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Neuropharmacology 46 (2004) 895–903
NEURO PHARMACOLOGY
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Role of hippocampal oxidative stress in memory deficits induced by sleep deprivation in mice

R.H. Silva [a,*], V.C. Abílio [a], A.L. Takatsu [a], S.R. Kameda [a], C. Grassl [a], A.B. Chehin [a], W.A. Medrano [a], M.B. Calzavara [a], S. Registro [a], M.L. Andersen [b], R.B. Machado [b], R.C. Carvalho [a], R. de A. Ribeiro [a], S. Tufik [b], R. Frussa-Filho [a]

[a] *Department of Pharmacology, Universidade Federal de São Paulo (UNIFESP), Rua Botucatu, 862 Ed. Leal Prado, CEP 04023-062 São Paulo, SP, Brazil*
[b] *Department of Psychobiology, Universidade Federal de São Paulo (UNIFESP), Rua Botucatu, 862 Ed. Leal Prado, CEP 04023-062 São Paulo, SP, Brazil*

Received 28 August 2003; received in revised form 14 November 2003; accepted 25 November 2003

Abstract

Numerous animal and clinical studies have described memory deficits following sleep deprivation. There is also evidence that the absence of sleep increases brain oxidative stress. The present study investigates the role of hippocampal oxidative stress in memory deficits induced by sleep deprivation in mice. Mice were sleep deprived for 72 h by the multiple platform method—groups of 4–6 animals were placed in water tanks, containing 12 platforms (3 cm in diameter) surrounded by water up to 1 cm beneath the surface. Mice kept in their home cage or placed onto larger platforms were used as control groups. The results showed that hippocampal oxidized/reduced glutathione ratio as well as lipid peroxidation of sleep-deprived mice was significantly increased compared to control groups. The same procedure of sleep deprivation led to a passive avoidance retention deficit. Both passive avoidance retention deficit and increased hippocampal lipid peroxidation were prevented by repeated treatment (15 consecutive days, i.p.) with the antioxidant agents melatonin (5 mg/kg), *N*-tert-butyl-α-phenylnitrone (200 mg/kg) or vitamin E (40 mg/kg). The results indicate an important role of hippocampal oxidative stress in passive avoidance memory deficits induced by sleep deprivation in mice.

Keywords: Sleep deprivation; Memory; Oxidative stress; Mice

1. Introduction

There is considerable evidence that sleep plays an important role in memory processes (for review, see Maquet, 2001). Clinical data have shown that the deprivation of sleep causes deficits in several forms of learning/memory (Tilley and Empson, 1978; Cochran et al., 1994; Karni et al., 1994; Fluck et al., 1998; Harrison and Horne, 2000; Mednick et al., 2002). In addition, numerous studies have demonstrated that sleep deprivation in laboratory animals produces memory deficits in several behavioral models, such as avoidance tasks (Harris et al., 1982; Smith and Kelly, 1998; Bueno et al., 1994; Guart-Masso et al., 1995), Morris water maze task (Smith and Rose, 1996; Youngblood et al., 1997, 1999) and radial maze task (Smith et al., 1998).

The mechanisms responsible for the occurrence of memory deficits following sleep deprivation are not clearly understood. In this respect, one of the theories to explain the changes in the cerebral function that follow sleep deprivation proposes that normal sleep would revert oxidative stress by removing the reactive oxygen species that were produced during the wake period. In short, sleep deprivation would reduce the

* Corresponding author. Department of Pharmacology, Universidade Federal de São Paulo (UNIFESP), Rua Botucatu, 862 Ed. Leal Prado, CEP 04023-062 São Paulo, SP, Brazil. Tel.: +55-11-55494122; fax: +55-11-55792752.
E-mail address: regina.farm@epm.br (R.H. Silva).

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doi:10.1016/j.neuropharm.2003.11.032

antioxidant defenses (Reimund, 1994). Indeed, increases in hypothalamic and thalamic oxidative stress levels were found in sleep-deprived rats (D'Almeida et al., 1998, 2000). Furthermore, Maquet et al. (2002) suggested that the proposed restorative function of sleep might involve the elimination of toxic compounds (e.g. free radicals) and the replenishment of energy stores.

