Chanana and Kumar, J Sleep Disord Ther 2017, 6:1
DOI: 10.4172/2167-0277.1000258

Journal of
Sleep Disorders & Therapy

Research Article Open Access

An Insight into Mechanisms underlying Sleep Deprivation Induced Cognitive Dysfunction

Priyanka Chanana and Anil Kumar*

Pharmacology Division, University Institute of Pharmaceutical Sciences, UGC Centre of Advanced Study, Panjab University, Chandigarh, India

Abstract

Introduction: Sleep has been found to influence both acquisition and consolidation of memory; therefore, sleep deprivation is often linked to multiple anomalies in cognitive paradigms giving rise cognitive dysfunctions.

Objective: The present study has been designed with an aim to decipher plausible mechanistic interplays involved in the induction of cognitive dysfunction as a direct consequence of sleep deprivation.

Methods: Male *laca* mice were sleep deprived for 24-hours using grid suspended over water method on alternate days extended over the total protocol duration of 21 days. Various neurobehavioral memory evaluating parameters followed by biochemical, acetylcholinesterase (AChE) activity, mitochondrial respiratory enzyme complex activities (I-IV) and histopathological examinations of the mice brain were monitored.

Results: Sleep deprivation of 24-hours on alternate days for 21 days significantly impaired cognitive performance in both memory evaluating paradigms (Morris water maze test and elevated plus maze test) as compared to naïve animals. Additionally animals subjected to sleep deprivation also demonstrated conditions of elevated oxidative stress, impaired mitochondrial enzyme complex activities, increased aceytlcholineestaerase activity, as well as histopathological alterations pertaining to hippocampal and thalamo-cortical regions of mice brain as compared to naïve animals. However, chronic piracetam (100 mg/kg) treatment showed significant protective effect against sleep deprivation induced cognitive dysfunction, oxidative damage, mitochondrial respiratory enzyme complex insuffeciencies, increased acetylcholinesterase activity as well as neuromorphological alterations.

Conclusion: The present study suggests mechanistic interplay between oxidative stresses; mitochondrial impairment as well as acetylcholineesterase activity may influence hippocampal and cortical neuronal survival and thus lead to precipitation of sleep deprivation induced cognitive deficits.

Keywords: Cognition; Hippocampus; Mitochondrial insufficiency; Oxidative stress; Sleep deprivation

Introduction

Extensive research has portrayed the role played by sleep dynamics in nurturing of cognitive profiles via influencing both acquisitions as well as consolidation paradigms of memory formation [1-5]. It has been validated that sleep affects almost every individual component of cognition such as stimulus detection, information encoding, working memory, etc. Against this background, sleep deprivation [SD] is entitled to cause cognitive deficits. The direct effect of SD over cognitive paradigms may be manifested either via decreased alertness and attention lapses as well as slowed responses owing to sleep debt. SD has also led to deleterious structural and functional instabilities of selective encephalic structures controlling cognitive processes which may partially explain the cognitive impairments precipitated by SD [6,7]. The same has been investigated by using potent non-invasive neurophysiological techniques such as Transcranial Magnetic Stimulation (TMS) which specifically evaluates primary motor cortex (M1) and cortico spinal tract excitability in vivo. The significant alterations observed in key characteristics of single and paired TMS techniques (as a consequence of SD) clearly demonstrate significant variations in cortical excitability as well as functionality of inter-neuronal circuitry in the primary motor cortex brought and thus aptly explain pathophysiological substrates of SD induced cognitive impairments [8-10].

Though all this has been well researched but still, there remain several lacunae in the molecular mechanistic interplays that may facilitate SD mediated phenotypes of cognitive deficits. The present study is an endeavor towards the same.

Several plausible mechanisms may be purported for the SD induced cognitive lapses. SD has been directly linked to precipitating situations of elevated oxidative stress [11-13]. SD has been found to be linked to not only elevated generation of free radicals but also depletion in the activity of endogenous antioxidant enzymes [12]. Also, nitric oxide (NO) is thought to be involved in memory consolidation processes in the hippocampus during sleep [14]. Additionally, SD has also been found to be associated with mitochondrial respiratory enzyme complex insufficiencies as well as *Bax* activation [15], directly leading to situations of energy deficits as well as indirect activation of apoptotic responses. This suggests that both conditions of oxidative damage and energy deficits may influence neuromorphological changes as well survival of neurons specifically in areas of the brain responsible for memory encoding and consolidation, particularly the hippocampus [16], which may be partially held responsible for cognitive deficits precipitated as a direct consequence of SD. Furthermore, SD has also demonstrated to increase acetylcholinesterase (AChE) activity, which may probably lead to deficits in memory tasks [17,18].

***Corresponding author:** Anil Kumar, Pharmacology Division, University Institute of Pharmaceutical Sciences, UGC Centre of Advanced Study, Panjab University, Chandigarh, India, Tel: 911722534106; Fax: 91 172 2543101; E-mail: kumaruips@yahoo.com

Received October 21, 2016; **Accepted** February 22, 2017; **Published** March 02, 2017

Citation: Chanana P, Kumar A (2017) An Insight into Mechanisms underlying Sleep Deprivation Induced Cognitive Dysfunction. J Sleep Disord Ther 5: 258 doi: 10.4172/2167-0277.1000258

Based on this background, the present study was designed with an aim to evaluate the possible crosstalks and mechanistic interplays between pivotal molecular cascades of oxidative damage, mitochondrial enzyme complex insuffeciencies, acetylcholineesterase activity as well as neuromorphological changes in memory controlling encephalic regions to be put forward as cardinal etiological links between SD and induction of cognitive dysfunction.

Material and Method

Animals

Male *laca* mice (22 g to 30 g) bred in Central Animal House (CAH) facility of the Panjab University, Chandigarh, India were used. The animals were housed under standard laboratory conditions and maintained under natural light-dark cycles. The experimental protocol was approved by Institutional Animal Ethics Committee (PU/IAEC/S/14/99) of Panjab University and carried out in accordance with the guidelines of Committee for the Purpose of Control and Supervision of Experimentation on Animals (CPCSEA), Government of India and Indian National Science Academy.

Sleep Deprivation and protocol design

Animals were sleep-deprived for 24-hours (every alternate day) across the 21 days protocol by using modified grid suspended over water method developed by Shinomiya et al. [19]. Briefly, animals in a group of six to eight were sleep deprived by placing on a grid floor (29 × 15 × 7 cm) inside the polyacrylic cages (38 × 23 × 10 cm), filled with water upto 1 cm below the grid surface for 24-hours (every alternate day for 21 days, to comply with clinical situations). The stainless steel rods of the grid (3 mm wide) were set 2-cm apart from each other. Food and water were provided ad libitum. As soon as the animal tried to sleep, it lost its muscle tone and thereby grip over the grid which made it fall in water and thus awakened the animal. The process was continued for total of 21 days. During the course of the protocol behavioral estimations like locomotor activity was ascertained on day 0, 7, 14 and 21; elevated plus maze performance was evaluated on day 15, 16 and 21 as well as morris water maze test performance was evaluated from day 17th to day 21st.

