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INCREASED APOPTOSIS IN RAT BRAIN AFTER RAPID EYE MOVEMENT SLEEP LOSS

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Abstract—Rapid eye movement (REM) sleep loss impairs several physiological, behavioral and cellular processes; however, the mechanism of action was unknown. To understand the effects of REM sleep deprivation on neuronal damage and apoptosis, studies were conducted using multiple apoptosis markers in control and experimental rat brain neurons located in areas either related to or unrelated to REM sleep regulation. Furthermore, the effects of REM sleep deprivation were also studied on neuronal cytoskeletal proteins, actin and tubulin. It was observed that after REM sleep deprivation a significantly increased number of neurons in the rat brain were positive to apoptotic markers, which however, tended to recover after the rats were allowed to undergo REM sleep; the control rats were not affected. Further, it was also observed that REM sleep deprivation decreased amounts of actin and tubulin in neurons confirming our previous reports of changes in neuronal size and shape after such deprivation. These findings suggest that one of the possible functions of REM sleep is to protect neurons from damage and apoptosis.

Key words: bcl-2/bax, cytoskeletal proteins, electron and confocal-microscopy, Hoechst, REM sleep deprivation, TUNEL.

Rapid eye movement (REM) sleep is a unique physiological phenomenon present at least across mammalian species (Frank, 1999). Ontogeny studies showed that it is maximum in the pre-term (immature) babies and gradually reduces in the newborn and with ageing (Roffwarg et al., 1966; Segawa, 1999). REM sleep or total sleep (that includes REM sleep) has significant developmental and life sustaining functions to the extent that its loss is fatal. REM sleep is reduced in neurodegenerative diseases viz. Parkinson's and Alzheimer's diseases (Christos, 1993; Baumann et al., 2005). Rapid eye movement sleep deprivation (REMSD) reduces brain maturation, spatial memory acquisition (Youngblood et al., 1997) and memory consolidation (Graves et al., 2003). Conflicting findings on neuronal loss have been reported after total sleep loss (that includes REM sleep loss as well). Some did not find any neuronal loss (Cirelli et al., 1999; Hipolide et al., 2002); however, others reported degeneration of neurons (Bentivoglio and Grassi-Zucconi, 1997; Eiland et al., 2002). Several other studies showed that sleep plays a significant role in the development of neuronal connectivity and its loss has adverse effects on neuronal growth and development (Oksenberg et al., 1996; Shaffery et al., 1998; Jha et al., 2005).

REM sleep loss affects neuronal responsiveness (Mallick et al., 1990, 1991) and brain excitability (Cohen et al., 1970; Mallick et al., 1999, 2002). This could be induced by alterations in several physico-chemico-physiological factors associated with REM sleep loss (Vogel, 1975; Kushida et al., 1989; Gulyani et al., 2000; Zenko et al., 2000; Mallick et al., 1999, 2005). Changes in at least some of these factors e.g. Na^+,K^+-ATPase (Gulyani and Mallick, 1993; Mallick et al., 2000), norepinephrine (NE) (Porkka-Heiskanen et al., 1995; Shouse et al., 2000; Majumdar and Mallick, 2003), calcium ions (Mallick and Gulyani, 1996), membrane fluidity (Mallick et al., 1995) might affect neuronal size, structure and well-being (Vaillend et al., 2002; Spafford et al., 2004), which in turn might play a significant role in REMSD associated alterations in neuronal survival, longevity and structural integrity. The latter may be supported by our recent findings that REMSD alters neuronal size and shape (Majumdar and Mallick, 2005). Therefore, we proposed that one of the functions of REM sleep is to maintain neuronal integrity, longevity and survivability. We hypothesized that since REMSD affects process(es) that are vital for neuronal health and well-being, it would affect neuronal survival as well. Further, owing to its previously reported effects on neuronal structural integrity, it would also affect the structural proteins actin and tubulin. Therefore, in this study, effects of varying periods of REMSD were studied *in situ* on several cellular degeneration and apoptotic markers and neuronal cytoskeletal proteins.