Increased brain oxidative stress seems to have an important role in cognitive impairment caused by normal aging and neurodegenerative diseases. Administration of antioxidant agents has been shown to improve such deficits (Carney et al., 1991; Carrillo et al., 1993; Knoll et al., 1994; Markesbery, 1997; Small, 1998; Kontush, 2001). However, the participation of brain oxidative stress in sleep-induced memory deficiency has not yet been investigated. Thus, the aim of the present study is to investigate this possibility, by verifying: (1) whether the same protocol of sleep deprivation would produce increased oxidative stress in hippocampus and memory deficits assessed in a passive avoidance task (demonstrated to be related to the hippocampal function; Kim and Fanselow, 1992; Izquierdo and Medina, 1993) and (2) the effects of the administration of different antioxidant agents on memory deficits and hippocampal oxidative stress induced by sleep deprivation.

2. Materials and methods

2.1. Subjects

Three-month-old Swiss EPM-M1 male mice (weighing 30–35 g) were housed under conditions of controlled temperature (22–23 °C) and lighting (12 h light, 12 h dark; lights on at 7 am). Food and water were available ad libitum throughout the experiments. Animals used in this study were maintained in accordance with the guidelines of the Committee on Care and Use of Laboratory Animal Resources, National Research Council, USA.

2.2. Drugs

Melatonin (MEL; Sigma-Aldrich, St. Louis, MO) and vitamin E (α-tocopherol; Sigma-Aldrich, St. Louis, MO) were suspended in a 3% Tween 80 vehicle and *N*-tert-butyl-α-phenylnitrone (PBN; Sigma-Aldrich, St. Louis, MO) was dissolved in a 3% propylene glycol vehicle. The vehicles were given to control groups. All substances were given i.p. at a volume of 10 ml/kg of body weight. The doses were determined considering previous studies showing effective actions of these antioxidant agents on behavioral models (Fredriksson et al., 1997; Milivojevic et al., 2001; Abílio et al., 2002, 2003; Reis et al., 2002).

2.3. Sleep deprivation procedure

The method of sleep deprivation used was an adaptation of the multiple platform method, originally developed for rats (Nunes and Tufik, 1994). Groups of 4–6 animals were placed in water tanks (41 × 34× 16.5 cm), containing 12 platforms (3 cm in diameter) each, surrounded by water up to 1 cm beneath the surface, for 72 h. In this method, the animals are capable of moving inside the tank, jumping from one platform to the other. Stress-control animals were submitted to the same procedure, except the platforms were 10 cm in diameter, and control animals were maintained in their home cages in the same room. Food and water were made available through a grid placed on top of the water tank.

2.4. Sleep parameters recording

Before carrying out the experimental protocols, the duration of paradoxical sleep and slow-wave sleep was evaluated for efficiency of the adopted sleep deprivation method (Huber et al., 2000). The states of wakefulness, paradoxical sleep and slow-wave sleep were identified and scored by a combination of electrocorticography, electromyogram and standard criteria (Timo-Iaria et al., 1970) using Polysmith Neurotronics software (Gainesville, FL, USA). The duration of paradoxical sleep and slow-wave sleep was obtained in minutes thorough the experimental 24 h periods.

Nine animals were anesthetized with sodium thiopental anesthesia (60 mg/kg i.p) and sterile surgical and stereotaxic procedures were employed for implant placement. The cranium was exposed and two burr holes were drilled (1.5 mm lateral to midline and 2.0 mm anterior to bregma) and over the right parietal cortex (2.0 mm lateral to midline and 2.5 mm posterior to bregma) according to the atlas of Franklin and Paxinos (1997). The electrocorticography electrodes (two stainless-steel screws) were inserted into these holes by just touching the dura, while minimizing surgical trauma. Nickel–chromium fine wire electrodes for electromyogram recording were implanted into the dorsal neck muscles. The electrodes were soldered to a socket containing four pins and covered with dental acrylic. After a 7-day surgery recovery period, the recording sessions were carried out over 4 days: baseline (in home cage) and the 3 days of SD (in water tanks). Electrocorticography and electromyogram signals were amplified using a Nihon Koden Co. (Tokyo, Japan) model QP 223-A (acquisition of digital signal) and filtered at 0.3–100 Hz (electrocorticography) and 30–300 Hz (electromyogram). The sampling frequency was set at 200 Hz and the polysomnographic recording was archived to DVD-R discs for off-line sleep staging and analysis. The electrocorticography/electromyogram

traces were visually examined and scored in periods of 30 s.