S.No.	Group name	Treatment (mg/kg)
1	Naïve	Animals not subjected sleep deprivation [vehicle treated]
2	SD	Sleep deprived animals [subjected to sleep deprivation of 24 hours on alternate days for 21 days and vehicle treated]
3	Pira (100)	Piracetam (100 mg/kg) administered in sleep deprived animals for 21 days

Table 1: Treatment groups.

Drugs and Treatment Schedule

Animals were randomly divided into different treatment groups (n=10 to 12) as described in (Table 1). The entire study has been performed as per the schematic plan given in the protocol as described in (Figure 1). Across the total protocol duration of 21 days animals were exposed to standardized treatments once daily, based on their treatment groups. While animals in the naive and sleep deprived control groups were treated with vehicle i.e. normal saline i.p; the animals in the standard group were treated with Piracetam (100 mg/kg i.p; prepared in normal saline). Piracetam (Pira) was purchased from CDH, India. The treatments (vehicle as well as standard) were administered to animals in volume of 1 ml/100 g of bodyweight.

Behavioral assessments

Measurement of locomotor activity: To rule out the interference of change in motor activity in the parameters of learning and memory, locomotor activity of animals was evaluated on weekly basis (i.e. day 0, 7, 14 and 21). The locomotor activity was recorded by using an actophotometer (IMCORP, Ambala, India). The instrument consisted of a closed arena or activity chamber in which each animal was placed for total of 8 minutes (3 minutes for habituation and exploration as well as 5 minutes for evaluation of actual locomotor activity). Animals's total activity (ambulation + rearing counts) was recorded and expressed in terms of total photo beam counts cut for 5 min per animal [20].

Assessment of cognitive performance (Morris Water Maze Task): Individual animals were tested for spatial memory using the Morris water maze (MWM) test paradigm [21,22]. The apparatus consisted of

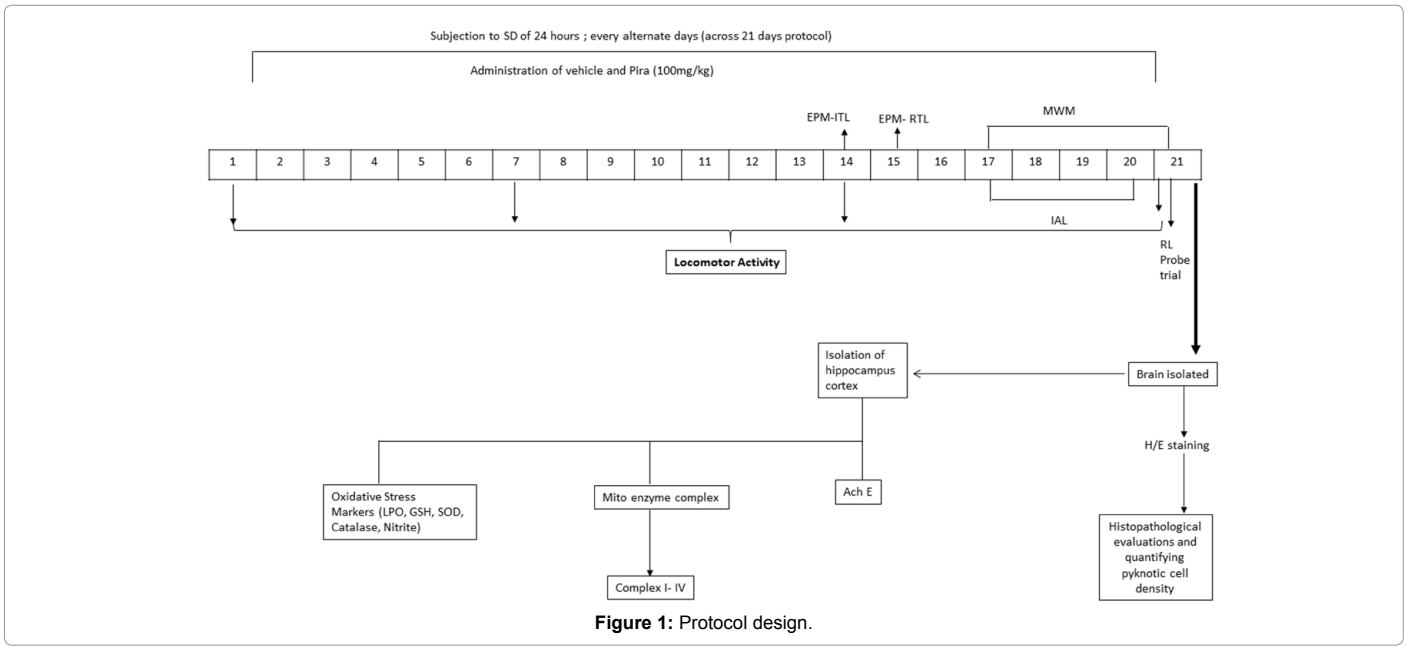

Figure 1: Protocol design.

a circular water tank (120 cm diameter, 30 cm high, filled to a depth of 20 cm with water at 28 ± 1°C). The tank was divided into four equal quadrants (Q1 to Q4) and water in the tank was made opaque with a nontoxic white dye. A platform (4.5 cm in diameter) was placed in one quadrant of the pool (Q4), 1 cm above the water level during the acquisition phase and position of the platform was kept unaltered all through the acquisition phase. The tank was located in a large room with presence of several brightly colored visible clues external to the maze which could be easily used by the mice for spatial orientation and further for memory consolidation. The task was carried out for five consecutive days (17^{th} to 21^{th} day, four days of acquisition and one day of retention trial). During each trial, the position of the clues remained unchanged and animal was subjected to four such consecutive trials (corresponding to each quadrant).

Acquisition phase (training): For each trial, individual mice was smoothly put into the water at one of the four starting positions (corresponding to each quadrant) to locate the submerged platform (maintained at Q4) and allowed to stay on the platform for next 20 seconds. If animal failed to find the platform within 90 seconds, same was gently guided onto the platform. Latency time to locate the hidden platform (known as initial acquisition latency (IAL) also known as escape latency time (ELT) was noted as parameter of acquisition or learning using a video-graphed computer tracking system maintained by EthoVision software (Noldus Information Technology, Wageningen, Netherlands).

Retention phase (Probe Trial): To assess the extent of memory consolidation after learning, retention trial was performed [23] on day 21 wherein the animal was placed into the pool for a total duration of 90 seconds as in the training trial, with the absence of hidden platform from the Q4 quadrant of the pool. Various parameters such as time spent in target quadrant [TSTQ] and number of crossings across the platform area were measured using above mentioned computer tracking system [24].

