Progress in Neuro-Psychopharmacology & Biological Psychiatry 39 (2012) 23–30

Contents lists available at SciVerse ScienceDirect

Progress in Neuro-Psychopharmacology & Biological Psychiatry

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

Effect of chronic sleep restriction and aging on calcium signaling and apoptosis in the hippocampus of young and aged animals

Luciane de Souza [a,1], Soraya S. Smaili [b,2], Rodrigo P. Ureshino [b,2], Rita Sinigaglia-Coimbra [c,3], Monica L. Andersen [a,1], Guiomar S. Lopes [b,2], Sergio Tufik [a,*]

[a] Departamento de Psicobiologia, Universidade Federal de São Paulo/UNIFESP, Rua Napoleão de Barros, 925, Vila Clementino, 04024-002, São Paulo, SP, Brazil
[b] Departamento de Farmacologia, UNIFESP, Rua Três de Maio, 100, Vila Clementino, 04044-020, São Paulo, SP, Brazil
[c] Centro de Microscopia Eletrônica, UNIFESP, Rua Botucatu 862, Edif, Ciências Biomédicas, Térreo, Vila Clementino, São Paulo, SP, Brazil

ARTICLE INFO

Article history:
Received 31 October 2011
Received in revised form 25 January 2012
Accepted 31 January 2012
Available online 7 February 2012

Keywords:
Sleep restriction
Sleep deprivation
Calcium
Hippocampus
Apoptosis

ABSTRACT

Aging leads to progressive deterioration of physiological function and diminished responses to environmental stress. Organic and functional alterations are frequently observed in elderly subjects. Although chronic sleep loss is observed during senescence, little is known about the impact of insufficient sleep on cellular function in aging neurons. Disruption of neuronal calcium (Ca^{2+}) signaling is related to impaired neuronal function and cell death. It has been hypothesized that sleep deprivation may compromise neuronal stability and induce cell death in young neurons; however, it is necessary to evaluate the impact of aging on this process. Therefore, the aim of this study was to evaluate the effects of chronic sleep restriction (CSR) on Ca^{2+} signaling and cell death in the hippocampus of young and aged animals. We found that glutamate and carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP) induced a greater elevation in cytosolic Ca^{2+} ($[Ca^{2+}]_c$) in hippocampal slices from aged rats subjected to CSR compared to age-matched controls. Interestingly, aged-matched controls showed a reduced Ca^{2+} response to glutamate and FCCP, relative to both CSR and control young animals. Apoptotic nuclei were observed in aged rats from both treatment groups; however, the profile of apoptotic nuclei in aged CSR rats was highly variable. Bax and Bcl-2 protein expression did not change with aging in the CSR groups. Our study indicates that aging promotes changes in Ca^{2+} signaling, which may also be affected by CSR. These age-dependent changes in Ca^{2+} signaling may increase cellular vulnerability during CSR and contribute to Ca^{2+} signaling dysregulation, which may ultimately induce cell death.

1. Introduction

Several studies have examined the regulatory mechanisms of sleep and the impact of sleep deprivation (SD) on biological systems. Sleep facilitates learning and memory and, consequently, synaptic plasticity (for reviews, see Frank, 2006; Walker, 2008). Sleep loss induces changes in behavior, as well as in synaptic and membrane excitability in the hippocampus and cortex (McDermott et al., 2003; Winters et al., 2011). In addition, some studies have shown genetic damage (Andersen et al., 2009) or cell death (Biswas et al., 2006) in response to sleep loss. Furthermore, in the hippocampus, SD affects glutamate content (Cortese et al., 2010) and glutamate receptor functions by altering the surface expression of N-methyl-D-aspartate (NMDA) receptor subunits (Chen et al., 2006; McDermott et al., 2003). Glutamate receptor activation promotes Ca^{2+} mobilization, which triggers signaling cascades that impact multiple neuronal functions. However, prolonged overstimulation of glutamate receptors and Ca^{2+} overload are associated with excitotoxicity and apoptosis (Alberdi et al., 2010; Ferreiro et al., 2008; Tan et al., 1998; Ureshino et al., 2010).

Aging has also been associated with changes in glutamatergic neurotransmission and a deterioration of learning and memory (for reviews, see Segovia et al., 2001; Lister and Barnes, 2009). Evidence has been accumulated over the last decade indicating that these functional changes are due to modifications of synaptic connectivity and intracellular signaling (Toescu and Verkhratsky, 2007; Tonkikh et al., 2006).

Abbreviations: AG, Aged; AG-SR, Aged sleep-restricted; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; ANOVA, Two-way analysis of variance; CA2, *cornu ammonis* 2; CA3, *cornu ammonis* 3; CSR, Chronic sleep restriction; FCCP, Carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone; NMDA, N-methyl-D-aspartate; SD, Sleep deprivation; REM, *Rapid eye movement*; TUNEL, Terminal deoxynucleotidyl transferase dUTP nick end labeling; YG-CTL, Young adult control; YG-SR, Young adult sleep-restricted.

* Corresponding author at: Departamento de Psicobiologia, Universidade Federal de São Paulo/UNIFESP, Rua Napoleão de Barros, 925, Vila Clementino, 04024-002, São Paulo, SP, Brazil. Tel.: +55 11 2149 0155; fax: +55 11 5572 5092.

E-mail addresses: lugyn96@yahoo.com.br (L. de Souza), ssmaili@farm.epm.br (S.S. Smaili), rodrigo_ureshino@yahoo.com.br (R.P. Ureshino), rita.sinigaglia@unifesp.br (R. Sinigaglia-Coimbra), mandersen@psicobio.epm.br (M.L. Andersen), guiolopes@terra.com.br (G.S. Lopes), stufik@psicobio.epm.br (S. Tufik).

[1] Tel.: +55 11 2149 0155; fax: +55 11 5572 5092.
[2] Tel.: +55 11 5576 4449; fax: +55 11 5576 4449.
[3] Tel.: +55 11 5576 4528.

0278-5846 © 2012 Elsevier Inc. Open access under the Elsevier OA license.
doi:10.1016/j.pnpbp.2012.01.018

Thus, age-dependent changes in Ca^{2+} signaling may contribute to memory deficits and cell death. Indeed, some authors have postulated that age-induced changes in Ca^{2+} signaling may lead to synaptic dysfunction and neurodegeneration (Hajieva et al., 2009; Toescu, 2005; Toescu and Verkhratsky, 2004; Tonkikh et al., 2006). Moreover, mitochondrial oxidative stress may to impair mitochondrial function, causes alterations of mitochondrial Ca^{2+} responses and promote the release of pro-apoptotic proteins, triggering apoptosis (Pivovarova et al., 2004; for review Marchi et al., 2012). The proteins of the Bcl-2 family regulate mitochondrial outer membrane permeability and the apoptotic process (Kuwana and Newmeyer, 2003).

It is well known that most elderly individuals experience a gradual decline in cognitive function and sleep quality (Bonnet and Rosa, 1987; Espiritu, 2008; Plassman et al., 2008). Therefore, it is important to understand the cellular mechanisms underlying age-dependent alterations in synaptic function. For studies focusing on the effects of sleep reduction, chronic sleep restriction (CSR) is more clinically relevant than total sleep deprivation, as humans are more likely to experience the former. In modern society, the time spent sleeping has become shorter, leading to a population that is chronically sleep deprived (Bonnet and Arand, 1995; Broman et al., 1996; Tufik et al., 2009). For this reason, the study of impact of sleep loss on a healthy aging population is important; therefore, the aim of this study is to investigate the effects of CSR on Ca^{2+} signaling and apoptosis in young and aged rats.