2.5. Passive avoidance task

In passive avoidance experiments, an adaptation of the method previously described for rats (Silva et al., 1996, 1999) was used. The apparatus employed was a two-way shuttle-box provided with a guillotine door placed between the modular testing chambers. One chamber is illuminated by a 40 W bulb, while the other remained in the dark. In the training session, the animals were individually placed in the illuminated chamber, facing away from the guillotine door. When the animal entered the darkened chamber, the door was noiselessly lowered and a 0.5 mA foot shock was applied for 2 s through the grid floor. In the test sessions, the animals were again placed in the illuminated chamber, but no foot shock was applied. Latency to step through was recorded in each session.

The passive avoidance task has been used by several research groups to study learning and memory in rodents. In this paradigm, decreased latency to step through in the test session is usually presented by animals with several kinds of cognitive deficits, such as those induced by pharmacological manipulations (Silva et al., 1999; Santucci and Shaw, 2003; Isomae et al., 2003), consequent to neural lesions (Isomae et al., 2003), related to aging (Silva et al., 1996; Fiore et al., 2002; Yasui et al., 2002) or due to sleep deprivation (Moreira et al., 2003).

2.6. Hippocampus dissection

The animals were killed by decapitation and the brain was removed immediately and washed with ice-cold saline. After that, the hippocampus was quickly dissected and weighed. The whole procedure takes no longer than 3 min and brain tissue was always maintained on ice. Hippocampi were stored at a temperature of −80 °C for biochemical analysis.

2.7. Determination of glutathione levels

Glutathione is a tripeptide that plays an important role in protecting cells against damage produced by free radicals. During this process, oxidation of glutathione occurs. Thus, glutathione exists both in reduced and in oxidized forms (Shaw, 1998). In this way, reductions in glutathione levels as well as increases in oxidized/reduced glutathione ratio have been proposed as a sensitive index of oxidative stress (Toborek and Henning, 1994; Bains and Shaw, 1997; D'Almeida et al., 2000; Afzal et al., 2002).

Hippocampi were homogenized in 0.5 M perchloric acid (1:50, w:v). The homogenate was centrifuged at 2130*g* for 10 min, at a temperature of 4–5 °C. An aliquot of the supernatant was neutralized with 1.75 M K_3PO_4 and centrifuged at 11,800*g* for 3 min. After this centrifugation, the obtained supernatant was used for both oxidized (GSSG) and total (reduced + oxidized, GSH) glutathione determinations. For GSH, an aliquot of the supernatant was added to the standard glutathione assay mixture containing 0.1 M phosphate/0.05 EDTA buffer, pH = 7.0, DTNB and glutathione reductase. For GSSG determination, another aliquot of the supernatant was added to a standard mixture containing 0.5 M phosphate buffer (pH = 6.8), NADPH and glutathione reductase. The rate of reaction was expressed as the change in absorbancy at 412 nm, 25 °C, per 3 min, for GSH, and at 340 nm, 30 °C, per 16 min, for GSSG (Tietze, 1969). For each pool, GSSG/GSH ratio was calculated.

2.8. Measurement of hippocampal lipid peroxidation

One of the consequences of an oxidative stress process is an increase in lipid peroxidation. In this respect, lipid peroxidation is caused by an attack of free radicals upon cell membrane lipids (Halliwel and Gutteridge, 1999). Measurement of malondialdehyde (MDA), the most abundant product arising from lipid peroxidation (Kagan, 1988), has been extensively used an index of oxidative stress (Halliwel and Gutteridge, 1999; Komatsu and Hiramatsu, 2000; Gluck et al., 2001; Abílio et al., 2002).