Elevated Plus Maze Task: The elevated plus maze (EPM) consisted of two opposite black open arms (16 x 9 x 5 cm), crossed with two closed walls of the same dimensions of 12 cm height. The arms were connected with a central square of dimensions 5 × 9 × 5 cm. The entire maze was elevated to a height of 25 cm from the floor. Both acquisition and retention of memory processes were assessed as previously described. Acquisition of memory was tested on day 15 of study protocol. Animal was placed individually at one end of the open arm facing away from the central square. The time taken by the animal to move from the open arm to the closed arm was recorded as the initial transfer latency (ITL; noted on day 15). Cut off time was set to be 90 seconds and if the animal could not enter closed arm within 90 seconds, same was guided to the closed arm and the ITL recording was noted as 90 seconds. Animal was allowed to explore the maze for 30 seconds after recording the ITL and then returned to the home cage. Retention of memory was assessed on day 16^{th} (1^{st} RTL) and 21^{st} (2^{nd} RTL) by placing the mouse again in an open arm and ascertaining the time taken to cross over to the closed arm [25].

Biochemical estimations

Dissection and homogenization: Thereupon, after the last behavioral test, animals were randomized into distinct sets; the first set was used for the biochemical assays (n=5/group). For biochemical analysis, animals were sacrificed by decapitation. Whole brain from each animal was put over ice and dissected into hippocampus and cerebral cortex sections. For antioxidant enzyme activity estimation, a 10% (w/v) tissue homogenates were prepared in 0.1 M phosphate buffer (pH 7.4). The homogenates were centrifuged at 10,000 × g at 4°C for 15 min. Aliquots of supernatants were separated and used for biochemical estimations.

Lipid peroxidation assay: The quantitative measurement of lipid peroxidation in the whole brain was measured in accordance with the method of Wills [26]. The amount of malondialdehyde (MDA) concentration formed was measured by reaction with thiobarbituric acid at 532 nm using Perkin Elmer lambda 20 UV-vis spectrophotometer (Norwalk, CT, USA). The results were expressed as nano moles of MDA per milligram protein using the molar extinction coefficient of chromophore (1.56 × 10 M/cm) and represented as percentage of naive.

Estimation of nitrite levels: The nitrite accumulation in the homogenate supernatant (an indicator of the production of nitric oxide (NO load), was determined via a colorimetric assay using Greiss reagent (0.1% N-(1-napthyl) ethylene diaminedihydrochloride, 1% sulfanilamide and 2.5% phosphoric acid) as per the method reported by Green and co-workers [27]. Equal volumes of supernatant and Greiss reagent were mixed, incubated for 10 min at room temperature; consequently the absorbance of reaction mixture was read at 540 nm using a spectrophotometer (Perkin Elmer lambda 20 UV-Visible spectrophotometer, Norwalk, CT, USA). The concentration of nitrite in the supernatant was determined from a standard curve. The results were expressed as mg/ml and represented as percentage of naïve.

Estimation off reduced glutathione: Reduced glutathione (GSH) was estimated according to the method described by Ellman [28]. 1.0 ml of homogenate was precipitated using 1.0 ml of 4% sulfosalicylic acid by keeping the mixture at 4°C for 1 hour and the samples were immediately centrifuged at 1200 g for 15 min at 4°C. The assay mixture contained 0.1 ml of supernatant, 2.7 ml of phosphate buffer (pH 8.0) and 0.2 ml of 0.01 mL M-dithiobisnitrobenzoic acid. The yellow colour developed was read immediately at 412 nm using spectrophotometer (Perkin Elmer lambda 20 UV-Vis spectrophotometer (Norwalk, CT, USA). The results were expressed as nano moles of GSH per milligram of protein and represented as percentage of naive.

Estimation off catalase: Catalase activity was assayed by the method reported by Luck [29], wherein the breakdown of hydrogen peroxide (H_2O_2) was measured at 240 nm. Briefly, the assay mixture consisted of 3 ml of H_2O_2 phosphate buffer (1.25×10^{-2} M H_2O_2) and 0.05 mL of supernatant of tissue homogenate (10%). The change in absorbance was recorded at 240 nm using UV-Vis spectrophotometer (Perkin Elmer lambda 20 spectrophotometer (Norwalk, CT, USA). The results were expressed as micromole H_2O_2 decomposed per milligram of protein/min and represented as percentage of naive.

Superoxide Dismutase activity: Superoxide dismutase (SOD) activity was assayed by the method of Kono [30]. According to the method, reduction of nitro blue tetrazolium (NBT) was inhibited by the SOD and the change in absorbance was recorded for 2 minutes at 30 seconds intervals by measuring absorbance at 560 nm using Perkin Elmer Lambda 20 spectrophotometer. The results were expressed as units/mg protein and represented as percentage of naive.

Protein estimation: The total protein content was measured according to Biuret method described by Lowry and his team [31]. The amount was calculated using a standard curve wherein bovine serum albumin was used as the protein standard.

Mitochondrial enzyme complex estimations: The brain samples

of second set of animals were used for evaluation of mitochondrial respiratory enzyme complex activities as described by Berman and Hastings [32]. The whole brain was sectioned into hippocampi and cortical regions and individual sections were then homogenized in isolation buffer (215 mM mannitol, 75 mM sucrose, 0.1% BSA, 20 mM HEPES, 1 mM EGTA, and pH 7.2). Homogenates were centrifuged at 13,000 g for 5 minutes at 4°C. Pellets were re-suspended in isolation buffer with ethylene glycol tetra acetic acid (EGTA) and spun again at 13,000 g at 4°C for 5 min. The resulting supernatants were transferred to new tubes and topped off with isolation buffer with EGTA and again spun at 13,000 g at 4°C for 10 min. Pellets containing pure mitochondria were re-suspended in isolation buffer without EGTA.

Nicotinamide Adenine Dinucleotide (NADH) Dehydrogenase activity: The quantitative analysis of Complex-I was done spectrophotometrically by the method mentioned by King and Howard [33]. The method involved catalytic oxidation of NADH to NAD^+ with subsequent reduction in cytochrome c. The reaction mixture contained 0.2 M glycyl glycine buffer pH 8.5, 6 mM NADH in 2 mM glycyl glycine buffer and 10.5 mM cytochrome c. After addition of requisite amount of solubilised mitochondrial sample to reaction mixture, absorbance change was recorded at 550 nm for 2 minutes and the results were expressed as nano moles of NADH oxidized per minute per milligram protein and represented as percentage of naïve.

Succinate Dehydrogenase (SDH) activity: The spectrophotometric quantification of Complex-II/ SDH was done according to method reported by King [34]. The reaction mixture contained 0.2 M phosphate buffer (pH 7.8), 1% BSA, 0.6 M succinic acid, and 0.03 M potassium ferricyanide. The reaction was initiated by the addition of mitochondrial sample and recording absorbance change at 420 nm for 2 minutes. The results were expressed as nano moles of SDH per milligram of protein and represented as percentage of naïve.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT or mitochondrial redox activity) assay: This method is used to evaluate the mitochondrial redox activity via the conversion of MTT tetrazolium salt to formazan crystals by mitochondrial respiratory chain reactions in isolated mitochondria by the method of Liu et al. [35]. The blue formazan crystals so formed were then further solubilised with dimethylsulphoxide and corresponding absorbance was measured by an ELISA reader at 580 nm filter. The results were expressed number of viable cells per well and represented as percentage of naïve.