2. Material and methods

2.1. Animals

A total of 80 male Wistar rats (38 young adult rats, 3–4 months old; 42 aged rats, 22–23 months old) were used in the experiments. These animals were obtained from our animal facility (CEDEME-Universidade Federal de São Paulo/UNIFESP) and this study was approved by the Ethical Committee of the UNIFESP (CEP 0365/08). The rats used in this study were fed ad libitum and were maintained and treated in accordance with the guidelines established by the Ethical and Practical Principles of the Use of Laboratory Animals (Andersen et al., 2004b).

2.2. Chronic sleep restriction (CSR) protocol

Rats were sleep deprived using the multiple platform (flower pot) procedure (Coenen and van Luijtelaar, 1985; Van Hulzen and Coenen, 1981). The protocol consists of placing rats in a tiled water tank containing platforms (10 cm in diameter) raised 1 cm above the water surface. This allows the rats to move around by leaping from one platform to another. The temperature of the water inside the tanks was similar to the ambient room temperature (22 ± 1 °C). During paradoxical, or *rapid eye movement* (REM) sleep, rats fall off the platform due to muscular atonia and are awakened. An intermediate-sized (10 cm) platform was used because aged rats are slightly larger than young ones, as previously described (Andersen et al., 2004a).

To evaluate the effect of repeated sleep deprivation in young adult and aged rats, we used the CSR protocol developed by Machado et al. (2005). In that protocol, the rats were kept on the platforms for 18 h (beginning at 16:00 h) and allowed to sleep for 6 h in their individual home cages (10:00–16:00 h) every day for 21 days. Therefore, while the rats were on the platform (18 h), they were deprived of paradoxical sleep and when they were in the home cage (6 h), they were allowed to sleep freely (sleep opportunity period). Thus, this protocol permits partial compensation for sleep loss. We reasoned that this procedure should mimic sleep fragmentation due to repeated awakenings and would be a useful tool to investigate the effects of SD. However, in contrast to the Machado protocol (2005), the animals in our study were subjected to the protocol for only 15 days because aged animals begin to show difficulties in maintaining their posture on the platform after 2 weeks. All animals were exposed to a habituation period on the platforms for 1 h per day for 3 days prior to beginning the CSR protocol. Young and aged animals were randomly assigned to control and sleep-restricted groups: young adult control (YG-CTL), young adult sleep-restricted (YG-SR), aged (AG), and aged sleep-restricted (AG-SR). Both control groups (age-matched controls) were maintained in cages in the same room as the experimental groups for the duration of the study.

Throughout the study, the experimental room was maintained at a controlled temperature (22 ± 1 °C) with a 12:12 h light–dark cycle (lights on at 07:00 h). Food and water were provided ad libitum and the water in the tank was changed daily. Liquid nutritional supplement (Sustagen®, Brazil) was administered to attenuate the tiring and weakening effects of the CSR protocol (see further details in de Souza et al., 2010).

2.3. Preparation of rat hippocampal slices

After the 15th day of sleep restriction, the rats were removed from the water tank (10:00 h) and returned to their cage until they were euthanized (13:00 h). During this interval (10:00–13:00 h), the animals groomed themselves, ate and slept. This time period was consistent for all animal groups. The rats were euthanized and their brains were removed and immediately immersed in oxygenated artificial cerebrospinal fluid (aCSF; 1.2 mM KH_2PO_4, 4.7 mM KCl, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$, 1.2 mM $MgSO_4$, and 11 mM glucose, pH 7.4) supplemented with sucrose solution (220 mM). Using a vibratome (1000 Plus Sectioning System; Campden Instruments Inc., U.S.A.), the brains were sliced into 200-μm coronal sections that included the cortex and hippocampus (Smaili et al., 2008). The hippocampus was dissected and the slices were transferred to oxygenated incubation solution and protected from light. After washing, the sections were mounted in a Leiden chamber with 1 ml aCSF and kept at 37 °C for the duration of the experiments.

2.4. Ca^{2+} measurement in rat hippocampal slices

For Ca^{2+} measurements, the slices were incubated at room temperature for 30 min with Fura2-AM (10 μM), a ratiometric Ca^{2+} dye (Grynkiewicz et al., 1985), plus 0.01% pluronic acid F-127. During the experiments, the samples were maintained at 37 °C. To elevate cytosolic calcium ($[Ca^{2+}]_c$), we used 2 drugs: glutamate (1 mM), which activates ionotropic and metabotropic glutamate receptors and carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP; 2.5 μM), which disrupts mitochondrial membrane potential and causes the release of mitochondrial Ca^{2+}. The number of slices collected in each experiment was equivalent to the number of rats, i.e., 1 slice was obtained from each rat.

A dose–response curve was used to determine the ideal concentration of glutamate (1 mM) that would evoke a $[Ca^{2+}]_c$ rise in both young and aged hippocampal slices. In the FCCP experiment, oligomycin (100 μg/ml) was added to prevent excessive ATP consumption by (reversible) ATP-synthase (Budd and Nicholls, 1996; Smaili et al., 2000). In hippocampal slices from young adult rats ($n = 3$), FCCP was also tested in Ca^{2+}-free medium (0 Ca^{2+}) to exclude the possibility that the rise in $[Ca^{2+}]_c$ was due to extracellular Ca^{2+} mobilization.

Variations in $[Ca^{2+}]_c$ were evaluated by real-time high-resolution fluorescence microscopy (Nikon TE 300; Nikon, Japan) coupled to a CCD camera (Quantix 512 or CoolSnap; Princeton Instruments, Monmouth Junction, U.S.A.). Images were acquired and analyzed using BioIP software (Anderson Eng.). Basal Ca^{2+} levels were set as the average of the first 19 images and fluorescence traces were normalized for comparison. The data were normalized using the $(F - F_0)/F_0 \times 100$ formula, where F_0 represents the basal Ca^{2+} level. Calibrations were performed with digitonin (1 mM), which was added at the end of

each experiment. Responses represent the normalized fluorescence values as a percent of maximum fluorescence (Rmax = 100%). For glutamate and FCCP, Ca^{2+} measurements were taken at the peak Ca^{2+} response induced by each drug.

The total numbers of rats from each group used to evaluate the effects of glutamate were as follows: YG-CTL, n = 5; YG-SR, n = 6; AG, n = 6; AG-SR, n = 6. The total numbers of rats from each group used to evaluate the effects of FCCP were as follows: YG-CTL, n = 5; YG-SR, n = 5; AG, n = 7; AG-SR, n = 7.

2.5. Apoptosis detection using the TUNEL assay

Perfusion was started approximately 1 h after the CSR protocol; however, the animals (control and sleep restricted) were randomly perfused 1 at a time (2–3 animals per h). The animals were anesthetized and transcardially perfused with saline followed by 4% formaldehyde in 0.1 M phosphate-buffered saline (pH 7.4). The brains were removed for paraffin embedding and coronally sectioned at 5 μm. A terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed according to the manufacturer's protocol (Bortner et al., 1995; Apop Tag Plus; Chemicon, U.S.A.). Briefly, brain slices were deparaffinized in xylene and dehydrated in ethanol. The samples were treated with proteinase K (20 μg/ml) for 15 min and then endogenous peroxidase activity was quenched with an application of 1% H_2O_2 for 20 min. Next, the samples were incubated in an equilibration buffer for 10 min before being treated with terminal deoxynucleotidyl transferase at 37 °C for 1 h. To stop the reaction, wash buffer was applied (15 min) to the samples, which were then incubated with anti-digoxigenin-peroxidase antibody (30 min). After washing, slides were developed with 3,30-diaminobenzidine (DAB; 0.5%) and counterstained with hematoxylin (Gavrieli et al., 1992). Images were captured using a Nikon E8000 microscope (Nikon, Japan) coupled to a Cool Snap Pro Color camera and analyzed with Image Pro Express software (Media Cybernetics, U.S.A). The number of TUNEL-positive cells in the pyramidal layer of hippocampal slices were counted hemilaterally under a 40× objective and an average of 2 slices (5 μm each) were collected from each rat (coordinates: interaural between 5.20 mm and 5.86 mm; bregma between −3.80 mm and −3.14 mm; Paxinos and Watson, 1998). The data are expressed as the percentage of TUNEL-positive *cornu ammonis* 2 and 3 (CA2 and CA3, respectively) pyramidal neurons to the total number of cells (apoptotic cells/total number of pyramidal cells × 100). The numbers of rats from each group used to evaluate apoptosis were as follows: YG-CTL, n = 4; YG-SR, n = 5; AG, n = 5; AG-SR, n = 6.