Hippocampi were homogenized in ice-cold 0.1 M phosphate buffer (1:50, w:v). A duplicate of each sample was used to determine MDA by measurement of fluorescent product formed from the reaction of this aldehyde with thiobarbituric acid, as described by Tanizawa et al. (1981). The results are expressed as μmol MDA/g tissue, calculated by plotting the obtained fluorescence (excitation at 315 nm, emission at 553 nm) against an MDA concentration standard curve.

2.9. Statistical analysis

All data were compared by Kruskal–Wallis analysis of variance followed by Mann–Whitney *U*-test.

2.10. Experimental design

2.10.1. Experiment I: Effects of sleep deprivation or stress-control procedure on passive avoidance performance

Groups of 9–12 mice were submitted to sleep deprivation, stress-control procedure or kept in their home cage for 72 h. Immediately after this period, the animals were submitted to the passive avoidance training session. The test session was performed 72 h later.

Between the behavioral sessions, all the animals were kept in their home cages.

2.10.2. Experiments II and III: Effects of sleep deprivation or stress-control procedure on hippocampal total glutathione, GSSG/GSH ratio and lipid peroxidation levels

Groups of 6–7 (experiment II) or 9–10 (experiment III) mice were submitted to sleep deprivation, stress-control or cage-control procedures for 72 h. Immediately after this period, hippocampi were dissected for glutathione (experiment II) or lipid peroxidation (experiment III) assays.

2.10.3. Experiment IV: Effects of long-term administration of antioxidant agents on passive avoidance deficits induced by sleep deprivation

Groups of 12–16 mice were treated with tween vehicle, propylene glycol vehicle, 40 mg/kg vitamin E, 5 mg/kg MEL or 200 mg/kg PBN, once a day, for 15 consecutive days. From day 13 to 16, half of the animals in each group were submitted to sleep deprivation or kept in their home cages (control) for 72 h. Thus, mice were both drug-treated and sleep deprived on days 13, 14 and 15. Immediately after this period (day 16–24 h after the last injection), the animals were submitted to the passive avoidance training session. The test session was performed on day 19. No drug treatment was performed from days 16 to 19. Between the behavioral sessions, all the animals were kept in their home cages.

2.10.4. Experiment V: Effects of long-term administration of antioxidant agents on the increase in hippocampal lipid peroxidation induced by sleep deprivation

Groups of 8–11 mice were treated with tween vehicle, propylene glycol vehicle, 40 mg/kg vitamin E, 5 mg/kg MEL or 200 mg/kg PBN, once a day, for 15 consecutive days. From day 13 to 16, half of the animals treated with each vehicle solution and all animals treated with the antioxidant agents were submitted to sleep deprivation while the remaining vehicle-treated animals were kept in their home cages (control) for 72 h. Immediately after this period (24 h after the last injection), hippocampi were dissected for lipid peroxidation assay.

3. Results

3.1. Sleep parameters recording

The results showed that duration of slow-wave sleep ($H = 23.46$; $p < 0.0001$) and paradoxical sleep ($H = 20.13$; $p < 0.0005$) in all deprivation days was significantly shorter than baseline values (Table 1). Therefore, the protocol of sleep deprivation employed markedly decreased both slow-wave sleep and paradoxical sleep under our laboratory conditions.

Table 1
Sleep parameters in mice submitted to 72 h of sleep deprivation

	Baseline	Day 1	Day 2	Day 3
SWS	558.5 ± 29.8	125.5 ± 9.5*	83.9 ± 9.9*	101.5 ± 10.8*
PS	55.0 ± 4.6	2.1 ± 0.6*	2.9 ± 0.7*	2.9 ± 1.0*

Values are mean ± SE of time (min) spent in slow-wave sleep (SWS) and paradoxical sleep (PS) during baseline and deprivation periods of 24 h (days 1, 2 and 3).