Cytochrome Oxidase activity: Cytochrome oxidase activity was assayed in brain mitochondria according to the method developed by Sottocasa et al. [36]. The assay mixture contained 0.3 mM reduced cytochrome C in 75 mM phosphate buffer. The reaction was started by the addition of solubilized mitochondrial sample and the changes in absorbance were recorded at 550 nm for 2 minutes (at intervals of 30 seconds each) and the results represented as percentage of naive.

Acetyl Cholinesterase (AChE) activity assay: AChE activity (marker of cholinergic activity in the brain) was assessed in the hippocampal and cortical regions by the method of Ellman et al. [37]. The assay mixture contained 0.05 ml of supernatant, 3 ml of sodium phosphate buffer (pH 8), 0.1 ml of acetylthiocholine iodide and 0.1 ml of DTNB (Ellman reagent). The change in absorbance was measured for 2 min at 30 seconds interval at 412 nm using a Perkin Elmer UV–Vis spectrophotometer. Results were expressed as micromoles of acetylthiocholine iodide hydrolyzed per minute per mg protein.

Histopathological Analysis by Hematoxylin and Eosin Staining (H&E) Staining

Tissue sections preparation: Third set of animals were scarified immediately after last behavioural test and perfused transcardially with cold phosphate buffered saline (0.1 M, pH 7.4) via the ascending aorta. The whole brain was dissected out and fixed overnight in the same buffer containing 10% (v/v) paraformaldehyde. The brain was then washed with 0.1 M PBS (pH 7.4) for 1 hour, dehydrated in alcohol, and consecutively embedded in paraffin wax to form blocks. Freezing microtome was used to obtain serial coronal sections (5 µm to 10 µm thickness) of whole brain.

Haematoxylin and Eosin (H&E) staining: The brain sections (5 µm to 10 µm) thick were de-waxed and stained with haematoxylin and eosin. Briefly, sections were immersed in the filtered haematoxylin solution for 1 min followed by rinsing with tap water. Then, the sections were immersed in eosin stain for 1 min to 2 min and rinsed thoroughly with tap water followed by dehydration in ascending alcohol solutions (50%, 70%, 80%, 95%, 97% and 100% alcoholic solutions). Now the sections were mounted on labelled slides. The stained sections were viewed under a binocular microscope (Nikon Eclipse 80i, Nikon Instruments Inc., USA) at 40× targeting the thalamo-cortical and hippocampal regions of the brain for the presence of morphological changes and thereby photographed. The thalamo-cortical encephalic area was specifically analyzed for the presence and density of pyknotic nuclei. The number of such pyknotic nuclei per square pixel were then quantified using computer based image processing and analysis (Image J 1.42q, NIH, USA).

Statistical Analysis

The behavioral assessment data was analyzed using two-way analysis of variance (ANOVA) followed by Bonferroni's test. The time spent in target quadrant, number of platform crossings, biochemical estimations, mitochondrial activity estimations, AchE activity as well as pyknotic cell density in the thalamo-cortical encephalic regions were separately analyzed by one way ANOVA followed by Tukey's post hoc test. In all the tests, criterion for statistical significance was considered to be $p<0.05$. All statistical procedures were carried out using sigma stat Graph Pad Prism (Graph Pad prism Software, version 5, San Diego, CA).

Results

Effect of SD on locomotor activity

As observed on day 0, 7, 14 and 21, the mean scores of locomotor activity (ambulation+rearing count per 5 minutes) did not differ significantly between naïve; sleep deprived and piracetam treated groups of animals depicting a rather stable locomotor activity on each day. However, a trend of gradual decrease in locomotor activity across the duration of entire protocol (day 7^{th}, 14^{th}, and 21^{st}) was observed (Figure 2).

Effect of SD on performance in Morris Water Maze (MWM) test paradigm: Maze acquisition trial (Day 17 to 20)

The cognitive performance was assessed using the MWM performance. Mean escape latency/ initial transfer latency (IAL) (to find the submerged platform) was found to be invariable amongst all the groups on the first day of training (i.e. day 17). However, mean escape/transfer latency gradually decreased in all groups on subsequent days of training period from day 18 to day 20 in MWM test. Further, animals exposed to SD of 24 hours on alternate days for 21 days exhibited a significantly poor cognitive performance (depicted by increased mean escape latency) from day 17 to 20 as compared to the naive group (Figure 3). However, treatment with piracetam (100 mg/

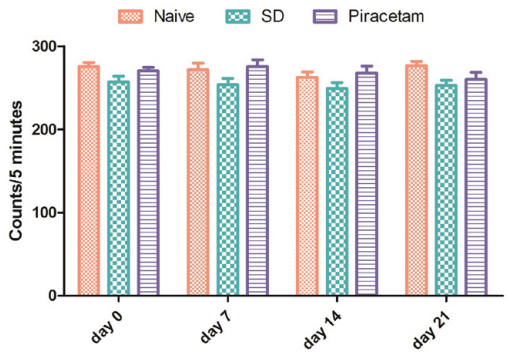

Figure 2: Effect of SD on locomotor activity. Values expressed as mean ± SEM. SD: Sleep deprived for 24-hours every alternate day for 21 days; Pira: Piracetam.

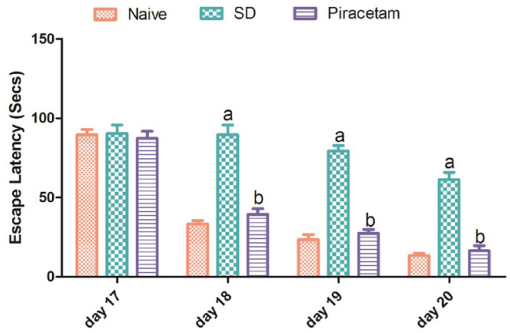

Figure 3: Effect of SD on mean escape latency in morris water maze (MWM) test paradigm. Values are expressed as mean ± SEM. [a]p<0.05 as compared with naive, [b]p< 0.05 as compared with SD, (Two-way ANOVA followed by Bonferroni's test). SD: Sleep deprived for 24 hours every alternate day for 21 days; Pira: Piracetam.

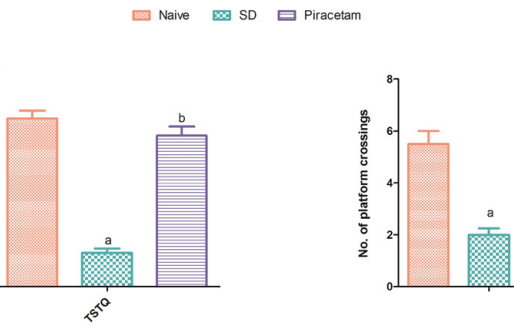

Figure 4: Effect of SD on time spent in target quadrant [TSTQ] and number of crossings across the platform area in morris water maze test. Values are expressed as mean ± SEM. [a]p<0.05 as compared with naive, [b]p<0.05 as compared with SD, (One-way ANOVA followed by Tukey's test). SD: Sleep deprived for 24-hours every alternate day for 21 days; Pira: Piracetam.

kg) significantly improved the cognitive performance (decreased mean escape latency) from second day of the task (day 18) uptil last day of training (day 20) as compared to the SD control group.