2.6. Western blot analysis of Bcl-2 and Bax

Hippocampi from both hemispheres were minced and homogenized in ice-cold lysis buffer (50 mM Tris, 150 mM NaCl, 0.05% deoxycholate, 1% NP-40, 1 mM EDTA, 0.1% SDS and protease inhibitors, pH 8.0) with an Ultra-Turrax homogenizer. Total protein concentration was determined using the Bradford (1976) assay method and 50 and 100 μg of protein were used for Bcl-2 and Bax labeling, respectively. The proteins were electrophoresed on 15% SDS polyacrylamide gels and transferred onto nitrocellulose membranes. To block nonspecific labeling, membranes were blocked with 5% nonfat dry milk for 1 h for Bcl-2 or 2 h for Bax and then incubated with anti-Bcl-2 (1:200, N-19 sc-492; Santa Cruz Biotechnology, U.S.A.), anti-Bax (1:100, Δ21 sc-6236; Santa Cruz Biotechnology, U.S.A.) or anti-β-actin (1:1000, A5060; Sigma, U.S.A.) primary antibodies in 1% bovine serum albumin at 4 °C overnight. After washing, goat anti-rabbit IgG secondary antibody (1:20,000; ImmunoPure, Thermo Scientific, U.S.A.) in 1% bovine serum albumin was added and allowed to incubate for 1 h. After additional washing, the membranes were developed using an ECL detection system and labeled bands were captured on autoradiographic film. Membranes were then stripped and relabeled with anti-β-actin antibody. Densitometric analyses were performed after autoradiographic films were scanned using Quick Scan Win 2000 software. The results were normalized to the β-actin band intensity in each experiment and plotted (mean ± standard deviation (SD)) as percentage of β-actin. The numbers of rats from each group used for Western blotting were as follows: YG-CTL, n = 4; YG-SR, n = 4; AG, n = 4; AG-SR, n = 4.

2.7. Statistical analyses

The values shown are expressed as the mean ± SD. Homogeneity of variance was assessed by the Bartlett test and normal data distribution by the Kolmogorov–Smirnov test. Two-way analysis of variance (ANOVA) was used to assess the variables of age (adult or aged rat) and condition (sleep-restricted or not sleep-restricted). The Duncan post-hoc test was used to compare values of the different groups. When the Bartlett test showed absence of homogeneity of variance, the data were analyzed using the Kruskal–Wallis ANOVA followed by the Mann–Whitney *U*-test. $p<0.05$ was considered statistically significant.

3. Results

3.1. Effects of glutamate on $[Ca^{2+}]_c$ in the hippocampus of young and aged rats

To evaluate the effects of glutamate on $[Ca^{2+}]_c$ following CSR in hippocampal slices of young and aged rats, 1 mM glutamate was bath-applied and the fluorescence was measured for 20 min. As shown in Fig. 1, glutamate caused a similar $[Ca^{2+}]_c$ increase in both the dentate gyrus (DG) and CA3 of sleep-restricted and control young adult animals (DG: YG-CTL, 21.6 ± 9.1; YG-SR, 21.5 ± 6.3; CA3: YG-CTL, 19.4 ± 4.5; YG-SR, 23.6 ± 5.5). In the AG group, a smaller increase in $[Ca^{2+}]_c$ was observed (DG: 3.8 ± 2.5; CA3: 4.4 ± 2.2) compared to the AG-SR group (DG: 12.0 ± 4.8; CA3: 14.1 ± 5.3). ANOVA revealed a significant age effect in the DG [$F(1,20) = 34.3$; $p<0.01$] with a significant interaction [$F(1,20) = 5.65$; $p<0.05$] (Fig. 1B1). The Duncan test revealed significant differences between the AG and YG-CTL groups and between the AG and AG-SR groups. In the CA3, an ANOVA revealed significant effects of age [$F(1,20) = 43.9$; $p<0.01$] and condition (sleep-restricted or not sleep-restricted) [$F(1,20) = 12.8$; $p<0.01$] but there was not a significant interaction [$F(1,20) = 3.87$; $p = 0.06$] (Fig. 1B2).

3.2. Evaluation of mitochondrial Ca^{2+} content in the hippocampus of young and aged rats

As described earlier, hippocampal sections from young and aged rats were loaded with Fura2-AM and FCCP (2.5 μM) was added to examine mitochondrial Ca^{2+} content. In the DG, FCCP induced a similar increase in fluorescence ratio in both young adult groups (YG-CTL: 21.1 ± 6.7; YG-SR: 23.9 ± 5.9). However, a smaller fluorescence increase was detected in the AG group (10.5 ± 3.8) compared to the young adult groups. In the AG-SR group, the FCCP response (15.2 ± 4.3) was greater than in the AG group. An ANOVA revealed a significant FCCP response that was age-dependent [$F(1,20) = 21.07$; $p<0.01$] (Fig. 2B1). The effect of condition (sleep-restricted or not sleep-restricted) was not significant [$F(1,20) = 3.19$; $p = 0.08$] and there was no significant interaction [$F(1,20) = 0.19$; $p = 0.6$].

The CA3 area exhibited the same pattern of Ca^{2+} response as the DG [YG-CTL (22.3 ± 5.2), YG-SR (23.8 ± 4.9), AG (11.6 ± 2.9) and AG-SR (18.0 ± 2.7)] (Fig. 2B2). In the CA3, an ANOVA revealed significant differences that were age-dependent [$F(1,20) = 26.6$; $p<0.01$] and condition [$F(1,20) = 6.02$; $p<0.05$]; however, the interaction was not significant [$F(1,20) = 2.28$; $p<0.14$]. In slices treated with Ca^{2+}- free solution, similar responses were observed after the addition of FCCP