* $p < 0.001$ compared to baseline values (Kruskal–Wallis analysis of variance and Mann–Whitney *U*-test).

3.2. Experiment I: Effects of sleep deprivation or stress-control procedure on passive avoidance performance

No differences were found in latency to enter the dark chamber in any of the groups in the training session (Fig. 1A). In the test session, the sleep-deprived

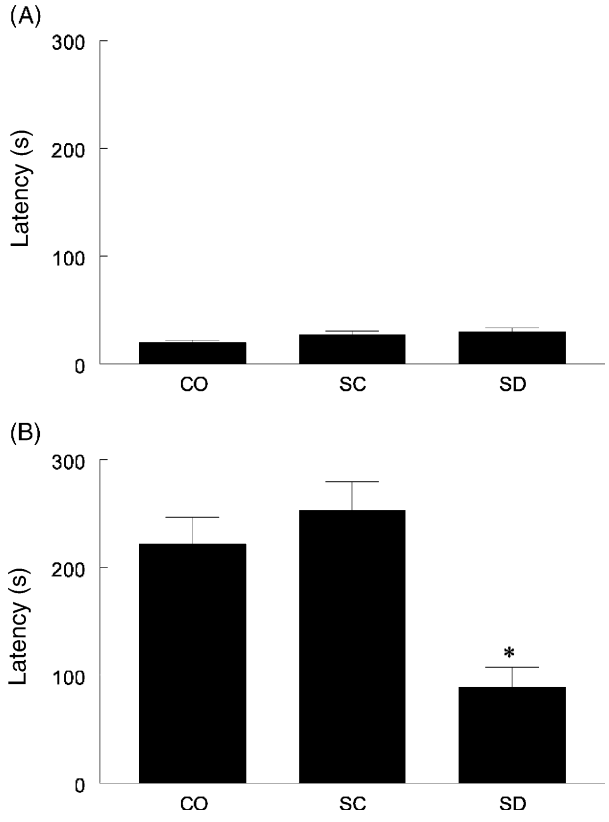

Fig. 1. Latency (s) to enter the dark chamber in the training (A) and test (B) sessions of a passive avoidance task (mean ± SE) presented by mice kept in their home cages (CO), submitted to the stress-control protocol (SC) or to sleep deprivation (SD) for 72 h prior to the training session. *$p < 0.001$ compared to CO and SC groups (Kruskal–Wallis analysis of variance and Mann–Whitney *U*-test).

group latency to enter the dark chamber significantly decreased when compared to the other two groups ($H = 14.11$; $p < 0.001$), whereas the stress-control group was not significantly different from the control group (Fig. 1B).

3.3. Experiment II: Effects of sleep deprivation or stress-control procedure on hippocampal total glutathione and GSSG/GSH ratio

As shown in Fig. 2, the sleep-deprived (but not the stress-control) group presented significantly decreased hippocampal total glutathione level ($H = 6.86$; $p < 0.05$) (Fig. 2A), as well as significantly increased GSSG/GSH ratio ($H = 7.59$; $p < 0.05$) (Fig. 2B) when compared to cage-control animals.

