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Behavioral and genetic effects promoted by sleep deprivation in rats submitted to pilocarpine-induced *status epilepticus*

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ABSTRACT

The interaction between sleep deprivation and epilepsy has been well described in electrophysiological studies, but the mechanisms underlying this association remain unclear. The present study evaluated the effects of sleep deprivation on locomotor activity and genetic damage in the brains of rats treated with saline or pilocarpine-induced *status epilepticus* (SE). After 50 days of pilocarpine or saline treatment, both groups were assigned randomly to total sleep deprivation (TSD) for 6 h, paradoxical sleep deprivation (PSD) for 24 h, or be kept in their home cages. Locomotor activity was assessed with the open field test followed by resection of brain for quantification of genetic damage by the single cell gel electrophoresis (comet) assay. *Status epilepticus* induced significant hyperactivity in the open field test and caused genetic damage in the brain. Sleep deprivation procedures (TSD and PSD) did not affect locomotor activity in epileptic or healthy rats, but resulted in significant DNA damage in brain cells. Although PSD had this effect in both vehicle and epileptic groups, TSD caused DNA damage only in epileptic rats. In conclusion, our results revealed that, despite a lack of behavioral effects of sleep deprivation, TSD and PSD induced genetic damage in rats submitted to pilocarpine-induced SE.

1. Introduction

Epilepsy is one of the most common neurological disorders, affecting people of all ages [7]. In addition, epilepsy and seizures affect many physiological patterns in humans and animals, such as sleep architecture [5,15]. Sleep disruption results in differential expression of many genes, including genes related to the sleep-wake cycle [9], and promotes extensive DNA breakage in healthy rodents [3]. Of note, DNA fragmentation in the rhinal cortex and hippocampus of rats with epilepsy has been reported [12]. Beyond molecular alterations, sleep loss has been shown to modify behavioral parameters, such as locomotor activity [1]. Stewart and Leung [24] demonstrated an increase in ambulation after the induction of *status epilepticus* (SE) in rats. The authors suggested that seizures modulate spontaneous locomotor activity by disrupting the normal function of the suprachiasmatic nucleus. Thus, previous research has shown that both sleep deprivation and a preclinical model of epilepsy can induce DNA damage and hyperactivity in rodents [1,3].

Sleep deprivation is one of the most important triggers of seizures in epilepsy patients [5,15,26]; however, the pathophysiological mechanisms involved in this harmful association have been unexplored. In this sense, more information, beyond the findings of electrophysiological studies, could provide a starting point for future clinical trials of treatments for epilepsy [16].

Considering the prevalence of sleep deficits in patients and animals with epilepsy, and the influence of sleep deprivation on locomotor activity and DNA damage, the aim of present study was to examine the effects of sleep loss (total and selective) on motor behavior and neuronal genotoxicity in rats submitted to pilocarpine-induced SE.

2. Materials and methods

All procedures in the present study complied with the recommendations in Animal Models as Tools in Ethical Biomedical Research [4]. The study was approved by the Ethical Committee of Universidade Federal de São Paulo (CEP 1363/10). The

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experiments were performed in male adult Wistar-Hannover rats aged 60 days at the beginning of the study.

2.1. Pilocarpine-induced status epilepticus

Status epilepticus was induced with a single dose of pilocarpine (350 mg/kg, i.p.) 30 min after injection of methylscopolamine (0.1 mg/kg, s.c.) (PILO group). The vehicle group (SAL) was injected with saline (0.9% NaCl, i.p.). Only rats that displayed convulsive and intermittent seizures after pilocarpine injection were included in this study. Seizure activity was monitored behaviorally and terminated after 3 h of convulsive SE with an i.p. injection of diazepam (10 mg/kg). Fifty days after the SE episode, pilocarpine-treated rats were assigned randomly to total sleep deprivation (TSD + PILO), paradoxical sleep deprivation (PSD + PILO) or control (kept in their home cages; CTRL + PILO) groups. Rats in the SAL group were distributed into groups submitted to the same conditions (TSD + SAL, PSD + SAL, and CTRL-SAL). The numbers of animals used were $n = 11–14$ for the open field test and $n = 6$ for the single cell gel electrophoresis assay.