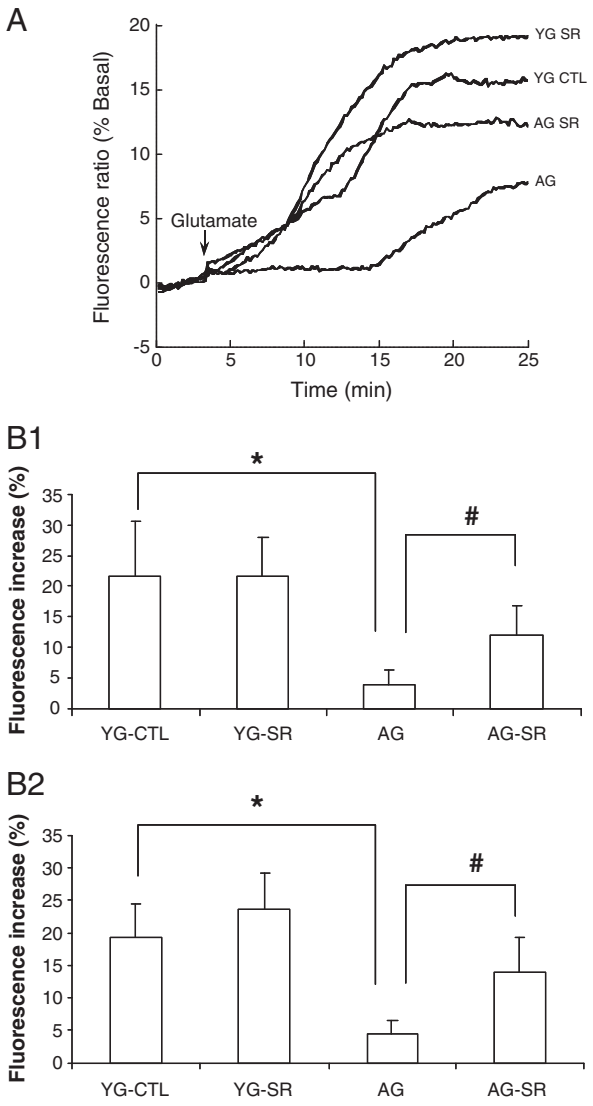

Fig. 1. Ca^{2+} responses after stimulation with glutamate in the hippocampus of young and aged rats. A: Typical Ca^{2+} traces evoked by glutamate (1 mM). B1: Histogram (mean ± SD) comparing the maximum Ca^{2+} increase evoked by glutamate in DG of young and aged rats (sleep-restricted or not sleep-restricted). B2: Histogram (mean ± SD) comparing the maximum Ca^{2+} increase evoked by glutamate in CA3 area of young and aged rats. Percentages are expressed as fluorescence ratio (340/380) relative to the maximum fluorescence (Rmax = 100%) and normalized to the basal fluorescence.* denotes that glutamate induced a small increase in the maximum response in aged rats as compared to young animals ($p<0.01$). # denotes that glutamate induced a significantly greater increase in the maximum response in sleep-restricted aged rats as compared to aged rats ($p<0.01$). Abbreviations in all figures: YG-CTL (young control group; n = 5); YG-SR (young sleep-restricted group; n = 6); AG (aged group; n = 6); AG-SR (aged sleep-restricted group; n = 6); $p<0.05$.

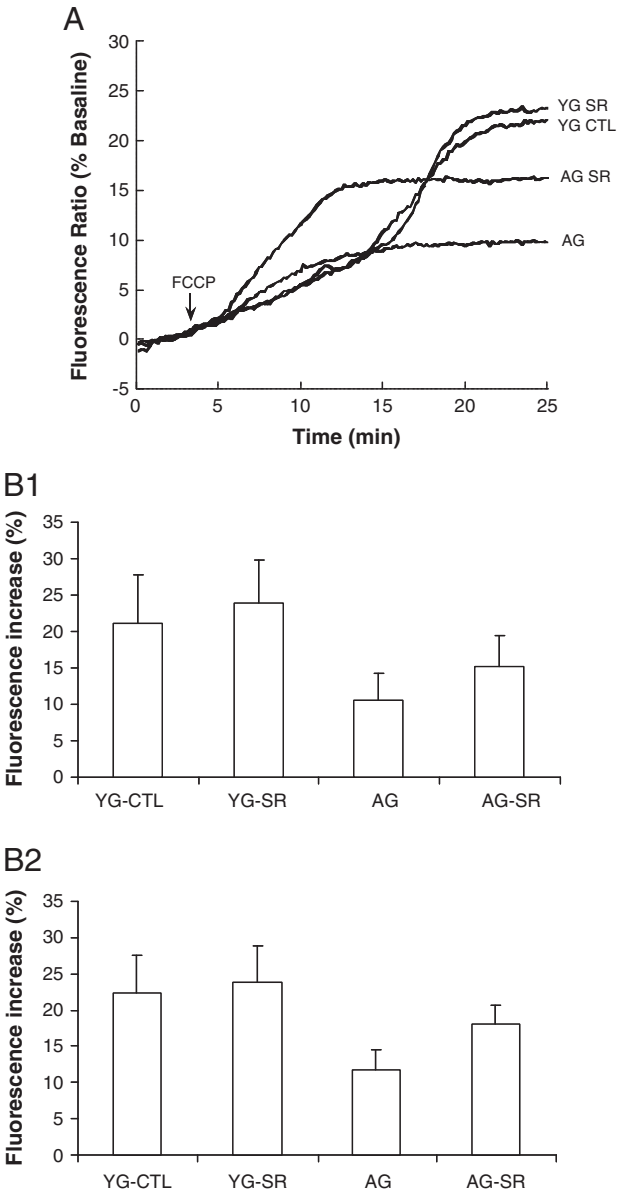

Fig. 2. Ca^{2+} responses after stimulation with FCCP in the hippocampus of young and aged rats. A: Typical Ca^{2+} traces evoked by FCCP (2.5 μM). B1: Histogram (mean ± SD) comparing the maximum Ca^{2+} increase evoked by FCCP in DG area of young and aged rats (sleep-restricted or not sleep-restricted). An ANOVA revealed a significant FCCP response that was age-dependent ($p<0.01$); however neither the effect of condition (sleep-restricted or not sleep-restricted) ($p=0.08$) nor interaction ($p=0.6$) were significant. B2: Histogram (mean ± SD) comparing the maximum Ca^{2+} increase evoked by FCCP in CA3 area of young and aged rats. There were significant differences dependent on age ($p<0.01$) and condition ($p<0.05$); the interaction ($p<0.14$) was not significant. Percentages were expressed as ratio (340/380) values relative to the maximum fluorescence (Rmax = 100%) and normalized to the basal fluorescence. YG-CTL (n = 5); YG-SR (n = 5); AG (n = 7); AG-SR (n = 7); $p<0.05$.

(19.3 ± 1.8) in the CA3 (data not shown), indicating that only mitochondrial Ca^{2+} was mobilized.

3.3. Detection of apoptosis by TUNEL staining in the hippocampus of young and aged rats

To determine the effects of aging and the CSR protocol on apoptosis, we performed TUNEL staining in the hippocampus of young and aged rats. We detected apoptotic nuclei, mainly in the pyramidal layer of the CA2 and CA3 regions, in both aged groups (AG: 2.2 ± 0.7; AG-SR: 4.0 ± 2.3); however, apoptosis in the hippocampi of both young groups was almost undetectable. The non-parametric Kruskal–Wallis test revealed a significant difference in TUNEL staining between the aged and young groups ($p<0.05$) (Fig. 3). A representative image demonstrating typical staining of an apoptotic nucleus using the TUNEL method is shown in Fig. 4.

3.4. Bcl-2 and Bax expression in the hippocampus of young and aged rats

To determine the expression levels of apoptosis-regulating proteins Bcl-2 and Bax, we performed Western Blot analysis of hippocampal lysates from young and aged rats. Band intensities were normalized to β-actin. As shown in Fig. 5, the ANOVA showed that there were no statistically significant differences in Bcl-2 or Bax levels

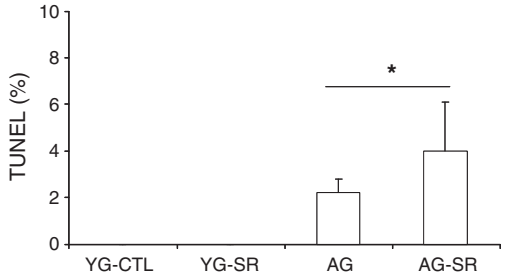

Fig. 3. Detection of apoptotic cells in hippocampal slices (CA2–CA3 pyramidal layer) of young and aged rats. Hippocampal slices (5 μm) from young and aged rats were subjected to TUNEL staining. Histogram (mean ± SD) showing morphometric analysis of TUNEL-positive cells in hippocampus of young and aged animals. The non-parametric Kruskal–Wallis test revealed difference between the aged group (sleep-restricted or not sleep-restricted) and young group. * denotes a significant increase in TUNEL-positive nuclei in aged rats ($p<0.05$). YG-CTL (n = 4); YG-SR (n = 5); AG (n = 5); AG-SR (n = 6); $p<0.05$.

between the different age groups, [$F(1,13)=0.02$; $p=0.87$] and [$F(1,12)=1.1$; $p=0.31$], respectively.