Maze retention trial (Day 21)

The probe or retention trial, performed after removal of the hidden platform from the MWM tank was used to evaluate the degree of memory consolidation attained by the animals after four days of training in the acquisition phase of MWM task and whether SD has any effect on degree of memory consolidation. Animals subjected to 24 hours SD on alternate days across the 21 day protocol significantly failed to recall the initial location of the hidden platform in Q4 as evident from lesser time spent in the target quadrant (TSTQ) quadrant and decreased number of crossings across the platform area as compared to naïve group on the day of probe trial (day 21) (Figure 4). Additionally treatment with piracetam (100 mg/kg) for 21 days significantly improved the cognitive performance and intensified memory consolidation (as evidenced by increased TSTQ and increase number of platform area crossings) as compared to control SD group.

Effect of SD on Elevated Plus Maze (EPM) task performance

The present study depicted no significant difference in mean initial transfer latency (ITL; day 15) between naïve, SD and piracetam treated groups. Further, SD of 24 hours on alternate day basis resulted in significant increase in first and second retention transfer latencies (1st and 2nd RTL; day 16 and 21 respectively) as compared to naive group, demonstrating SD induced memory impairment (Table 2). However, treatment with piracetam (100 mg/kg) for 21 days significantly improved the cognitive performance and depicted a decrease in both first and second retention latencies as compared to the control SD animals.

Effect of SD on oxidative stress parameters

As depicted in Table 3, exposing animals to SD of 24-hours on alternate days for a total period of 21 days significantly elevated levels of oxidative stress markers (increase in lipid peroxidation and nitrite levels) as well as depleted endogenous antioxidant activities (decreased reduced glutathione and catalase as well as superoxide dismutase (SOD) activity) in both hippocampi and cortical encephalic regions. Therefore, chronic SD may precipitate conditions of oxidative damage. Additionally, treatment with piracetam (100 mg/kg) for 21 days led to significant antioxidant like property and demonstrated depletion in lipid peroxidation and nitrite levels as well as increase in reduced glutathione, catalase as well as SOD activity (Table 3).

Effect of SD on mitochondrial respiratory enzyme complex activities

As depicted in Figure 5, SD of 24 hours on alternate days extended over a duration of 21 days, significantly depleted the mitochondrial respiratory enzyme complex activities (NADH dehydrogenase, succinate dehydrogenase, cytochrome oxidase activities as well as cell viabilities) in hippocampus and cortex regions of brain in comparison to the naïve group. Nevertheless, chronic treatment with piracetam (100 mg/kg) for 21 days significantly refurbished mitochondrial enzymes complex activities and increased cell viability as compared to SD control animals (Figure 5).

Effect of SD on acetylcholineesterase (AChE) activity

Sleep debt generated as a consequence of SD of 24 hours on alternate days within the 21 days protocol significantly increased AChE activity in hippocampus and cortex regions as compared to the naive group (without SD). However, upon treatment with piracetam (100 mg/kg) for 21 days, a significant attenuation in AChE activity was observed, which was found to be significant as compared to control (SD) vehicle treated animals (Figure 6).

Effect of SD on neuronal morphology and pyknotic cell density as depicted by histopathological sections of mice brains

Histopathological evaluation of hippocampi and thalamocortical

S.No.	Group name	ITL (Day 1)	RTL1 (Day 16)	RTL2 (Day 21)	% deficit in 1st and 2nd RTL
1	Naïve	78.21 ± 4.11	23.32 ± 1.00	27.15 ± 1.72	14.10 ± 2.51
2	SD	79.32 ± 3.61	39.57 ± 4.00[a]	77.36 ± 4.85[a]	48.84 ± 3.01
3	Pira (100)	76.14 ± 4.17	25.16 ± 0.85[b]	32.00 ± 1.50[b]	21.37 ± 2.65

Values are expressed as mean ± SEM. [a]p< 0.05 as compared with naive, [b]p< 0.05 as compared with SD, (Two-way ANOVA followed by Bonferroni's test). Pira: piracetam; SD: Sleep deprived for 24-hours every alternate day for 21 days.

Table 2: Effect of SD on cognitive performance in Elevated Plus Maze task.

Treatment group (mg/kg)	Brain Region	LPO (nmol MDA/mg of protein) (% of Naive)	Nitrite (µmol/ml) (% of Naive)	GSH (nmol of GSH/mg Pr) (% of Naive)	Catalase (µM of H_2O_2/min/ mgpr) (% of Naive)	SOD (Units/mg of protein) (% of Naive)
Naïve	Cortex	0.0179 ± 0.002 (100)	257.51 ± 3.52 (100)	0.802 ± 0.01 (100)	0.670 ± 0.043 (100)	67.58 ± 1.67 (100)
	Hippocampus	0.0231 ± 0.001 (100)	230.62 ± 2.79 (100)	0.896 ± 0.02 (100)	0.619 ± 0.016 (100)	78.32 ± 2.67 (100)
SD	Cortex	0.0949 ± 0.002a (530.16)	689.32 ± 5.62a (267.68)	0.139 ± 0.01a (17.33)	0.127 ± 0.029a (22.52)	25.63 ± 1.27a (37.92)
	Hippocampus	0.1243 ± 0.003a (538.09)	764.65 ± 6.61a (3331.54)	0.176 ± 0.01a (19.64)	0.159 ± 0.014a (27.59)	31.86 ± 1.01a (40.67)
Pira (100)	Cortex	0.0218 ± 0.001b (121.78)	300.65 ± 3.62b (116.75)	0.718 ± 0.02b (89.56)	0.626 ± 0.012b (90.07)	63.52 ± 2.16b (93.99)
	Hippocampus	0.0316 ± 0.002b (136.79)	361.81 ± 3.51b (156.89)	0.817 ± 0.03b (91.25)	0.592 ± 0.036b (95.87)	70.52 ± 3.11b (90.04)

Values are expressed as mean ± SEM. [a]p<0.05 as compared with naive, [b]p<0.05 as compared with SD, (One-way ANOVA followed by Tukey's test). Pira: piracetam; SD: Sleep deprived for 24 hours every alternate day for 21 days.

Table 3: Effect of SD on oxidative stress parameters.

