ABSTRACT. Clinical experience suggests that restless legs syndrome (RLS), periodic leg movement (PLM), and attention-deficit hyperactivity disorder (ADHD) may co-occur in both children and adults. The purpose of the present study was to provide an electrocorticography and electromyography evaluation of the spontaneously hypertensive rat (SHR) to investigate the potential of this rat strain as an animal model of RLS–PLM. Initial work focused on evaluating sleep patterns and limb movements during sleep in SHR, having normotensive Wistar rats (NWR) as control, followed by comparison of two treatments (pharmacological–dopaminergic agonist treatment and nonpharmacological–chronic physical exercise), known to be clinically beneficial for sleep-related movement disorders. The captured data strengthened the association between SHR and RLS–PLM, revealing a significant reduction on sleep efficiency and slow wave sleep and an increase on wakefulness as compared to the NWR group, effects that have characteristics as strikingly consistent with RLS–PLM. The pharmacological and nonpharmacological manipulations validated these results. The present findings suggest that the SHR may be a useful putative animal model to study sleep-related movement disorders mechanisms.

Keywords: attention-deficit hyperactivity disorder, periodic leg movement, rats, restless legs syndrome, sleep disorders

Clinical studies have suggested an intimate link between attention-deficit hyperactivity disorder (ADHD), restless legs syndrome (RLS), and periodic leg movement (PLM; Chervin et al., 2002; Cortese et al., 2005; Gaultney, Terrell, & Gingras, 2005). One possible hypothesis for this association is that the disorders are different manifestations of a common CNS pathology (Chervin et al., 2002).

Children with RLS and a subset of children with ADHD may share a common dopaminergic deficit. This concept is supported by neuroimaging studies (Wetter, Eisensehr, & Trenkwalder, 2004) that suggest the involvement of dopaminergic pathways in addition to pharmacological evidence for the therapeutic efficacy of dopaminergic agonists, which are recommended as the first-line pharmacological therapy for the symptomatic relief of RLS (Chesson et al., 1999; Hening, 2004). As a nonpharmacological treatment, exercise has also been the subject of clinical research, and both acute and chronic physical exercise have been shown to reduce RLS–PLM symptoms significantly (Aukerman et al., 2006; De Mello, Silva, Esteves, & Tufik, 2002; Esteves, de Mello, Pradella-Hallinan, & Tufik, 2009; Hening, 2004), due to its relationship with the dopaminergic and opioid system.

According to Picchietti, England, Walters, Willis, and Verrico (1998), leg discomfort in some RLS-afflicted children affects their ability to sit for extended periods of time during the school day. The need to walk to relieve this discomfort may lead to inattention in a subgroup of patients. However, large cross-sectional and longitudinal studies using rigorous standard diagnostic criteria for RLS, PLM, and ADHD are necessary to better understand the relationships among these disorders. Furthermore, animal models are important tools to verify hypotheses and decipher the details of pathophysiological mechanisms, including the connections among genes, biology, and disease.

Based on behavioral and neurobiological data, the spontaneously hypertensive rat (SHR) is currently the best validated (Sagvolden, Russell, Aase, Johansen, & Farshbaf, 2005) and the most widely studied (Arime, Kubo, & Sora, 2011) animal model of ADHD. Regarding the validity of this model, the SHR strain mimics the behavioral characteristics of ADHD described in children: impairment of sustained attention without obvious sensory deficiencies, motor impulsiveness, and hyperactivity (Adriani, Caprioli, Granstrem, Carli, & Laviola, 2003; Hunziker, Saldana, & Neuringer, 1996; Sagvolden, 2000; Sagvolden et al., 2005).

The purpose of the present study was to provide a comparative electrocorticography and electromyography evaluation between SHR and normotensive Wistar rats (NWR) to investigate the potential value of the SHR rat strain as an animal model of PLM–RLS. The effects of dopaminergic treatment and chronic physical exercise on the alterations of the electrocorticographic and electromyographic signals recorded during sleep presented by SHR were also investigated.