4. Discussion

In this study, we examined the relationship between neuronal Ca^{2+} signaling, apoptotic markers and chronic sleep loss in aged rats. We hypothesized that CSR would affect Ca^{2+} signaling and apoptosis differentially in young adult and aged animals. The CSR protocol led to alterations in calcium signaling in aged rats that were not present in young rats. CSR increased $[Ca^{2+}]_c$ in aged sleep-restricted rats compared to age-matched controls. However, there was a smaller increase in $[Ca^{2+}]_c$ in the aged group compared to both young adult groups. TUNEL-positive cells were detected primarily in the CA2 and CA3 regions of aged (non-sleep-restricted) rats and aged sleep-restricted rats. However, due to the high variability in the aged sleep-restricted group, the numbers were not significantly different between the two groups. Bcl-2 and Bax protein expression were not altered by either increased age or the CSR protocol.

4.1. Glutamate induces a smaller increase in the Ca^{2+} fluorescence ratio in hippocampal slices of aged rats

The effects of glutamate were investigated because there is evidence that sleep loss induces changes in glutamatergic signaling. In addition, this compound is involved in excitotoxic brain damage. In our study, we observed a smaller increase in the Ca^{2+} fluorescence ratio following the addition of glutamate in the aged group (non-sleep-restricted) in comparison with the other groups. The fluorescence ratio was greater in both young adult groups regardless of sleep condition. This reduced response observed in the aged group may be related to a reduction in hippocampal glutamatergic receptor density (Magnusson et al., 2010; Segovia et al., 2001). A reduction in NMDA receptor subunit expression may explain some of the NMDA receptor function changes observed during aging (Magnusson, 1998; Magnusson et al., 2002). The loss of hippocampal NMDA and AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptors significantly correlates with age-related declines in learning (Magnusson, 1998).

4.2. Reduction in mitochondrial Ca^{2+} content in hippocampal slices of aged rats

Paralleling the smaller increase in Ca^{2+} fluorescence ratio following glutamate addition in the aged group, we also observed a smaller elevation in $[Ca^{2+}]_c$ after FCCP addition. In addition to receptor-mediated Ca^{2+} changes during aging, another possible explanation is a reduction in Ca^{2+} stores, which may influence glutamate responses and cell metabolism. Following FCCP addition, a smaller increase in $[Ca^{2+}]_c$ was observed in the aged group, relative to both young adult groups. The age-dependent reduction in $[Ca^{2+}]_c$ has been shown to be related to mitochondrial $[Ca^{2+}]_c$ buffering (Murchison et al., 2004). The mitochondria participate in intracellular Ca^{2+} buffering and dysfunctional mitochondrial metabolism may be involved in age-dependent changes in Ca^{2+} homeostasis (Xiong et al., 2002, 2004). Chronic depolarization of mitochondrial membranes or alterations in mitochondrial ultrastructure may be responsible for age-dependent changes in mitochondrial function (Lopes et al., 2004; Xiong et al., 2002, 2004).

4.3. Chronic sleep restriction induces an increase in the Ca^{2+} fluorescence ratio after glutamate and FCCP addition to hippocampal slices from aged rats

The CSR paradigm induced an increase in $[Ca^{2+}]_c$ after stimulation with glutamate and FCCP in hippocampal slices from aged rats. In young adult animals, the glutamate and FCCP response was similar in control and sleep-restricted rats. In young adult animals, SD induces a reduction in hippocampal AMPA receptor phosphorylation (Hagewoud et al., 2010), decreases synaptic transmission and long-term potentiation (Ravassard et al., 2009) and leads to alterations in

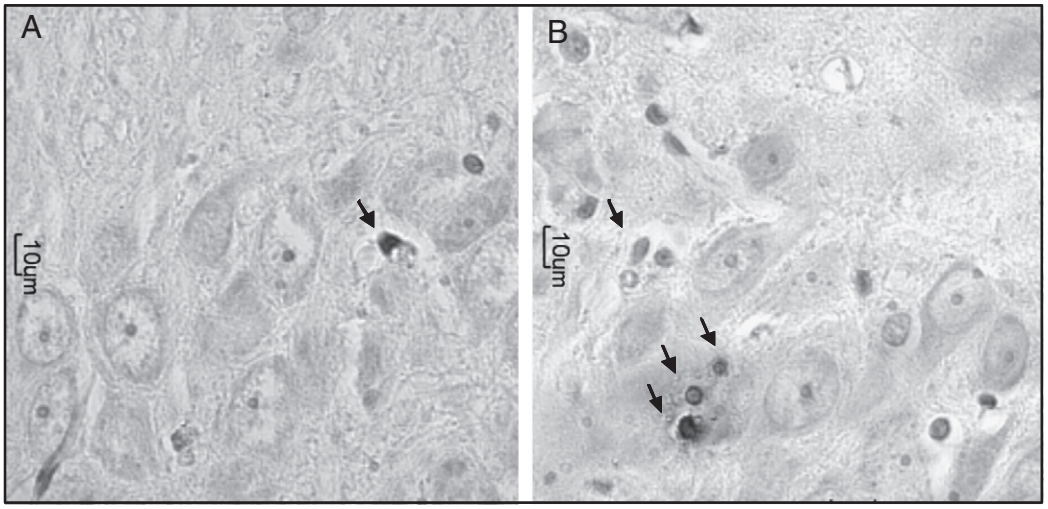

Fig. 4. TUNEL staining in hippocampus sections (5 μm) of aged rats. There were stained cells in both groups of aged rats, (A) stained scattered was most common, however, (B) detection of multiples apoptotic nuclei it was observed in AG-SR group.