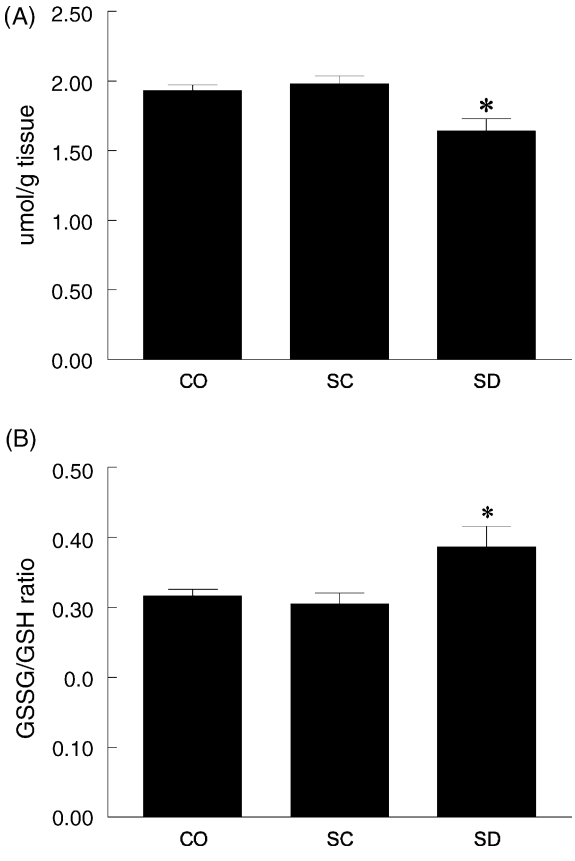

Fig. 2. Hippocampal levels of total glutathione (A) and GSSG/GSH ratio (B) of mice kept in their home cages (CO), submitted to the stress-control protocol (SC) or to sleep deprivation (SD) for 72 h prior to the sacrifice (mean ± SE). *$p < 0.05$ compared to CO and SC groups (Kruskal–Wallis analysis of variance and Mann–Whitney U-test).

3.4. Experiment III: Effects of sleep deprivation or stress-control procedure on hippocampal lipid peroxidation levels

As shown in Fig. 3, hippocampal level of lipid peroxidation was significantly increased in the sleep-deprived group when compared to both cage-control and stress-control animals ($H = 7.25$; $p < 0.005$). In addition, stress group was not significantly different from the cage-control group.

Since no differences were found between stress-control and cage-control animals in hippocampal glutathione and lipid peroxidation levels, neither in passive avoidance performance, only CO groups were included in experiments IV and V. In addition, in these experiments, no difference was found between TW vehicle and PG vehicle-treated animals, so the data from both groups were taken together for statistical comparisons.

3.5. Experiment IV: Effects of long-term administration of antioxidant agents on passive avoidance deficits induced by sleep deprivation

No differences were found in latency to enter the dark chamber in any of the groups in the training session (Fig. 4A). In the test session, sleep-deprived vehicle animals latency to enter the dark chamber significantly decreased when compared to the control-vehicle group ($H = 23.43$; $p < 0.0001$). The control animals treated with vitamin E, MEL and PBN were not significantly different from controls treated with vehicle. The sleep-deprived animals treated with vitamin E, MEL and PBN were not significantly different from the respective control groups, and their latencies to enter the dark chamber were significantly longer when compared to the sleep-deprived group treated with vehicle (Fig. 4B).

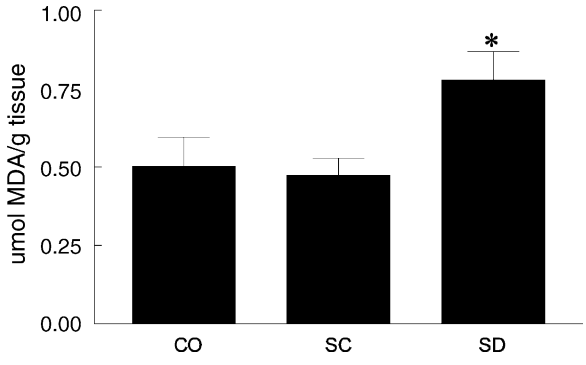

Fig. 3. Hippocampal levels of lipid peroxidation of mice kept in their home cages (CO), submitted to the stress-control protocol (SC) or to sleep deprivation (SD) for 72 h prior to the sacrifice (mean ± SE). *$p < 0.05$ compared to CO and SC groups (Kruskal–Wallis analysis of variance and Mann–Whitney U-test).