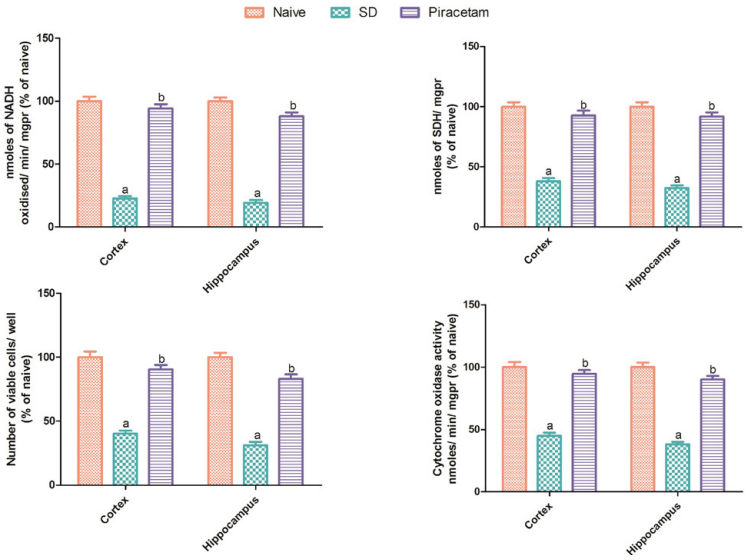

Figure 5: Effect of SD on mitochondrial respiratory enzyme complex activities. Complex-I (NADH dehydrogenase activity); Complex-II (Succinate dehydrogenase activity); Complex-III (MTT/ mitochondrial redox activity); Complex IV (cytochrome c oxidase activity). Values are expressed as mean ± SEM. [a]p<0.05 as compared with naive, [b]p<0.05 as compared with SD, (Two-way ANOVA followed by Bonferroni's test). SD: Sleep deprived for 24 hours every alternate day for 21 days; Pira: Piracetam.

regions of mice brain was conducted under light microscopy. Representative images of brain sections from different groups of animals have been shown in Figure 7A. Brains of naïve animals showed comparatively undamaged and optimum sized neuronal cells as compared to sleep deprived animals. However, disorganization of various cell layers, increased level of cellular spongiosis and increased density of pyknotic cells per 128800 square pixels (signifying activation of neuroinflammatory and apoptotic responses), was observed specifically in hippocampi and thalamo-cortical regions of the brains of sleep deprived animals. However, continued treatment with piracetam (100 mg/kg) significantly attenuated these neuroinflammatory alterations as well as reduced number of pyknotic cells, per 128800 square pixels of area compared to the control SD group, signifying the protective effect of piracetam against neuromorphological alterations in discrete brain areas precipitated as a direct outcome of chronic SD (Figures 7 and 8).

Discussion

The link between SD and other neuropsychiatric disorders like anxiety like behaviour, depression, psychosis and cognitive dysfunction have been suggested to be bidirectional. Both acute and chronic SD has been found to be intricately associated with change in sleep patterns and cognitive deficits both in preclinical as well clinical studies [16,38-41]. Furthermore, single paired and repetitive TMS studies have put forward effect of SD on neurophysiological patterns of cortical excitability which prove to be another etiological target in SD induced cognitive deficits. However, the absence of complex reproducibility of results may be due to heterogeneity in the design

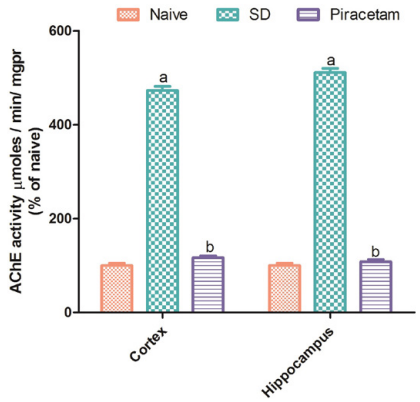

Figure 6: Effect of SD on acetylcholineesterase activity. Values are expressed as mean ± SEM. [a]p<0.05 as compared with naive, [b]p<0.05 as compared with SD, (Two-way ANOVA followed by Bonferroni's test). SD: Sleep deprived for 24 hours every alternate day for 21 days; Pira: Piracetam.

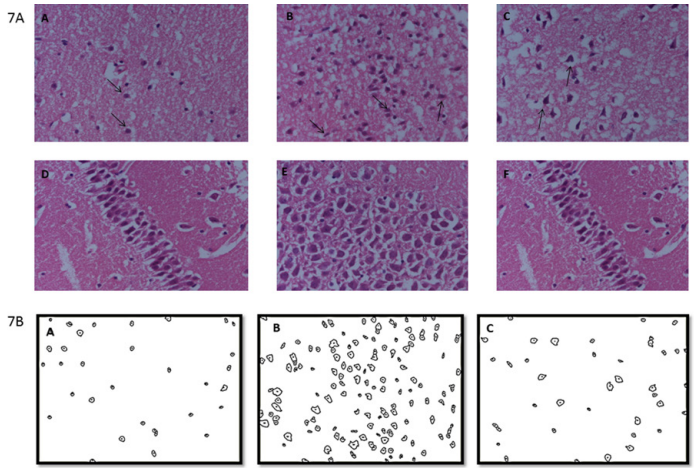

Figure 7A and 7B: Effect of SD on neuronal morphology as depicted by histopathological sections of sleep deprived mice brains. A-C: Thalamocortical regions; D-F: hippocampal regions. A: Naive; B: Sleep deprived (control); C: Piracetam (100); D: Naive; E: Sleep deprived (control); F: Piracetam (100); The pictographs of histopathological sections as modified by software for processing; showing outliners of pyknotic cells; A: Naive; B: Sleep deprived (control); C: Piracetam (100).

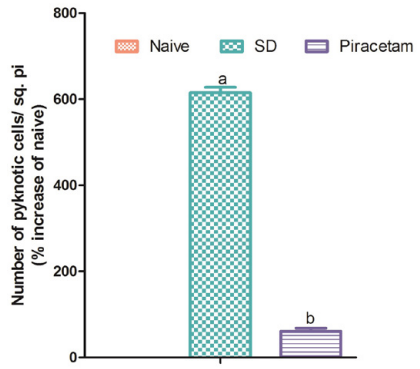

Figure 8: Effect of SD on pyknotic cell expression per 1228800 square pixels of area in thalamo-cortical regions of mice brains; Values are expressed as mean ± SEM. [a]p<0.05 as compared with naive, bp<0.05 as compared with SD, (One-way ANOVA followed by Tukey's test). SD: Sleep deprived for 24-hours every alternate day for 21 days; Pira: Piracetam.

of experimental protocols and number of subjects in each study. Studies have demonstrated that while single TMS characteristics like MEP latency or MEP size are found to be least affected by conditions of SD (clearly suggesting that SD least affects the structural integrity of cortico-spinal pathway), the characteristics of paired TMS are subjectively altered by SD (signifying pathophysiological correlates of cortical intraneuronal excitability) [10]. SD has shown to bring about a decrease in short latency intracortical inhibition (SICI), Short latency Afferent Inhibition (SAI), decrease in intra cortical facilitation (ICF), as well as cortical silent period (CSP), which may signify altered cortical excitability and thus precipitate memory deficits [8,9,42-45]. Besides, alterations in most of the above mentioned TMS characteristics as a