Method

Animals

A total of 27 three-month-old male SHRs and 15 three-month-old EPM-1 NWRs were used in the three experiments. All of the animals were provided from our own colony by the Department of Psychobiology at the Universidade Federal de São Paulo, Brazil. The SHR founders were purchased from Charles River Laboratories (Munich, Germany). The body weights of the rats were between 260 and 310 g. All of the experimental procedures were submitted to and approved by
the Ethics Committee of Universidade Federal de São Paulo (#1408/09), and the rats used in this study were maintained and treated in accordance with the Ethical and Practical Principles of the Use of Laboratory Animals guidelines (Andersen et al., 2004). The rats were kept individually in transparent cages, under controlled 12-hr light–dark cycle conditions (lights on at 7:00 a.m.) at a temperature of 23 ± 2°C, with unrestricted access to water and food.

**Design**

**Experiment 1: Evaluation of sleep patterns and leg movements in SHRs and NWRs.**

Electrodes were surgically implanted into seven SHR and five NWR rats. One week after surgery, the sockets were connected via flexible recording cables to a polygraph and computer. The rats were habituated to the apparatus with the wires attached over the course of two days and sleep recording data were collected during a 24-hr period including 12 hr of light and 12 hr of dark lighting conditions.

**Experiment 2: Pharmacological intervention (dopaminergic agonist).**

Electrodes were surgically inserted into 15 SHRs and five NWRs. One week after surgery, after the rats were habituated to the apparatus with the wires attached, the course of two days, the animals were distributed into four subgroups of five animals each: (a) NWR (without saline or drug), (b) SHR (CTRL; without saline or drug), (c) SHR (saline), and (d) SHR (drug). Data were collected from the sleep recordings for 24 hr (12 hr of light and 12 hr of dark). At 3 a.m. (Dean, Allen, O’Donnell, & Earley, 2006; Lopes, Esteves, Frussa-Filho, Tufik, & de Mello, 2012), data collection was briefly interrupted to dose the animals with the appropriate injection of saline or drug, after which collection of the data on the sleep and leg movement recordings of the animals continued. Sleep and leg movement recording for the noninjected animals was collected continuously. In these pharmacological experiments, a between-subjects protocol was preferred to a within-subjects one because it was necessary to evaluate the effect of the pharmacological agent at a specific time point to ensure that the only experimental variable was the pharmacological intervention. Importantly, a group of noninjected SHR animals was included to exclude possible effects of stress due to the injection procedure. In addition, a noninjected NWR group was also included to confirm the effects of strain on the experimental parameters evaluated.

**Experiment 3: Nonpharmacological intervention (chronic physical exercise).**

Electrodes were surgically inserted into five SHRs and five NWRs. As with the previously described experiments, after seven days of recovery, data for the sleep and movement parameters were acquired for 24 hr (12 hr of light and 12 hr of dark). After the baseline sleep recording, the animals underwent four weeks of physical exercise, after which a second set of data was collected.

**Experimental Protocols**

**Electrocorticography and Electromyogram Recording**

Two bipolar electrodes (California Fine Wire EUA, Grover Beach, CA; 0.008-mm diameter) were placed inside the skull through small holes drilled into the right lateral frontoparietal region and the left medial frontoparietal region in order to monitor the bipolar electrocorticogram (ECoG). For PLM analysis using electromyogram (EMG), electrodes (California Fine Wire EUA, 0.003-mm diameter) were implanted in the dorsal muscle of the neck and in each hind limb. The free ends of the electrodes were soldered to a custom-made socket, which was attached to the skull with acrylic dental cement. All rats received penicillin (20,000 U in 0.1 ml im) and sodium diclofenac (25 mg/ml ip) following surgery. One week after surgery, the head-socket was connected via flexible recording cables (Neurotec, Itajubá, MG, Brazil) and a commutator to a polygraph and a computer. The recordings were performed using a Nihon Kohden Co. (Tokyo, Japan) digital polygraph (Model QP-223A). Sleep recordings were monitored during the light and dark periods. The ECoG traces were visually and manually scored in 10-s periods.