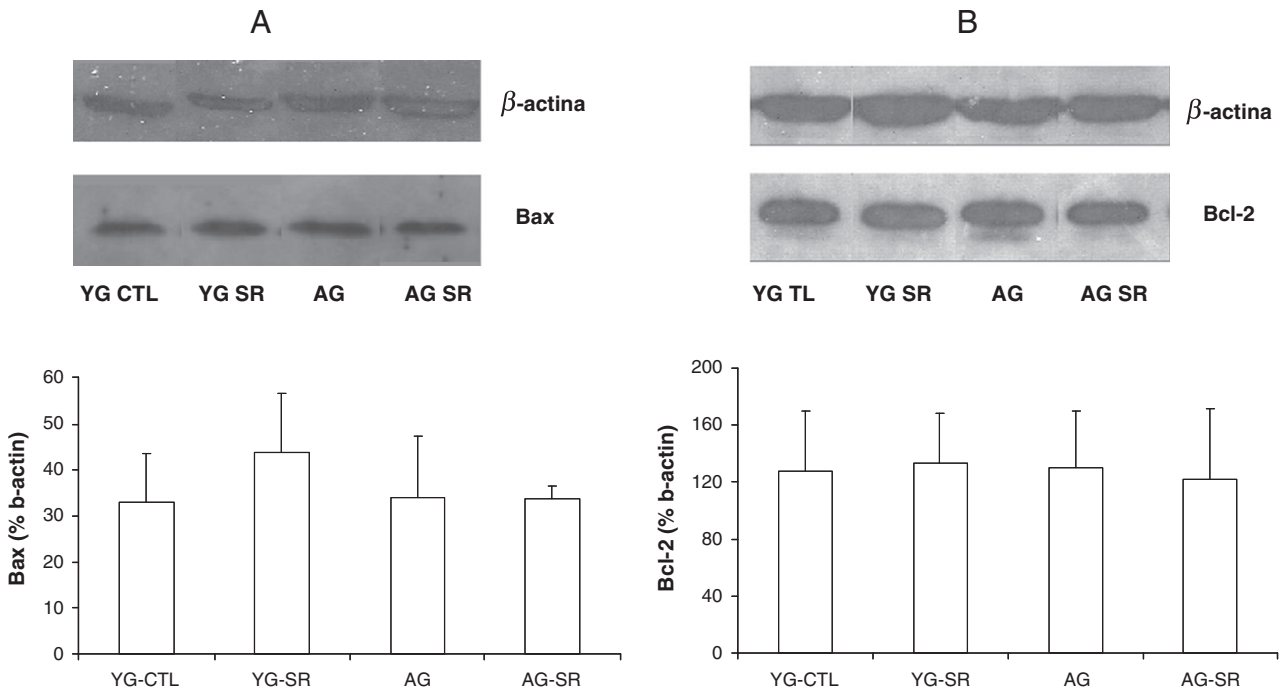

Fig. 5. Expression of Bax and Bcl-2 proteins in the hippocampus of young and aged rats. Lysates prepared from the hippocampus of young and aged rats were subjected to Western Blot analysis with anti-Bax and anti-Bcl-2 antibodies. The blots were reprobed with anti-β-actin antibody. The relative expression levels of Bax and Bcl-2, as normalized to β-actin's densitometric values, are shown in histograms (mean ± SD). The ANOVA did not show any significant difference between the groups. YG-CTL (n = 4); YG-SR (n = 4); AG (n = 4); AG-SR (n = 4); $p < 0.05$.

NMDAR surface expression (Kopp et al., 2006; McDermott et al., 2006). The recovery of sleep post-SD rescues these deficits (Kopp et al., 2006; Ravassard et al., 2009). In fact, previous studies in our laboratory have shown that during CSR, young adult animals adapted and slept more during the period of rest (6 h sleep) (see details in Machado et al., 2005). In addition, the Ca^{2+} experiments were performed 3 h after the animals were removed from the platform (on the 15th day of the CSR protocol), which may be enough time for young neurons to recover and buffer the increase in $[Ca^{2+}]_c$. Therefore, the absence of a difference in Ca^{2+} increase between the young groups may be due to their capability of adapting to the chronic sleep restriction protocol (de Souza et al., 2010; Machado et al., 2005). Unfortunately, all the studies examining SD and glutamate receptors were conducted with young rats or for short periods of SD (Kopp et al., 2006; McDermott et al., 2003, 2006; Ravassard et al., 2009; Tadavarty et al., 2009; Tartar et al., 2010), which limits their comparison to our study. Furthermore, it remains to be determined how glutamate receptor signaling is affected during chronic sleep reduction.

A previous study in our laboratory demonstrated that unlike young rats (Machado et al., 2005), aged rats did not recover their sleep deficit during the sleep opportunity period (6 h) (de Souza et al., 2010). Therefore, chronic sleep loss due to aging may be primarily responsible for the glutamatergic signaling-induced increase in $[Ca^{2+}]_c$ in the hippocampal slices of aged sleep-restricted rats. In fact, aged neurons exhibit decreased Ca^{2+} buffering capacity and a delayed ability to restore basal $[Ca^{2+}]_c$ following KCl stimulation (Verkhratsky and Toescu, 1998; Xiong et al., 2002). Hippocampal neuronal cell culture models showed greater sensitivity to a glutamate challenge in senescent neurons than in young neurons (Abramov and Duchen, 2010; Brewer et al., 2007). Moreover, aging is associated with changes in the threshold for mitochondrial permeability transition pore (mPTP) opening, which releases stored Ca^{2+} from mitochondria (Abramov and Duchen, 2010), as well as the ability to maintain intracellular ATP content under pathophysiological conditions (Joo et al., 1999). The changes induced by the CSR protocol may also be related to physical stress (Papale et al., 2005; Tufik et al., 1995) and/or sleep loss during aging. Physical stress is an inherent feature of the SD protocol and aged animals may be more vulnerable than young animals, mainly because also can contribute to oxidative stress (Naidoo et al., 2008; Singh et al., 2008).

4.4. Apoptotic nuclei are increased in the hippocampus of aged rats (sleep-restricted and non-sleep-restricted)

Previously, it was suggested that sustained waking may damage neurons through excitotoxicity and lead to cell death (Biswas et al., 2006; Cirelli et al., 1999; Hipólide et al., 2002; Naidoo et al., 2008; Novati et al., 2012). However, the first two studies did not find any neuronal loss in young adult animals after SD (Cirelli et al., 1999; Hipólide et al., 2002). Notably, it was recently shown that chronic partial sleep deprivation reduces the sensitivity of the nucleus basalis magnocellularis to excitotoxic insult promoted by NMDA (Novati et al., 2012). However, Biswas and collaborators (2006) observed that long-term REM sleep loss (6–10 days) does lead to increased apoptosis in some nuclei in the rat brain. In young and aged mice, basal expression of pro-apoptotic factors increased with aging and SD produced additional increases in old, but not young, animals (Naidoo et al., 2008).

Our study did not find any statistically significant difference in the number of apoptotic nuclei in the hippocampus of young rats following CSR. In fact, apoptosis was almost undetectable in both young groups. Conversely, aged animals showed more TUNEL staining, mainly in the CA3 region, indicating a selective vulnerability to aging. Despite the absence of any significant difference, there was more variability in the aged sleep-restricted group, with some animals showing more vulnerability to neuronal death than others.

The delay in recovery from the rise in $[Ca^{2+}]_c$ following the addition of glutamate and FCCP in aged sleep-restricted rats may lead to increased susceptibility to excitotoxic damage and apoptosis (Ureshino et al., 2010). It is possible that excess mitochondrial Ca^{2+} promotes increases in reactive oxygen species production, which triggers apoptotic pathways (Ureshino et al., 2010). Oxidative stress was elevated in the hippocampus of sleep-deprived adult and aged animals and the aged

animals were more severely affected (Singh et al., 2008). A decrease in antioxidant defense mechanisms was observed in the hippocampus of adult rats after 21 days of SD (Süer et al., 2011). In our study, despite the relatively small increase in apoptotic nuclei in the aged sleep-restricted rats, the observed age-dependent alterations in Ca^{2+} signaling suggest that aging causes the organism to be more vulnerable to stressful conditions. However, other mechanisms must also underlie the increase in apoptosis observed in the hippocampus of aged rats because we did not observe an increase in mitochondrial Ca^{2+} or an increase in $[Ca^{2+}]_c$ after FCCP and glutamate stimulation.

4.5. Absence of change in Bax and Bcl-2 expression

The role of apoptotic proteins in sleep and aging in rats remains controversial. Our data did not show changes in expression levels of the anti-apoptotic protein Bcl-2 or the pro-apoptotic protein Bax during aging or following CSR. This is in agreement with data presented by Hipólide et al. (2002), who found no alterations in Bcl-2 or Bax mRNA in young rats following CSR using the classical platform technique. In contrast, Montes-Rodríguez et al. (2009) reported an increase in the Bcl-2/Bax ratio in the hippocampus of adult young rats subjected to acute sleep deprivation and a decrease in this ratio in other brain structures. Thees et al. (2005) demonstrated an increase in Bax expression without changes in Bcl-2 levels in the hippocampus of aged baboons, which correlated with an increased number of TUNEL-positive cells in this group. In other brain regions during aging, a reduction in Bcl-2 levels may lead to increased cell vulnerability (Ureshino et al., 2010). However, in our model of aging and CSR, we were unable to correlate changes in apoptotic protein expression with hippocampal apoptosis.