2.2. Total and paradoxical sleep deprivation procedures

After 50 days of SE, TSD was induced by a gentle handling protocol in TSD + PILO and TSD + SAL groups. This procedure was performed for 6 h (07:00–13:00), and consisted of either touching the animals with a brush or gently moving their cages whenever they closed their eyes. The animals were not disturbed during feeding and drinking.

In parallel, PSD was induced for 24 h using the modified multiple platform method. Briefly, the PSD procedure consisted of placing rats in a tiled water tank (106 cm × 40 cm × 30 cm) containing 14 circular platforms, each of 6.5 cm in diameter. The rats could thus move around inside the tank by jumping from one platform to another. When they reached the paradoxical phase of sleep, muscle atonia set in, and they fell into the water and awoke [2,3]. Based on pilot experiments, 24 h was established as a safe period of PSD in rats with epilepsy. The cage control group was maintained in the same room as the PSD group.

2.3. Open field test

Immediately after the sleep deprivation protocols (13:00–15:00), the open field test was applied to assess general motor behavior. Rats were placed in a circular arena (96 cm diameter), the floor of which was divided into 19 squares. Frequency of peripheral locomotion (number of entrances into the floor units close to the walls of the box), central locomotion (number of entrances into the floor units not close to the walls), total locomotion (number of floor units entered), and rearing (number of times the animals stood on their hind legs) were quantified by counting the number of inter-square lines crossed. Immobility (total time of lack of movement) was also measured.

2.4. Single cell gel electrophoresis (comet) assay

After behavioral analysis, the animals were euthanized by decapitation, a rapid and painless procedure carried out less than 1 min in adjacent room. Brains were rapidly removed, and the cerebral cortex was dissected and minced in 0.9% NaCl. The supernatant was removed and cellular suspensions were used in single cell gel electrophoresis (comet) assays conducted according Sasaki and colleagues [20] with some modifications. Cell suspensions (10 μL) were added to 120 μL of 0.5% low-melting-point agarose at 37 °C, layered onto a slide pre-coated with 1.5% regular agarose, and covered with a coverslip. After rapid agarose solidification in a refrigerator, the coverslips were removed and the slides were immersed in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris–HCl buffer, pH 10, 1% sodium sarcosinate with 1% Triton X-100 and 10% DMSO) for approximately 1 h. The slides were placed in alkaline buffer (pH > 13) for 20 min and then electrophoresed for 20 min at 0.7 V/cm, 300 mA. After electrophoresis, the slides were neutralized in 0.4 M Tris–HCl (pH 7.5), fixed in absolute ethanol and stored until analysis.

2.5. Genotoxicity data analysis

Following the comet assay procedure, a total of 50 randomly captured comets per animal (25 cells on each of two slides) [10] were examined blindly by an expert observer at 400× magnification under a fluorescence microscope (Olympus). The microscope was connected to an image analysis system (Comet Assay II, Perceptive Instruments, Haverhill, Suffolk, UK) calibrated previously according to the manufacturer's instructions. Undamaged cells have an intact nucleus without a tail, whereas damaged cells have the appearance of a comet. In the present study, DNA damage was analyzed based on the tail moment parameter (the product of the tail length and the fraction of DNA in the comet tail) [10].

2.6. Statistical methods

All variables were first tested for normality and none showed a normal distribution ($p > 0.05$ in the Shapiro-Wilk test). Thus, the data were converted into z-score. Once there were 2 factors in the current study: Sleep (CTRL, TSD and PSD) and Treatment (SAL and PILO), the variables were further analyzed with 2-way ANOVA followed by Tukey post hoc test when necessary. The level of significance was set at 5%. Data are reported as means ± SEM.