Sleep stages were identified and scored according to Timofari et al. (1970). The sleep parameters calculated were total sleep time, in minutes, during the recording period (TST); periods of wakefulness as a percentage of the recording period (WK); arousals as the number of awakenings during the recording period (ARS); non-REM as the periods that feature high delta content as a percentage of the recording period (slow-wave sleep [SWS]); and the percentage of all paradoxical sleep periods during the recording period (PS). Wakefulness percentages were calculated for three blocks of 4 hr during the dark period, yielding the following bins: 7–11 p.m., 11 p.m. to 3 a.m., and 3–7 p.m. (Lopes et al., 2012).

**Analysis of Limb Movements and Periodic Limb Movements**

The record of muscle activity (limb movement [LM]) was analyzed using EMG recording by placing electrodes in the two hind limbs (tibialis anterior muscle) to measure limb flexion and extension during SWS. Limb movements are associated with increased EMG amplitude, periodically (leg movement occurring in a higher frequency in a certain period of time) or in isolated occurrences, with average duration of LM during 3–9 s (Esteves et al., 2007).

**Pharmacological Intervention**

Pramipexole is a dopamine agonist of the nonergoline class that is widely approved for the treatment of RLS and Parkinson’s disease and most likely acts on D2/D3
receptors to produce its pharmacological effect in RLS (Becker, Ondo, & Sharon, 1998; Montplaisir, Nicolas, & Gomez-Mancilla, 1999). For pharmacological intervention, pramipexole, (0.1 ml/100 g body weight, dissolved in saline vehicle; Luo et al., 2011) and saline (0.1 ml/100 g body weight) were injected into the peritoneal cavity 10 min before the beginning accomplished using of the last dark period of the light/dark cycle (3–7 a.m.; Dean et al., 2006; Lopes et al., 2012).

Physical Exercise

The physical exercise was a treadmill (Columbus Instruments, Columbus, OH). After the surgical implantation of the electrodes and the recording of the baseline eCoG and EMG, both groups (NWR and SHR) were adapted to running on a treadmill for three days (10 min/day), at a speed of 12 m/min with no inclination. To quantify the rats’ performance, all of the animals were evaluated on a 5-point Likert-type scale (Dishman, Armstrong, Delp, Graham, & Dunn, 1988) ranging from 1 (the rat refused to run) to 5 (good runner [consistent runs in front of the mat]). The animals with a rating of three or more were included in the protocol of aerobic exercise and were subjected to an aerobic exercise program for four weeks at five days per week, totaling 20 sessions. The intensity of the physical exercise was moderate (speed of 21 m/min), corresponding to approximately 60% of the VO2 max (Arida, Scorza, dos Santos, Peres, & Cavalheiro, 1988). Each training session began with 5 min of warm up at a speed of 12–15 m/min, and was followed by a training session with duration of 30 min (20–22 m/min). The exercise intensity was identical for all of the animals.

Statistical Analysis

The data were statistically analyzed and are presented using the mean and standard deviation. For Experiment 1, Student’s t test was used to compare the sleep parameters between the SHR and NWR subjects, and repeated measures analysis of variance (ANOVA) followed by the Tukey test was used to compare the two strains for the three dark time blocks (7–11 p.m., 11 p.m. to 3 a.m., and 3–7 a.m. blocks). The Mann-Whitney test was performed for nonnormal variables, as based to the Shapiro-Wilk test. One-way ANOVA was used for Experiment 2 and a three-way ANOVA (Time × Group × Block) was used for Experiment 3. Multiple post hoc comparisons were performed using Tukey tests. Data that did not represent a normal distribution were logarithmically transformed. The statistical significance was set at p < .05.