5. Conclusions

Our data indicate that CSR induces alterations in Ca^{2+} signaling in aged rats and that although apoptosis is present in the hippocampus, apoptotic protein expression is not affected. Because chronic SD is quite common in our modern society, particularly during aging, it is important to evaluate changes in Ca^{2+} signaling during CSR to better understand the pathophysiological mechanisms associated with aging. The increase in $[Ca^{2+}]_c$ after CSR suggests that sleep loss perturbs Ca^{2+} signaling mechanisms that are compromised by aging.

Disclosure statement

This was not an industry supported study. The authors have indicated no financial conflicts of interest.

Acknowledgments

The authors gratefully acknowledge the invaluable assistance of Juliana Cini Perry and Walquíria Arruda. This work was supported by grants from Associação Fundo de Incentivo a Pesquisa (AFIP) and FAPESP (no 08/50424-3 to L.S. and CEPID no 98/14303-3 to S.T.). MLA and ST are recipients of the CNPq fellowship.

References

Abramov AY, Duchen MR. Impaired mitochondrial bioenergetics determines glutamate-induced delayed calcium deregulation in neurons. Biochim Biophys Acta 2010;1800(3):297–304.
Alberdi E, Sánchez-Gómez MV, Cavaliere F, Pérez-Samartín A, Zugaza JL, Trullas R, et al. Amyloid beta oligomers induce Ca2 + dysregulation and neuronal death through activation of ionotropic glutamate receptors. Cell Calcium 2010;47(3):264–72.
Andersen ML, Bignotto M, Papale LA, Tufik S. Age-related effects on genital reflexes induced by paradoxical sleep deprivation and cocaine in rats. Exp Gerontol 2004a;39:233–7.
Andersen ML, D'Almeida V, Ko GM, Kawakami R, Martins PJ, Magalhaes LE, et al. Ethical and Practical Principles on the Use of Laboratory Animals, Universidade Federal de São Paulo (editor), São Paulo; 2004b.
Andersen ML, Ribeiro DA, Bergamaschi CT, Alvarenga TA, Silva A, Zager A, Campos RR, Tufik S. Distinct effects of acute and chronic sleep loss on DNA damage in rats. Prog Neuropsychopharmacol Biol Psychiatry 2009;33(3):562–7.
Biswas S, Mishra P, Mallick BN. Increased apoptosis in rat brain after rapid eye movement sleep loss. Neuroscience 2006;142(2):315–31.
Bonnet MH, Arand DL. We are chronically sleep deprived. Sleep 1995;18(10):908–11.
Bonnet MH, Rosa RR. Sleep and performance in young adults and older normals and insomniacs during acute sleep loss and recovery. Biol Psychol 1987;25(2):153–72.
Bortner CD, Oldenburg NB, Cidlowski JA. The role of DNA fragmentation in apoptosis. Trends Cell Biol 1995;5:21–6.
Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal Biochem 1976;72:248–54.
Brewer LD, Thibault O, Staton J, Thibault V, Rogers JT, Garcia-Ramos G, Kraner S, Landfield PW, Porter NM. Increased vulnerability of hippocampal neurons with age in culture: temporal association with increases in NMDA receptor current, NR2A subunit expression and recruitment of L-type calcium channels. Brain Res 2007;1151:20–31.
Broman JE, Lundh LG, Hetta J. Insufficient sleep in the general population. Neurophysiol Clin 1996;26(1):30–9.
Budd SL, Nicholls DG. Mitochondria, calcium regulation, and acute glutamate excitotoxicity in cultured cerebellar granule cells. J Neurochem 1996;67(6):2282–91.
Chen C, Hardy M, Zhang J, LaHoste GJ, Bazan NG. Altered NMDA receptor trafficking contributes to sleep deprivation-induced hippocampal synaptic and cognitive impairments. Biochem Biophys Res Commun 2006;340:435–40.
Cirelli C, Shaw PJ, Rechtschaffen A, Tononi G. No evidence of brain cell degeneration after long-term sleep deprivation in rats. Brain Res 1999;840:184–93.
Coenen AM, van Luijtelaar EL. Stress induced by three procedures of deprivation of paradoxical sleep. Physiol Behav 1985;35(4):501–4.
Cortese BM, Mitchell TR, Galloway MP, Prevost KE, Fang J, Moore GJ, et al. Region-specific alteration in brain glutamate: possible relationship to risk-taking behavior. Physiol Behav 2010;99(4):445–50.
de Souza L, Andersen ML, Smaili SS, Lopes GS, Ho PS, Papale LA, Tufik S. Age-related changes during a paradigm of chronic sleep restriction. Behav Brain Res 2010;214(2):201–5.
Espiritu JR. Aging-related sleep changes. Clin Geriatr Med 2008;24(1):1-14.
Ferreiro E, Oliveira CR, Pereira CM. The release of calcium from the endoplasmic reticulum induced by amyloid-beta and prion peptides activates the mitochondrial apoptotic pathway. Neurobiol Dis 2008;30(3):331–42.
Frank MG. The mystery of sleep function: current perspectives and future directions. Rev Neurosci 2006;17(4):375–92.
Gavrieli Y, Sherman Y, Ben-Sasson SA. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J Cell Biol 1992;119(3):493–501.
Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J Biol Chem 1985;260(6):3440–50.
Hagewoud R, Havekes R, Novati A, Keijser JN, Van der Zee EA, Meerlo P. Sleep deprivation impairs spatial working memory and reduces hippocampal AMPA receptor phosphorylation. J Sleep Res 2010;19(2):280–8.
Hajieva P, Kuhlmann C, Luhmann HJ, Behl C. Impaired calcium homeostasis in aged hippocampal neurons. Neurosci Lett 2009;451(2):119–23.
Hipólide DC, D'Almeida V, Raymond R, Tufik S, Nóbrega JN. Sleep deprivation does not affect indices of necrosis or apoptosis in rat brain. Int J Neurosci 2002;112:155–66.
Joo HJ, Ma JY, Choo YG, Choi BK, Jung KY. Age-related alteration of intracellular ATP maintenance in the cell suspensions of mice cerebral cortex. Mech Ageing Dev 1999;110(1–2):1-12.
Kopp C, Longordo F, Nicholson JR, Lüthi A. Insufficient sleep reversibly alters bidirectional synaptic plasticity and NMDA receptor function. J Neurosci 2006;26(48):12456–65.
Kuwana T, Newmeyer DD. Bcl-2-family proteins and the role of mitochondria in apoptosis. Curr Opin Cell Biol 2003;15(6):691–9.
Lister JP, Barnes CA. Neurobiological changes in the hippocampus during normative aging. Arch Neurol 2009;66(7):829–33.
Lopes GS, Mora OA, Cerri P, Faria FP, Jurkiewicz NH, Jurkiewicz A, et al. Mitochondrial alterations and apoptosis in smooth muscle from aged rats. Biochim Biophys Acta 2004;1658(3):187–94.
Machado RB, Suchecki D, Tufik S. Sleep homeostasis in rats assessed by a long-term intermittent paradoxical sleep deprivation protocol. Behav Brain Res 2005;160:356–64.
Magnusson KR. Aging of glutamate receptors: correlations between binding and spatial memory performance in mice. Mech Ageing Dev 1998;104(3):227–48.
Magnusson KR, Nelson SE, Young AB. Age-related changes in the protein expression of subunits of the NMDA receptor. Brain Res Mol Brain Res 2002;99(1):40–5.
Magnusson KR, Brim BL, Das SR. Selective vulnerabilities of N-methyl-D-aspartate (NMDA) receptors during brain aging. Front Aging Neurosci 2010;2:11–25.
Marchi S, Giorgi C, Suski JM, Agnoletto C, Bononi A, Bonora M, De Marchi E, Missiroli S, Patergnani S, Poletti F, Rimessi A, Duszynski J, Wieckowski MR, Pinton P. Mitochondria-ros crosstalk in the control of cell death and aging. J Signal Transduct 2012;329635:1-17.
McDermott CM, LaHoste GJ, Chen C, Musto A, Bazan NG, Magee JC. Sleep deprivation causes behavioral, synaptic, and membrane excitability alterations in hippocampal neurons. J Neurosci 2003;23(29):9687–95.
McDermott CM, Hardy MN, Bazan NG, Magee JC. Sleep deprivation-induced alterations in excitatory synaptic transmission in the CA1 region of the rat hippocampus. J Physiol 2006;570(Pt 3):553–65.
Montes-Rodríguez CJ, Alavez S, Soria-Gómez E, Rueda-Orozco PE, Guzman K, Morán J, et al. BCL-2 and BAX proteins expression throughout the light–dark cycle and modifications induced by sleep deprivation and rebound in adult rat brain. J Neurosci Res 2009;87(7):1602–9.
Murchison D, Zawieja DC, Griffith WH. Reduced mitochondrial buffering of voltage-gated calcium influx in aged rat basal forebrain neurons. Cell Calcium 2004;36(1):61–75.

