the period during which RLS symptoms occur and during which LMs are present during sleep. Furthermore, physical exercise, regardless of previous 6-OHDA-induced A11 lesions, has been shown to reduce LMs in rats. However, exercise prior to the A11 lesion represented a significant protective factor for LMs.

These findings are consistent in demonstrating that a physical exercise manipulation had a beneficial effect on LM symptoms. However, further research is important to identify changes in the central nervous system that serve to improve these physiological patterns.

4. Experimental procedure

This experiment used 48 male rats of the Wistar strain. The body weights of the rats ranged between 260 and 310 g. All animals were bred in the animal facility of CEDEME (Universidade Federal de São Paulo, Brazil). All experimental procedures were submitted and approved by the Ethics Committee of Federal University of São Paulo (1408/09). Rats were kept individually in transparent cages under controlled 12-h light–dark cycle conditions (lights on at 7:00 h) at a temperature of 23±2°C with unrestricted access to water and food.

To verify that exercise has a protective effect with respect to movement disorders related to sleep, two experiments were conducted. In the first experiment, the animals underwent four weeks of exercise prior to A11 lesion to evaluate the protective effect of physical exercise in relationship to damage that is associated with 6-OHDA-induced A11 lesions. In the second experiment, the animals performed the exercise only after the A11 lesion to evaluate physical activity as treatment.

4.1. Groups

The experiment was subdivided into two parts:

1) Physical exercise before and after A11 lesion – Groups:
   - CTRL/PE (without A11 lesion – with physical exercise, n=6).
   - SHAM/PE (A11 lesion, PBS – with physical exercise, n=6).
   - A11 Lesion/NPE (A11 lesion, 6-OHDA – not physical exercise, n=6).
   - A11 Lesion/PE (A11 lesion, 6-OHDA – with physical exercise, n=6).
The physical exercise program began two weeks prior to the A11 lesion and the electrode insertion surgery and continued for up to 4 weeks post operatively. Only the CTRL group was not subjected to the A11 nuclei lesion surgery. Seven days after the surgery (electrodes implantation and A11 lesion), all groups underwent a 24-h sleep-recording session (12 h light and 12 h dark). After the final physical training session, all groups underwent a second 24-h sleep-recording session. The A11 lesion/NPE did not perform any physical exercise during the experiment.

2) Physical exercise after A11 lesion – Groups:
- CTRL/PE (without A11 lesion – with physical exercise, \(n=6\)).
- SHAM/PE (A11 lesion, PBS – with physical exercise, \(n=6\)).
- A11 Lesion/NPE (A11 lesion, 6-OHDA – not physical exercise, \(n=6\)).
- A11 Lesion/PE (A11 lesion, 6-OHDA – with physical exercise, \(n=6\)).

All groups were subjected to electrode insertion surgery. Only the CTRL group was not subjected to A11 nuclei lesion surgery. Seven days after surgery (electrodes implantation and A11 lesion), all groups underwent a 24-h sleep-recording session (12 h light and 12 h dark). After baseline sleep recording, the rats in the exercise groups underwent four weeks of physical training. After the final physical training session, all groups underwent a second 24-h sleep-recording session. The A11 lesion/NPE group did not perform any physical exercise during the experiment.

The experimental procedure was initiated according to the schedule that is shown in Fig. 4.

4.2. Experimental protocols

4.2.1. Surgical procedures

4.2.1.1. Electrode insertion. After being exposed to anesthesia (diazepam – 10 mg/kg, i.p. and ketamine – 90 mg/kg, i.p.), rats were subjected to a trichotomy of the upper region of the head and the hind limbs and then placed in a stereotaxic apparatus (David KopfTM). Stereotaxic coordinates were then demarcated. Four stainless steel micro screws (\(\varnothing 1.0\) mm) were carefully attached, only touching the dura mater (\(1 \pm 1.0\) mm depth). Two long bipolar cortical ipsilateral derivations were performed to record cortical electrical activity (ECoG). One pair of screw electrodes was located laterally to the sagittal plane to perform an ECoG recording with minimum theta activity (3 mm anterior to bregma, 1 mm lateral to the central suture; and 4 mm anterior to lambda, 1 mm lateral to the central suture). The other was located more medially to the sagittal plane to record the ECoG with maximum theta activity (3 mm anterior to bregma, 1 mm lateral to the central suture; and 4 mm anterior to lambda, 1 mm lateral to the central suture) (Ronsemberg et al., 1976; Bergmann et al., 1989). Two electrodes were implanted in the dorsal muscle of the neck (trapezius) and one in each of the hind limbs for electromyographic (EMG) analysis.

After the electrodes had been implanted, a connector was fastened with self-polymerizing dental acrylic adhesive to the skull of the rat.

The rats were administered 0.5 ml/Kg i.m. of poly-antibiotic suspension with streptomycin and penicillin (WYETHr Pen-tabioc) to prevent infection.

The rats were also administered 10 mg/Kg, i.m. sodium diclofenac to assist in postoperative analgesia and to reduce surgical wound inflammation.

The rats were then taken to a photo-thermo-stimulation unit for approximately 12 h to avoid the hypothermic shock often found during post-anesthesia period. After this period, they were taken to their cages and provided free access to water and food for another 7 days before any experimental intervention.