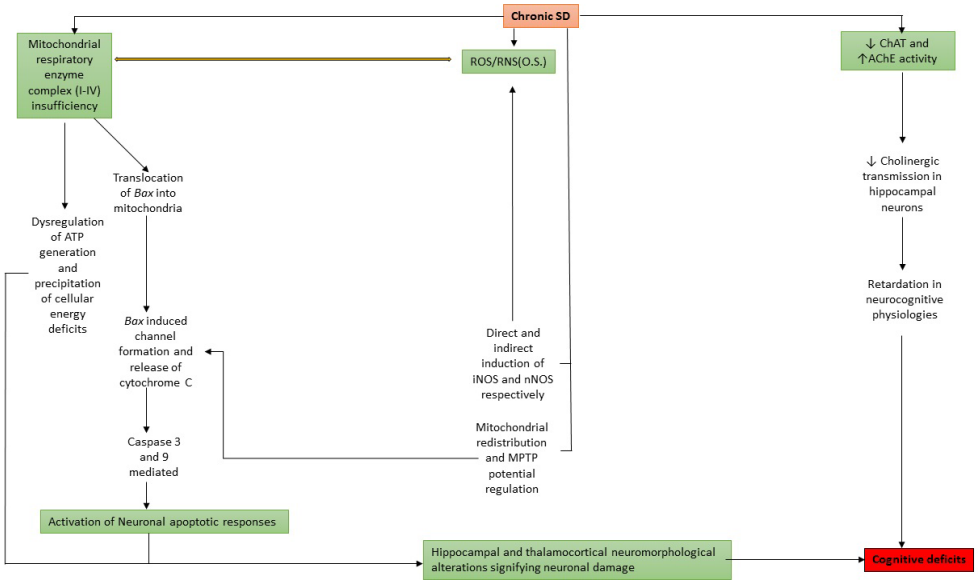

Figure 9: Figure tries to delineate the plausible etiological pathways involved in SD induced cognitive dysfunction. AChE: Acetylcholineesterase; ATP: Adenosine triphosphate; Bax: Bcl-2 associated X protein; ChAT: Choline acetyltranferase; iNOS: inducible nitric oxide synthase; nNOS: Neuronal nitric oxide synthase.

consequence of SD, have also been found to be significantly correlated with deficits in parameters of episodic verbal memory and executive functions in SD subjects, clearly signifying that SD may influence both encoding and consolidation of memory paradigms via alteration of cortical excitability profiles [8,9].

But the exact behavioral and biochemical mechanistic interplays exacerbated by SD that may consequently lead to cognitive deficits are largely unknown. In concordance with the previously mentioned reports, in the present study as well, SD created a sleep debt and induced significant alterations in cognitive functions. The study witnessed marked deviations in escape, transfer latencies as well as retention latencies in neurobehavioral memory evaluating paradigms like EPM as well as MWM. This clearly purports that SD interferes with complex physiologies involved in both acquisition as well as consolidation of cognitive paradigms. Additionally, the study utilized a differential protocol of SD wherein animals were exposed to 24 hours SD on every alternate day to be in line with the clinical situations of sleep management wherein acute and chronic SD behaviors are not witnessed.

Earlier reports have demonstrated acute SD of 72 or 96 hours to be intricately linked with depleted antioxidant enzymes profile as well as elevated production of oxidative free radicals precipitating conditions of oxidative damage [11,13,46]. In line with the acute SD conditions, in the present study as well exposure to alternate days SD over a total period of 21 days not only depicted increased lipid peroxidation, and nitrite production but also diminished glutathione reduction and antioxidant enzymes activities of SOD as well as catalase in discrete encephalic areas of hippocamapus as well as cortex. Additionally present study also depicted that SD led to significant depletions in activities of mitochondrial respiratory enzyme complexes and cellular viabilities in both hippocampus and cortex which may manifest situation of not only cellular energy deficits but also increased production of oxidative free radicals as well as activation of caspase dependent apoptotic responses. Therefore, the present study purports that vicious bidirectional crosstalks between oxidative stress and mitochondrial insufficiencies may hamper neuronal survival in specific encephalic areas controlling memory encoding and consolidation.

Acetylcholine is one of the major neurotransmitters that cardinally modulates memory formation and consolidation which is justified on grounds of earlier reports wherein decrease in the levels of acetylcholine has often led to decline in cognitive performances and and AChE inhibitors serve as targeted therapies for the treatment of dementia [47]. In the current study, the elevated levels of AChE in specific brain sections of hippocampus and cortex clearly put forward that the decrease in the levels of acetylcholine might serve as another etiological link behind the cognitive decline as a direct consequence of 24 hours SD every alternate day for 21 days. This observation has been in line with previous reports wherein acute SD has been found to be associated with increased AChE activity [17,48].

In order to reassure the above mentioned hypothesis that vicious interplays between oxidative damage, mitochondrial insufficiencies and consequent activation of apoptotic responses may serve as pivotal etiological links behind SD induced cognitive deficits, histopathological correlates pertaining to thalamo-cortical and hippocampal regions were evaluated. The results consolidified the fact that SD led to neuromorphological alterations as well as increased pyknotic cell density (via above mentioned cascades) signifying detrimental effects on neuronal survival in thalamo-cortical and hippocamapal regions, thereby leading to alterations in cognitive physiologies. Therefore, it can be purported that vicious and complex mechanistic interplays between oxidative free radicals, mitochondrial respiratory enzyme complex insufficiencies, AChE activity as well as activation of apoptotic responses may serve as cardinal interactive molecular cascades in SD induced cognitive dysfunction. But still confirmative molecular and sub cellular studies may be required to validate the above mentioned set of results. However, Figure 9 tries to elaborate the plausible molecular mechanistic interplays which may play cardinal role apart from other neuropathologies in SD induced cognitive deficits (Figure 9).

Conclusion

The present study suggests that chronic SD may directly head to substantial depletions in cognitive performances. Additionally, it was also suggested that oxidative stress, depleted mitochondrial respiratory enzyme complex activities and cellular survival, increased

AChE activity may bring out neuromorphological alterations in specific encephalic regions which may manifest alterations in cognitive physiologies and thereby serve as cardinal etiologies in SD induced cognitive dysfunction.

Acknowledgments

Authors would like to thank the University Grants Commission, New Delhi for providing financial support under the UGC- RFSMS [Research Fellowship in Sciences for Meritorious Students; award file number: F.5-94/2007(BSR)] scheme.