Results

Experiment 1: Evaluation of Sleep Patterns and Leg Movement in SHR and NWR Strains

Table 1 shows the sleep patterns presented by the SHRs during the light (12 hr) and dark (12 hr) periods. Student’s t test revealed a significantly lower sleep efficiency, t(10) = −2.62, p = .02, and SWS, t(10) = −4.09, p = .002, and a significantly greater wakefulness, t(10) = 2.90, p = .01, for the SHR group during the dark period, as compared to the NWR group. As for the limb movement parameter, the SHRs demonstrated a significantly greater frequency (highest number of times the muscle was active) when compared to the NWR group during the 12-hr light (Z = 2.51, p = .01) and 12-hr dark (Z = 2.35; p = .01) recording periods.

The repeated measures ANOVA revealed a significant interaction between the strain and three blocks of 4 hr during the dark period (7–11 p.m., 11 p.m. to 3 a.m., and 3–7 a.m.) for the percentage of wakefulness time parameter, F(2, 20) = 11.659, p < .001. Specifically, whereas the NWR group showed a progressive reduction in the percentage of wakefulness time before the light phase, the SHR group showed a significant increase in this parameter during the last dark block (Figure 1).

Experiment 2: Pharmacological Intervention (Dopaminergic Agonist)

The saline injection did not modify the percentage wakefulness or the number of limb movements. In contrast, the pramipexole injection significantly decreased the percentage wakefulness, F(3, 16) = 9.800, p < .001, and the limb

<table>
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<tr>
<th>TABLE 1. Sleep Pattern During Light (12 hr) and Dark Periods (12 hr) in the Normotensive Wistar Rat (NWR) and Spontaneously Hypertensive Rat (SHR) Groups</th>
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<td>Limb movement (events)</td>
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a p < .05 when compared to NWR (Student t test). b p < .05 when compared to NWR (Mann-Whitney test).
FIGURE 1. Percentage of wakefulness during blocks in the dark period (7–11 p.m., 11 p.m.–3 a.m., 3–7 a.m.) presented by SHR and NWR groups. Repeated measures analysis of variance followed by the Duncan test ($p < .05$). *$p < .05$ when compared to NWR. #$p < .05$ when compared to 7–11 p.m. and 11 p.m.–3 a.m. blocks.

FIGURE 2. Effects of injection procedure and 0.1 ml/100 g pramipexole on the percentage of wakefulness (A) and limb movements (B) of spontaneously hypertensive rats (SHRs) during the last block (3–7 a.m.) of the dark period. One-way analysis of variance followed by the Duncan test. *$p < .05$ when compared to NWR group. #$p < .05$ when compared to SHR (CTRL) and SHR (saline) groups. NWR = normotensive Wistar rats.
SHR: An Animal Model of RLS and PLM

FIGURE 3. Percentage of the wakefulness in the dark blocks (7–11 p.m., 11 p.m.–3 a.m., 3–7 a.m.) in the normotensive Wistar rat (NWR) and spontaneously hypertensive rat (SHR) groups before and after physical exercise. Repeated measures analysis of variance followed by the Duncan test. $^p < .05$ compared to the first block. $^#p < .05$ compared to SHR (3–7 a.m. block baseline). $^¥p < .05$ compared to NWR (3–7 a.m. block baseline). Results are expressed as $M \pm SD$.

Experiment 3: Nonpharmacological Intervention (Chronic Physical Exercise)

A three-way ANOVA (Time $\times$ Group $\times$ Block) for the percentage wakefulness revealed a significant effect of the time factor, $F(1, 16) = 6.571, p = .02$; the block factor, $F(1, 16) = 3.120, p = .05$; and the time by group by block interaction, $F(2, 32) = 9.688, p < .001$. Specifically, for the NWR group, the percentage wakefulness values during the third block (3–7 a.m.) were lower than those of the first block (7–11 p.m.) in both the baseline and after physical exercise. During the baseline recordings of the SHR group, the percentage wakefulness during the third block (3–7 a.m.) was higher than those of the first block (7–11 p.m.). However, after physical exercise, these values in the third block were lower than those of the first block. Thus, chronic physical exercise restored the normal (as observed in the NWRs) circadian cycle in the SHR group (Figure 3).