4.2.1.2. A11 nuclei lesion. The A11 lesion was made at the same time of the electrodes implantation. They were injected with 2 \(\mu\)L of 0.2% 6-OHDA in 0.01% ascorbic acid in both the right and left A11: ML 0.3 mm, DV 8 mm, AP – 3 mm from the bregma (bilateral diencephalic A11 dopaminergic nuclei) (Paxinos and Watson, 2007). The SHAM rats were similarly injected with phosphate-buffered saline (PBS).

4.2.2. Data Acquisition – electrocorticography and electromyographic

Polysonomnography (PSG) was recorded using a Nihon-Koden QP 223 polygraph (digital signal acquisition) using the following five channels: two for the EcoG, one for EMG of the cervical musculature, and two for the EMG of both hind
limbs. Electrocorticographic signals were amplified and low-pass filtered at 0.1 s (1.6 Hz), and electromyographic activity was low-pass filtered at 0.03 s (5.3 Hz). To ensure reproducibility, a single researcher scored all recordings, which were analyzed manually in a blinded fashion by using the Polysmith Software program® (Neurotronics, Gainesville, FL). Upon the conclusion of the analysis, sleep parameters were quantified using the Polysmith Software program®. The analysis was based upon the predominant amplitude and frequency of the tracing (Timo-Iaria et al., 1970), and considered 10-s epochs, which were classified according to the dominant state (that is, arousal, slow-wave sleep, or paradoxical sleep).

The analysis of sleep records was divided into two periods of 12 h each (light/dark) (Timo-Iaria et al., 1970), and also into six consecutive 4-h blocks that together constituted a 24-h day (7–11 am, 11–3 pm, 3–7 pm, 7–11 pm, 11–3 am and 3–7 am). This subdivision was described by Dean et al. (2006) as simulating a particular circadian time point that corresponds to the period during which RLS symptoms would maximally disturb sleep onset and progression in humans.

The following sleep parameters were examined: total sleep time (TST), sleep efficiency (SE – percentage of total sleep time during recording period), arousal, wakefulness (W – percentage of all periods of wake throughout the recording period), slow-wave sleep (SWS – percentage of all periods featuring high delta content during the recording period), paradoxical sleep (PS – percentage of all paradoxical sleep periods during the recording period) and limb movements (LM – number of rats moving limbs during SWS sleep).

4.2.3. Analysis of limb movements
Limb movements were analyzed with EMG by placing electrodes in the two hind limbs to register their flexion and extension during SWS. Limb movements are associated with increased EMG amplitude in isolated occurrences (Esteves et al., 2007).

4.2.4. Physical training
Both groups underwent adaptation for three days on a treadmill (Columbus Instruments) for 10 min/day at a speed of 12 m/min. To check their performance, all rats were evaluated on a scale of 1–5. The scale was defined as follows: 1 = Refused to run, 2 = below-average runner (sporadic, stop running or run in the wrong direction), 3 = average runner, 4 = above-average runner (runner consistent, occasionally runs at the end of the mat), 5 = good runner (consistently runs in front of the mat) (Dishman et al., 1988). Rats with a rating of 3 or more were included in the aerobic exercise protocol. This procedure was used to exclude non-runner rats and avoid possible stress to them.

After the surgical implantation of electrodes and the realization of the baseline EcOG, the rats were subjected to an aerobic exercise program for 4 weeks, 5 days per week, totaling 20 sessions. The intensity of physical exercise was moderate, corresponding to a speed of 20–22 m/min, equivalent to approximately 60% of VO₂ max (Arda et al., 1999).

Each training session began with 5 min of heating at 12–15 m/min. The duration of each training session was 30 min at 20–22 m/min. The exercise intensity was the same for all rats.

4.2.5. Perfusion and immunohistochemistry
All groups were perfused with 2% paraformaldehyde at 30 days after lesioning. The brains were removed, stored in fixative overnight at 4°C, dehydrated in 30% sucrose for 36 h, and frozen at 80°C. The brains were cryoprotected in dry ice and sectioned on a cryostat at 20°C. Serial coronal sections (30 lm) of diencephalic regions containing the A11 nuclei were cut. Adjacent pairs of sections were collected at systematically placed 30-lm intervals throughout the whole region. To examine whether the substantia nigra (SN) and ventral tegmental area (VTA) were affected by injection of 6-OHDA, the mesencephalon was also sectioned (30-lm sections). Representative sections were collected at systematically placed 200-lm intervals throughout the entire mesencephalon region. Tyrosine hydroxylase (TH) immunohistochemistry was performed as previously described (Ondo et al., 2000). Fig. 5 shows the illustration of A11 neurons.

4.3. Statistical analysis
Data with homogeneity of variance, as assessed by Levene’s test, were analyzed using a one-way ANOVA (sleep patterns, light and dark, each 12 h) and a repeated-measures ANOVA (blocks, 7–11 pm, 11 pm to 3 am, 3–7 am). Multiple post-hoc comparisons were performed using the Duncan test. Non-normality variables were assessed by the Kruskal–Wallis test for the LM and PLM. The results were expressed as the
mean ± standard error of the mean (SEM). The level of significance was set at p < 0.05.

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