Naidoo N, Ferber M, Master M, Zhu Y, Pack AI. Aging impairs the unfolded protein response to sleep deprivation and leads to proapoptotic signaling. J Neurosci 2008;28(26):6539–48.
Novati A, Hulshof HJ, Granic I, Meerlo P. Chronic partial sleep deprivation reduces brain sensitivity to glutamate N-methyl-D-aspartate receptor-mediated neurotoxicity. J Sleep Res 2012;21(1):3–9.
Papale LA, Andersen ML, Antunes IB, Alvarenga TA, Tufik S. Sleep pattern in rats under different stress modalities. Brain Res 2005;1060(1–2):47–54.
Paxinos G, Watson C. The rat brain in stereotaxic coordinates. 4th ed. San Diego: Academic Press; 1998.
Pivovarova NB, Nguyen HV, Winters CA, Brantner CA, Smith CL, Andrews SB. Excitotoxic calcium overload in a subpopulation of mitochondria triggers delayed death in hippocampal neurons. J Neurosci 2004;24(24):5611–22.
Plassman BL, Langa KM, Fisher GG, Heeringa SG, Weir DR, Ofstedal MB, et al. Prevalence of cognitive impairment without dementia in the United States. Ann Intern Med 2008;148(6):427–34.
Ravassard P, Pachoud B, Comte JC, Mejia-Perez C, Scoté-Blachon C, Gay N, et al. Paradoxical (REM) sleep deprivation causes a large and rapidly reversible decrease in long-term potentiation, synaptic transmission, glutamate receptor protein levels, and ERK/MAPK activation in the dorsal hippocampus. Sleep 2009;32(2):227–40.
Segovia G, Porras A, Del Arco A, Mora F. Glutamatergic neurotransmission in aging: a critical perspective. Mech Ageing Dev 2001;122(1):1-29.
Singh R, Kiloung J, Singh S, Sharma D. Effect of paradoxical sleep deprivation on oxidative stress parameters in brain regions of adult and old rats. Biogerontology 2008;9(3):153–62.
Smaili SS, Hsu YT, Youle RJ, Russell JT. Mitochondria in Ca^{2+} signaling and apoptosis. J Bioenerg Biomembr 2000;32(1):35–46.
Smaili SS, Rosenstock TR, Hsu YT. Evaluation of some cell death features by real time real space microscopy. Methods Enzymol 2008;442:27–50.
Süer C, Dolu N, Artis AS, Sahin L, Yilmaz A, Cetin A. The effects of long-term sleep deprivation on the long-term potentiation in the dentate gyrus and brain oxidation status in rats. Neurosci Res 2011;70(1):71–7.
Tadavarty R, Kaan TK, Sastry BR. Long-term depression of excitatory synaptic transmission in rat hippocampal CA1 neurons following sleep-deprivation. Exp Neurol 2009;216(1):239–42.
Tan S, Wood M, Maher P. Oxidative stress induces a form of programmed cell death with characteristics of both apoptosis and necrosis in neuronal cells. J Neurochem 1998;71(1):95-105.
Tartar JL, McKenna JT, Ward CP, McCarley RW, Strecker RE, Brown RE. Sleep fragmentation reduces hippocampal CA1 pyramidal cell excitability and response to adenosine. Neurosci Lett 2010;469(1):1–5.
Thees S, Hubbard GB, Winckler J, Schultz C, Rami A. Specific alteration of the Bax/Bcl2 ratio and cytochrome c without execution of apoptosis in the hippocampus of aged baboons. Restor Neurol Neurosci 2005;23(1):1–9.
Toescu EC. Normal brain ageing: models and mechanisms. Philos Trans R Soc Lond B Biol Sci 2005;360(1464):2347–54.
Toescu EC, Verkhratsky A. Ca^{2+} and mitochondria as substrates for deficits in synaptic plasticity in normal brain ageing. J Cell Mol Med 2004;8(2):181–90.
Toescu EC, Verkhratsky A. The importance of being subtle: small changes in calcium homeostasis control cognitive decline in normal aging. Aging Cell 2007;6(3):267–73.
Tonkikh A, Janus C, El-Beheiry H, Pennefather PS, Samoilova M, McDonald P, et al. Calcium chelation improves spatial learning and synaptic plasticity in aged rats. Exp Neurol 2006;197(2):291–300.
Tufik S, de Luca Nathan C, Neumann B, Hipólide DC, Lobo LL, de Medeiros R, et al. Effects of stress on drug-induced yawning: constant vs. intermittent stress. Physiol Behav 1995;58(1):181–4.
Tufik S, Andersen ML, Bittencourt LR, Mello MT. Paradoxical sleep deprivation: neurochemical, hormonal and behavioral alterations. Evidence from 30 years of research. An Acad Bras Cienc 2009;81(3):521–38.
Ureshino RP, Bertoncini CR, Fernandes MJ, Abdalla FM, Porto CS, Hsu YT, et al. Alterations in calcium signaling and a decrease in Bcl-2 expression: possible correlation with apoptosis in aged striatum. J Neurosci Res 2010;88(2):438–47.
Van Hulzen ZJ, Coenen AM. Paradoxical sleep deprivation and locomotor activity in rats. Physiol Behav 1981;27(4):741–4.
Verkhratsky A, Toescu EC. Calcium and neuronal ageing. Trends Neurosci 1998;21:2–7.
Walker MP. Cognitive consequences of sleep and sleep loss. Sleep Med 2008;9(Suppl 1):S29–34.
Winters BD, Huang YH, Dong Y, Krueger JM. Sleep loss alters synaptic and intrinsic neuronal properties in mouse prefrontal cortex. Brain Res 2011;420:1–7.
Xiong J, Verkhratsky A, Toescu EC. Changes in mitochondrial status associated with altered Ca^{2+} homeostasis in aged cerebellar granule neurons in brain slices. J Neurosci 2002;22(24):10761–71.
Xiong J, Camello PJ, Verkhratsky A, Toescu EC. Mitochondrial polarisation status and [Ca2 +]i signalling in rat cerebellar granule neurones aged in vitro. Neurobiol Aging 2004;25(3):349–59.