Regarding the total (light and dark) limb movements for the NWR and SHR groups before and after physical exercise, the repeated measures ANOVA revealed a significant effect of the time factor, $F(1, 8) = 37.008, p < .001$; the strain factor, $F(1, 8) = 33.059, p < .001$; and the interaction between the strain and time (baseline and after physical exercise), $F(1, 8) = 32.069, p = .001$. Importantly, the limb movements in the SHRs during the baseline period were at greater frequency (highest number of times the muscle was active) than those in the NWRs, but the limb movements in the SHRs were reduced after physical exercise (Figure 4).

Discussion

The reported association of ADHD with sleep disorders has stimulated a series of studies to examine the relationship between ADHD and PLM–RLS. Thus, analyzing the sleep patterns of the SHR strain is of critical importance to ascertain whether these animals have the appropriate characteristics to establish an animal model of PLM–RLS.

In the present study, the sleep pattern and LMs of the SHRs were compared to those of the NWRs. Our results suggest that the SHR strain may provide a potentially useful...
animal model for PLM/RLS. Pharmacological and nonpharmacological manipulations were performed to evaluate the validity of this animal model.

The sleep patterns were based on the simultaneous analyses of electrocorticographic and limb electromyographic signals. As a form of acute pharmacological manipulation, the animals were treated with pramipexole; as a nonpharmacological treatment, the animals were subjected to four weeks of physical exercise.

In a study performed by Kuo and Yang (2004), it was demonstrated that, in comparison to NWRs, SHRs may sleep less, with poorer sleep quality and a greater tendency to wake from quiet sleep (QS). Our results corroborated the findings of Kuo and Yang by demonstrating that the SHR group had a lower percentage of SE and SWS and an increase in the percentage of wakefulness during the dark period of the recordings.

The analysis of the percentage of wakefulness during the three blocks of the dark period (7–11 p.m., 11 p.m. to 3 a.m., and 3–7 a.m.) showed an increase in the percentage of wakefulness during the third block (3–7 a.m.) in the SHR group. Using an experimental model of dietary iron deficiency (that also implies in the pathogenesis of RLS–PLM) in mice, Dean et al. (2006) also demonstrated an increase in wakefulness during a particular circadian time point that corresponds to the period during which RLS symptoms would maximally disturb sleep onset and progression in humans. In a recent study by our group, the sleep-wake behavior and the presence of limb movements were evaluated in a rat model of RLS induced by lesions in the A11 dopaminergic nuclei produced with the neurotoxin 6-hydroxydopamine (6-OHDA). The results demonstrated that all of the A11-lesioned rats exhibited an increased percentage of wakefulness during the last block of the dark period, as would be expected for an animal model of this syndrome. In addition, these animals demonstrated increased frequencies of limb movements during the light and dark periods at all time points after inducing the lesions (Lopes et al., 2012).

Limb movements were detected during sleep in the SHR group during the light and dark periods. Spontaneous limb movements have been observed in narcoleptic dogs (Okura et al., 2001), aged rats (Baier, Winkelmann, Höhne, Lancel, & Trenkwald, 2002), and in animals with lesions in the A8 dopaminergic neurons (Lai & Siegel, 1997) and the spinal cord (Esteves, de Mello, Lancellotti, Natal, & Tufik, 2004). In addition, these phenomena are frequently associated with RLS (Ondo, Romanyshyn, Vuong, & Lai, 2004).

The association between RLS, PLM, and ADHD symptoms may have important consequences for the treatment of these conditions when they co-occur and when the standard treatments for ADHD symptoms are not effective (Cortese et al., 2005). There is evidence that a dopaminergic dysfunction (Wetter et al., 2004; Chesson et al., 1999) may contribute to all of these disorders. Dopaminergic agents are currently the first-line treatment for both RLS and PLM (Ondo et al., 2004) apart from physical exercise that act as a nonpharma-


Received January 11, 2013
Revised June 17, 2013
Accepted August 4, 2013