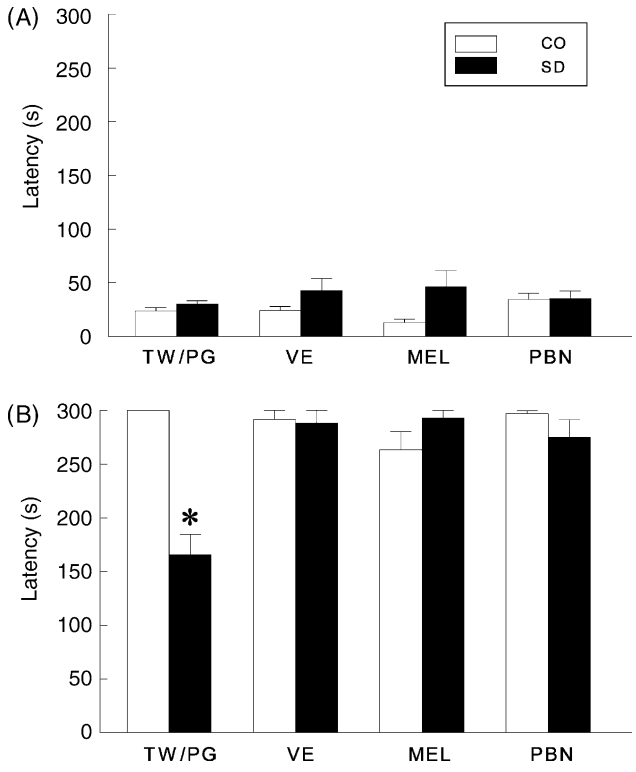

Fig. 4. Latency (s) to enter the dark chamber in the training (A) and test (B) sessions of a passive avoidance task (mean ± SE) presented by control (CO) or 72 h sleep-deprived (SD) mice that were treated i.p. with vehicle (TW/PG), 40 mg/kg vitamin E (VE), 5 mg/kg melatonin (MEL) or 200 mg/kg *N*-tert-butyl-α-phenylnitrone (PBN) once a day for 15 days. $^{*}p< 0.0001$ compared to all the other groups (Kruskal–Wallis analysis of variance and Mann–Whitney *U*-test).

3.6. *Experiment V: Effects of long-term administration of antioxidant agents on the increase in hippocampal lipid peroxidation induced by sleep deprivation*

As shown in Fig. 5, hippocampal level of lipid peroxidation was significantly increased in the sleep-deprived animals treated with vehicle, when compared to the control-vehicle group. Hippocampal levels of lipid peroxidation of sleep-deprived animals treated with vitamin E, MEL and PBN were not significantly different from the level of control group, and were significantly lower than that of sleep-deprived animals treated with vehicle ($H= 10.77$; $p< 0.05$).

4. Discussion

Results from experiment I corroborate previous studies that demonstrated memory deficits induced by sleep deprivation in animal models (see Introduction). The impaired performance of sleep-deprived animals seems to be related to sleep deprivation rather than the stress of the procedure, since there was no memory

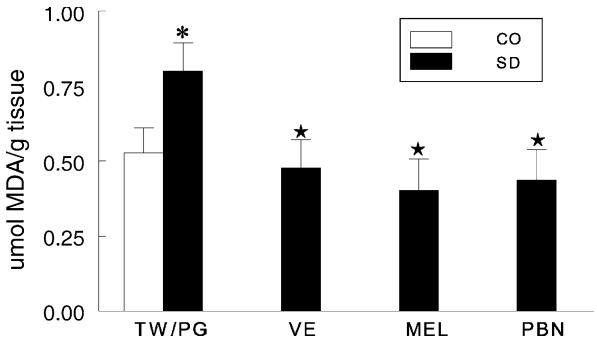

Fig. 5. Hippocampal levels of lipid peroxidation of control (CO) or 72 h sleep-deprived (SD) mice that were treated i.p. with vehicle (TW/PG), 40 mg/kg vitamin E (VE), 5 mg/kg melatonin (MEL) or 200 mg/kg *N*-tert-butyl-α-phenylnitrone (PBN) once a day for 15 days. $^{*}p< 0.05$ compared to CO–TTW/PG group. $^{\star}p< 0.05$ compaed to SD–TW/PG group (Kruskal–Wallis analysis of variance and Mann–Whitney *U*-test).