EXPERIMENTAL PROCEDURES

Animals and REMSD (Jouvet et al., 1964; Gulyani and Mallick, 1993)

All experiments were approved by the Institutional Animal Ethics Committee and conformed to NIH guidelines on the ethical use of animals. Efforts were made to minimize the number of animals used and their suffering. Experiments were conducted on inbred male Wistar rats (250–300 g) maintained with food and water *ad libitum* under 12-h light/dark cycle. Free-moving control (FMC)

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Abbreviations: ANOVA, analysis of variance; DAB, 3,3′diaminobenzidine; DPX, distrene plasticizer xylene; EM, electron microscopy; FITC, fluorescein isothiocyanate; FMC, free-moving control; IgG, immunoglobulin; LC, locus coeruleus; LDT, laterodorsal tegmentum; LPC, large platform control; LS, lateral septum; MPO, medial preoptic area; NE, norepinephrine; NGS, normal goat serum; PBS, phosphate-buffered saline; PPT, pedunculopontine tegmentum; REC, recovery; REM, rapid eye movement; REMSD, rapid eye movement sleep deprivation; TdT, terminal deoxynucleotidal transferase; TEM, transmission electron microscopy; TUNEL, TdT-mediated dUTP nick end labeling.

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Table 1. Mean (±SEM) change in amino-cupric-silver, stained neurons with respect to FMC values after 4, 6 and 10 days of REMSD

Area	4 Day REMSD (mean±SEM)	6 Day REMSD (mean±SEM)	10 Day REMSD (mean±SEM)
LC	17.10±1.35	52.40±2.31 [$F(1,5)=225.0$, $P<0.001$]	55.56±1.68 [$F(1,5)=489.7$, $P<0.001$]
LDT/PPT	19.96±2.47	52.00±1.80 [$F(1,5)=235.2$, $P<0.001$]	54.20±1.52 [$F(1,5)=337.0$, $P<0.001$]
MPO	19.23±2.67	50.23±3.20 [$F(1,5)=113.8$, $P<0.001$]	54.40±3.33 [$F(1,5)=132.3$, $P<0.001$]
LS	15.13±1.31	15.43±0.52	15.60±1.71

F and *P* values (ANOVA) indicated below the mean values. (Abbreviations are same as in the text)

rats were maintained in their normal dry home cages. Experimental rats were REM sleep deprived by the classical flowerpot method (Jouvet et al., 1964). For REMSD, rats were kept on a 6.5 cm diameter platform surrounded by water. In pilot studies, separate groups of rats were REM sleep deprived for 4, 6 and 10 days. As mentioned in the results that amino-cupric silver stain showed significantly more black stained neurons only after 6 and 10 days and not after 4 days of REMSD (Table 1). Hence, to inflict minimum discomfort, the rats were REM sleep deprived for 6 days only in this study. To rule out non-specific effects another group of control rats was maintained on larger (diameter 13 cm) platform (LPC) surrounded by water i.e. except the size of the platform rest all other conditions remained identical to that of the experimental animals. A fourth recovery group (REC) included those animals that had been REM sleep deprived for 6 days and then allowed to live in normal cages for 3 days to recover from lost REM sleep. Three days were allowed for REC because we had observed that such period was sufficient for the REMSD-induced changes in cellular morphology (Majumdar and Mallick, 2005) and enzyme activity (Gulyani and Mallick, 1993; Majumdar and Mallick, 2003) to return to the level of FMC. Thus, in each set, there was one rat each of FMC, REMSD, LPC and REC and three such sets were carried out in all the experiments. At the end of the experiments, the controls as well as the experimental rats were anesthetized with sodium pentobarbital (45 mg/kg, i.p.; Sigma, St. Louis, MO, USA). The brains were intracardially perfused with 0.1 M phosphate-buffered saline (PBS) and 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) (2% paraformaldehyde and 2% glutaraldehyde for electron microscopic studies). The brains were removed and fixed overnight in the same fixative. After fixation, the brains were cryoprotected in 30% sucrose in PBS. Thereafter, frozen sections (20–40 μm) through locus coeruleus (LC), laterodorsal tegmentum/pedunculopontine tegmentum (LDT/PPT), medial preoptic area (MPO) and lateral septum (LS) as per the atlas of Paxinos and Watson (1998) in conformity with our previous studies (Majumdar and Mallick, 2005) were cut using a Leica cryostat (Leica, Solms, Germany), collected in vials containing PBS and stored at 4 °C for staining. Due to the small size of the areas studied (LC, LDT/PPT, MPO, LS) and need for large number of sections of different thickness for each procedure it was necessary to use separate brains. Separate rat brains were used for different staining procedure except for bcl-2 and bax staining which were done in serial sections. The number of rats and sections used for each staining method have been mentioned in Tables 2 and 3. The thickness of sections used for different staining procedures was based on the requirements of individual protocols. 40 μm sections were used for immunohistochemistry (bcl-2, bax, actin and tubulin), 35 μm section for animo cupric silver staining and 20 μm sections for terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) and Hoechst staining procedures. For electron microscopy (EM), ultrathin sections were cut from 1 mm chucks of tissue (individually from each area) embedded in araldite.