3. Results

3.1. Open field test

The PILO group showed significant increase in peripheral ($F_{1,54} = 17.12$; $P < 0.001$), central ($F_{1,54} = 10.64$; $P < 0.01$) and total locomotion ($F_{1,54} = 22.09$; $P < 0.001$), and a reduction in immobility ($F_{1,54} = 24.75$; $P < 0.0001$) compared with the SAL group (Table 1). TSD and PSD did not affect locomotion or immobility in the

Table 1
Behavioral data from open field test.

	Peripheral locomotion	Central locomotion	Total locomotion	Immobility	Rearing
CTRL + SAL	21.2 ± 2.3	5.0 ± 1.5	26.2 ± 3.2	20.8 ± 3.0	12.7 ± 1.5
CTRL + PILO	89.7 ± 17.5*	15.3 ± 3.5*	105.1 ± 17.0*	2.1 ± 1.4*	7.9 ± 2.7
TSD + SAL	22.0 ± 5.6	6.1 ± 1.7	28.1 ± 6.3	17.7 ± 3.3	12.4 ± 3.0
TSD + PILO	91.4 ± 18.8*	16.8 ± 3.7*	108.2 ± 19.5*	0.2 ± 0.1*	14.0 ± 3.1
PSD + SAL	23.5 ± 5.4	7.3 ± 3.1	30.8 ± 7.3	16.0 ± 2.5	11.7 ± 2.5
PSD + PILO	92.8 ± 24.7*	19.2 ± 4.8*	112.1 ± 25.1*	6.4 ± 5.4	7.0 ± 1.5

Data are expressed as mean ± SEM.
* $p < 0.01$ compared to respective SAL group.

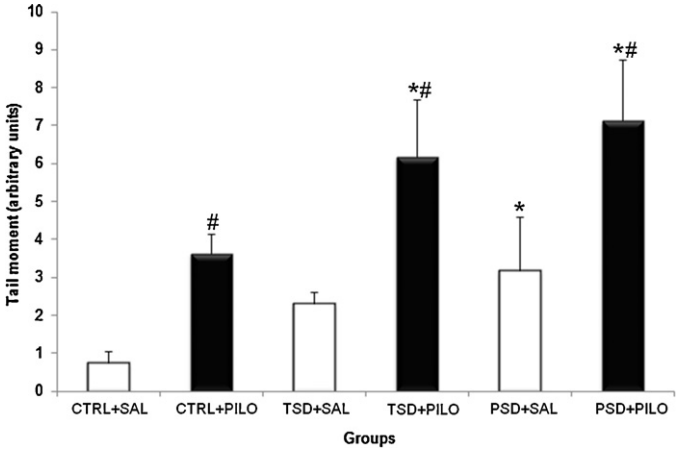

Fig. 1. DNA damage expressed as tail moment in brain cells. CTRL + SAL: SAL rats kept in their home cages; CTRL + PILO: PILO rats kept in their home cages; TSD + SAL: SAL rats submitted to TSD; TSD + PILO: PILO rats submitted to TSD; PSD + SAL: SAL rats submitted to PSD; PSD + PILO: PILO rats submitted to PSD. $^{*}P < 0.05$ when compared to respective control group. $^{\#}P < 0.05$ when compared with vehicle group. CTRL: home cage controls; PILO: rats submitted to pilocarpine-induced status epilepticus, PSD: paradoxical sleep deprivation, SAL: vehicle group, TSD: total sleep deprivation.

normal and epileptic groups ($F_{1,54} < 0.85$; $P > 0.43$). Furthermore, rearing behavior did not differ significantly between the groups ($F_{1,53} = 1.47$; $P = 0.23$).

3.2. Comet assay

The groups injected with pilocarpine demonstrated extensive genotoxic when compared to the SAL animals in all procedures ($F_{1,23} = 47.70$; $P < 0.001$) (Fig. 1). Sleep deprivation induced significant increase in the number of damaged cells in both SAL and PILO rats ($F_{1,23} = 11.51$; $P < 0.001$). The post hoc tests revealed that PSD, but not TSD, induced significant DNA damage in the SAL group ($P = 0.03$ vs. $P = 0.22$, respectively), whereas both TSD ($P = 0.02$) and PSD ($P = 0.003$) induced significant neuronal genotoxicity in PILO rats.