deficit in the stress-control group. In experiment II, it was shown that the same protocol of sleep deprivation that induced passive avoidance retention impairment in mice (a task dependent on the hippocampal function; Kim and Fanselow, 1992; Izquierdo and Medina, 1993) also produced an increase in hippocampal oxidative stress. This was demonstrated by decreased levels of glutathione, increased GSSG/GSH ratio and increased lipid peroxidation (proposed as sensitive indexes of oxidative stress; Kagan, 1988; Toborek and Henning, 1994; Bains and Shaw, 1997). The pattern of these alterations in indexes of oxidative stress reflects an imbalance in the normal equilibrium between formation of oxygen reactive species and antioxidant defense mechanisms which can lead to a potential cell damage. Our data corroborate previous reports showing that sleep deprivation induces increases in brain oxidative stress (D'Almeida et al., 1998, 2000; Ramanathan et al., 2002). In the studies of D'Almeida et al. (1998, 2000), a reduction in total glutathione levels was found in hypothalamus and thalamus, while decreased superoxide dismutase activity was found in hippocampus and brainstem by Ramanathan et al. (2002). Taken together with the present study, the existing findings suggest that sleep deprivation is followed by a decrease in brain antioxidant defenses, as well as an increase in indexes of oxidative stress. It has been suggested, however, that the brain is not uniformly affected by the effects of sleep deprivation (D'Almeida et al., 1998; Ramanathan et al., 2002). To the extent of our knowledge, the present study addresses for the first time lipid peroxidation levels in the hippocampus of sleep-deprived rodents. In addition, only one previous work investigated specifically hippocampal glutathione levels after sleep deprivation. In that study, glutathione levels were quantified in several brain areas of sleep-deprived rats, and no alteration in this parameter was found in

hippocampus (D'Almeida et al., 1998). Rats were sleep deprived for 96 h by the single platform technique. This method is similar to the method used here, but the animals are placed in individual cages, with one single platform. Discrepancies found in that and in the present study could be due to the differences in the sleep deprivation method, duration of sleep deprivation or still in animal species. Finally, only one parameter indicative of brain oxidative stress was addressed in that study. Nevertheless, despite also presenting methodological differences compared to the present work, the study of Ramanathan et al. (2002) strengthens the increase in hippocampal oxidative stress as a consequence of sleep deprivation.

The hippocampal increase in oxidative stress reported here seems to be, at least in part, responsible for the passive avoidance deficit induced in mice by sleep deprivation. Indeed, the repeated treatment with three different antioxidant agents reverted the deficit showed in the test session in sleep-deprived mice (experiment IV). Since the treatment was finished 24 h before the training session, and 96 h before the test session, it seems that the treatment had a preventive effect, reducing the prooxidant effects of sleep deprivation on hippocampal neurons, and therefore inhibiting memory impairment. Corroborating this hypothesis, the same protocol of antioxidant treatment prevented the increase in hippocampal lipid peroxidation induced by sleep deprivation (experiment V).

Both sleep disturbances and cognitive deficits have been reported to occur frequently among the aged population (Bartus et al., 1982; Lamour et al., 1994; Moe et al., 1995; Dykierek et al., 1998; Van Someren, 2000). In this respect, it is still unknown whether the sleep problems that commonly accompany aging are causally related to the concomitant cognitive impairment. However, considering the fact that increased oxidative stress has been implicated in the memory deficits related to senility (see Introduction), and so has the involvement of oxidative stress in memory deficits induced by sleep deprivation (demonstrated in the present study), one might speculate that there are intersections among the three factors associated to senility (cognitive impairment, sleep alterations and increased brain oxidative stress). This is an interesting working possibility that is currently under investigation in our laboratory.

In conclusion, the present results indicate an important role of hippocampal oxidative stress in memory deficits induced by sleep deprivation in mice.

Acknowledgements

This research was supported by fellowships from Fundação de Amparo a Pesquisa do Estado de São Paulo (proc. 01/10713-7 and FAPESP/CEPID proc. 98/14303-3), from Fundo de Auxílio ao Docente e Aluno da UNIFESP (FADA), from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and from Associação Fundo de Incentivo à Psicofarmacologia (AFIP). The authors would like to thank Ms. Teotila R.R. Amaral and Mr. Cleomar S. Ferreira for capable technical assistance.

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