Amino cupric silver staining (de Olmos et al., 1994)

The sections were stained with amino cupric silver as per the protocol of de Olmos et al. (1994). Briefly, 35 μm thick sections were pre-incubated at 50 °C for 50 min followed by cooling to room temperature for 2–3 h. Sections from experimental and control rat brains were taken in adjacent wells of a multi-welled plate and processed simultaneously in the same solutions so that all the conditions were maintained identically. They were subsequently incubated in diamine silver solution for 45 min at room temperature in a rotating platform. The sections were then transferred to reducer solution at 32 °C for 25 min to which 1.2 ml of the same diamine solution per 100 ml of reducer was added in four batches of 5 min each. Following this step, the sections were treated with 0.5% glacial acetic acid (Sigma) to stop further reduction. The sections were washed thoroughly in distilled water and then bleached first in a solution of 6% potassium ferricyanide (Merck & Co. Inc., Whitehouse Station, NJ, USA) and lactic acid (Sigma) and subsequently in a solution of 0.06% potassium permanganate (Merck & Co. Inc.) and 5% sulfuric acid. After washing in distilled water, sections were transferred to 2% sodium thiosulfate (Sigma) for stabilization. They were then treated with Rapid Fixer solution (Agfa Photo GmbH, Koln, Germany; diluted 1:6 times in deionized water) following which the sections became transparent. They were washed in distilled water, mounted on gelatin-coated slides, counterstained with 0.5% Neutral Red, dehydrated in ethanol and coverslipped with DPX mounting medium.

EM

EM was carried out in the National EM Facility of All-India Institute of Medical Sciences, New Delhi where the following protocol was followed. Tissue chucks approximately 1 mm around LC, LDT/PPT and MPO regions in the brain were cut and fixed for 18 h in 2% paraformaldehyde and 2% glutaraldehyde solution. They were transferred to PBS and subsequently processed for transmission electron microscopy (TEM). The tissues were postfixed in 1% osmium tetroxide (Sigma) for 2 h at 4 °C. The tissues were then dehydrated in grades of ethanol cleared in toluene and infiltrated with araldite resin and blocks were prepared by embedding in araldite. After polymerization of the embedding medium, ultra-thin sections were collected on metal grids, which were stained using uranyl acetate and lead citrate (both from Sigma). After washing in distilled water, the grids were stored in airtight Petri-plates for viewing under the TEM.

Immunohistochemistry (bcl-2, bax, actin and tubulin) (Majumdar and Mallick, 2003)

Sections were treated with 10% normal goat serum (NGS) for 2 h for blocking and then incubated in the primary antibody for 3 days at 4 °C. The antibody was diluted in 0.1 M PBS containing 5% NGS and 0.5% Triton X-100. Primary antibodies, anti-bcl-2 (1:300), anti-bax (1:300) and anti-tubulin (1:500), all three from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA and anti-actin (1:500) from Sigma were used in this study.

After washes in PBS, the sections were incubated in biotinylated goat anti-rabbit immunoglobulin (IgG), goat anti-mouse IgG or rabbit anti-mouse IgG (Vector Laboratories, Burlingame, CA, USA) at 1:200 for 18 h at 4 °C. Following this secondary antibody step, the sections were washed and placed in preformed avidin–biotin–peroxidase complex (ABC) (Elite Vectastain ABC kit, Vector Laboratories) at 1:400 dilution for 2 h at room temperature. For visualization of the reaction sites, the sections were treated with