4. Discussion

In the present study, we showed that acute sleep deprivation causes DNA damage in the brains of rats submitted to pilocarpine-induced SE. Although pilocarpine rats exhibited hyperactivity, this appeared not to be influenced by acute sleep deprivation.

The epilepsy-induced hyperactivity reported in the current study is consistent with earlier findings showing an augmentation of locomotor activity during the light period in rats with epilepsy [24]. In the present study, ambulation was measured between 1 PM and 3 PM. The hyperactivity observed in our study could be partially due to an abnormal sleep-wake cycle in rats with epilepsy, once these animals presented an increase of active wakefulness during the afternoon [15]. In fact, rats with epilepsy show altered sleep architecture and an abnormal distribution of sleep-wake phases compared with healthy rats [15]. In addition to epilepsy-induced sleep-wake alterations, lesions in many brain areas, including the piriform cortex, hippocampal formation and thalamus, could potentially explain the impairments in locomotor activity and other behaviors [25]. Furthermore, our data demonstrated that the pathological mechanisms involved in locomotor hyperactivity in rats submitted to pilocarpine-induced SE were not affected by acute sleep deprivation. In fact, TSD did not induce hyperactivity in mice and longer periods of PSD were required to produce behavioral alterations [1,19]. It is important to mention that, even with the absence of effect of sleep deprivation; locomotor activity is a final parameter resulting from a complex process and may not be sufficiently sensitive for this approach.

Our results revealed that epilepsy induces genetic damage in brain cells, and that sleep loss potentiates this damage. These findings demonstrate that genetic changes in the healthy and epileptic brains can begin even after just a few hours without sleep. Pilocarpine-induced SE has been shown to cause neuronal death in the brain [8,22,23] and internucleosomal DNA fragmentation has been observed in necrotic cells at 24 and 72 h following SE [8]. The results of the present study show that epilepsy also increases vulnerability to DNA damage. In addition, our observation of genotoxic effects of sleep deprivation is consistent with the results of previous studies reporting DNA damage in the brains of healthy rats after 24 h of PSD [3]. Moreover, nitric oxide-mediated regulation of sleep phases [21] and oxidative damage in mice deprived of sleep [13] may potentially be related to sleep deprivation-induced DNA damage [3].

Epilepsy clearly increased the amount of genotoxic damage after TSD and PSD. However, only PSD induced a detrimental effect in healthy rats. TSD and epilepsy combined produced detrimental effects, possibly as a result of the abnormal sleep-wake cycle in animals with epilepsy [15]. For instance, rats with spontaneous recurrent seizures show increased NREM sleep during the morning [15], the period during which rats were submitted to TSD. As a consequence, rats in the PILO group were exposed to a higher pressure for sleep compared with the vehicle group. In fact, during the period of TSD, epileptic rats showed no exploratory activity and the vast majority of animals with epilepsy showed extreme drowsiness throughout the entire experimental period (whereas healthy animals were more alert). However, it is possible that other variable, such as the high levels of corticosterone present in rats with epilepsy [26] and/or the stress induced by gentle handling [27] could corroborated with aforementioned result.

Few studies have investigated the differences in the molecular effects of total versus selective sleep loss. Lee et al. [14] reported that neither TSD nor PSD affected the stability of reference genes in brain and blood. In contrast, behavioral tests, such as tests of plus-maze discriminative avoidance or the passive avoidance task, revealed differences between the effects of PSD and TSD on state-dependent learning in mice [17].

In conclusion, we show that epilepsy induces marked locomotor hyperactivity and DNA damage in rats. However, the detrimental effect of sleep loss on brain cells was not accompanied by behavioral changes. In view of the fact that DNA damage is an important step in the events leading to genomic instability, our results emphasize the potential health risks associated with acute sleep deprivation in patients with epilepsy.

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