References

1. Stickgold R, Walker MP (2013) Sleep-dependent memory triage: evolving generalization through selective processing. Nat Neurosci 16: 139-145.
2. Walker MP, Stickgold R (2004) Sleep-dependent learning and memory consolidation. Neuron 44: 121-133.
3. Walker MP (2009) The role of sleep in cognition and emotion. Ann NY Acad Sci 1156: 168-197.
4. Havekes R, Vecsey CG, Abel T (2012) The impact of sleep deprivation on neuronal and glial signaling pathways important for memory and synaptic plasticity. Cell Signal 24: 1251-1260.
5. Abel T, Havekes R, Saletin JM, Walker MP (2013) Sleep, plasticity and memory from molecules to whole-brain networks. Curr Boil 23: R774-788.
6. Dorrian J, Rogers NL, Dinges DF (2005) Psychomotor vigilance performance: Neurocognitive assay sensitive to sleep loss (Doctoral dissertation, Marcel Dekker).
7. Durmer JS, Dinges DF (2005) Neurocognitive consequences of sleep deprivation. Semin Neurol 25: 117-129).
8. Nardone R, Bergmann J, Kunz A, Christova M, Brigo F, et al. (2012) Cortical afferent inhibition is reduced in patients with idiopathic REM sleep behavior disorder and cognitive impairment: a TMS study. Sleep Med 13: 919-925.
9. Chee MW, Chuah LY (2008) Functional neuroimaging insights into how sleep and sleep deprivation affect memory and cognition. Curr Opin Neurol 21: 417-423.
10. Lanza G, Cantone M, Lanuzza B, Pennisi M, Bella R, et al. (2015) Distinctive patterns of cortical excitability to transcranial magnetic stimulation in obstructive sleep apnea syndrome, restless legs syndrome, insomnia, and sleep deprivation. Sleep Med Rev 19: 39-50.
11. Gopalakrishnan A, Ji LL, Cirelli C (2004) Sleep deprivation and cellular responses to oxidative stress. Sleep 27: 27-35.
12. Singh R, Kiloung J, Singh S, Sharma D (2008) Effect of paradoxical sleep deprivation on oxidative stress parameters in brain regions of adult and old rats. Biogerontology 9: 153-162.
13. Khadrawy YA, Nour NA, Ezz HS (2011) Effect of oxidative stress induced by paradoxical sleep deprivation on the activities of Na+, K+-ATPase and acetylcholinesterase in the cortex and hippocampus of rat. Trans Res 157: 100-107.
14. Kalinchuk AV, Lu Y, Stenberg D, Rosenberg PA, Porkka-Heiskanen T (2006) Nitric oxide production in the basal forebrain is required for recovery sleep. J Neurochem 99: 483-498.
15. Andreazza AC, Andersen ML, Alvarenga TA, de-Oliveira MR, Armani F, et al. (2010) Impairment of the mitochondrial electron transport chain due to SD in mice. J Psychiatr Res 44: 775-780.
16. Silva RH, Abilio VC, Takatsu AL, Kameda SR, Grassl C, et al. (2004) Role of hippocampal oxidative stress in memory deficits induced by sleep deprivation in mice. Neuropharmacology 46: 895-903.
17. Benedito MA, Camarini R (2001) Rapid eye movement sleep deprivation induces an increase in acetylcholinesterase activity in discrete rat brain regions. Braz J Med Biol Res 34: 103-109.
18. Camarini R, Benedito MA (1997) Rapid eye movement (REM) sleep deprivation reduces rat frontal cortex acetylcholinesterase (EC 3.1.1.7) activity. Braz J Med Biol Res 30: 641-647.
19. Shinomiya K, Shigemoto Y, Okuma C, Mio M, Kamei C (2003) Effects of short-acting hypnotics on sleep latency in rats placed on grid suspended over water. Eur J Pharmacol 460: 139-144.
20. Kumar A, Kalonia H (2007) Protective effect of *Withania somnifera Dunal* on the behavioural and biochemical alterations in sleep-disturbed mice (grid over water suspended method). Indian J Experimental Biol 45: 524-528.
21. Morris R (1984) Developments of a water-maze procedure for studying spatial learning in the rat. J Neurosci Methods 11: 47-60.
22. Kumar A, Prakash A, Dogra S (2011) Centella asiatica attenuates D-Galactose-Induced Cognitive Impairment, Oxidative and Mitochondrial Dysfunction in Mice. Int J Alzheimer Dis 2011: 347569.
23. Tuzcu M, Baydas G (2006) Effect of melatonin and vitamin E on diabetes-induced learning and memory impairment in rats. Eur J Pharmacol 537: 106-110.
24. Mishra J, Chaudhary T, Kumar A (2014) Rosiglitazone synergizes the neuroprotective effects of valproic acid against quinolinic acid-induced neurotoxicity in rats: Targeting PPARγ and HDAC pathways. Neurotox Res 26: 130-151.
25. Misra S, Tiwari V, Kuhad A, Chopra K (2011) Modulation of nitrergic pathway by sesamol prevents cognitive deficits and associated biochemical alterations in intracerebroventricular streptozotocin administered rats. Eur J Pharmacol 659: 177-186.
26. Wills ED (1966) Mechanism of lipid peroxide formation in animal tissues. Biochem J 99: 667-676.
27. Green LC, Wagner DA, Glagowski J (1982) Analysis of nitrate, nitrite and [15N] nitrate in biological fluids. Anal Biochem 126: 131-138.
28. Ellman GL (1959) Tissue sulfhydryl groups. Arch Biochem Biophys 82: 48670-48677.
29. Luck H (1965) Catalase. Method of Enzymatic Analysis 885-894.
30. Kono Y (1978) Generation of superoxide radical during autoxidation of hydroxylamine and an assay for superoxide dismutase. Arch Biochem Biophys186: 189-195.
31. Lowry OH, Rosenberg NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin-phenol reagent. J Biol Chem 193: 265-275.
32. Berman SB, and Hastings TG (1999) Dopamine oxidation alters mitochondrial respiration and induces permeability transition in brain mitochondria. J. Neurochem 73: 1127-1137.
33. King TE, Howard RL (1967) Preparations and properties of soluble NADH dehydrogenases from cardiac muscle. Methods Enzymol 10: 275-294.
34. King TE (1967) Preparation of succinate dehydrogenase and reconstitution of succinate oxidase. Methods Enzymol 10: 322-331.
35. Liu Y, Peterson DA, Kimura H, Schubert D (1997) Mechanisms of cellular 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazoliniumbromide (MTT) reduction. J Neurochem 9: 581-593.
36. Sottocasa GL, Kuylenstierna B, Ernster L, Berqstrand A (1967) An electron-transport system associated with the outer membrane of liver mitochondria. J Cell Biol 32: 415-438.
37. Ellman GL, Courtney KD, Featherstone RM (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 7: 88-95.
38. Mueller AD, Meerlo P, McGinty D, Mistlberger RE (2013) Sleep and adult neurogenesis: implications for cognition and mood. Curr Top Beh Neurosci 25: 151-181.
39. Graves LA, Heller EA, Pack AI, Abel T (2003) Sleep deprivation selectively impairs memory consolidation for contextual fear conditioning. Learn Mem 10(3): 168-176.
40. Smith ME, McEvoy LK, Gevins A (2002) The impact of moderate sleep loss on neurophysiologic signals during working-memory task performance. Sleep 25(7): 784.
41. Xu ZQ, Gao CY, Fang CQ, Zhou HD, Jiang XJ (2010) The mechanism and characterization of learning and memory impairment in sleep-deprived mice. Cell Biochem Biophys 58: 137-140.
42. Kreuzer P, Langguth B, Popp R, Raster R, Busch V, et al. (2011) Reduced intra-cortical inhibition after sleep deprivation: a transcranial magnetic stimulation study. Neurosci Lett 493: 63-66.
43. Civardi C, Boccagni C, Vicentini R, Bolamperti L, Tarletti R, et al. (2001) Cortical excitability and sleep deprivation: a transcranial magnetic stimula- tion study. J Neurol Neurosurg Psychiatry 71: 809-812.

44. Scalise A, Desiato MT, Gigli GL, Romigi A, Tombini M, et al. (2006) Increasing cortical excitability: a possible explanation for the proconvulsant role of sleep deprivation. Sleep 29: 1595-1598.

45. Placidi F, Zannino S, Albanese M, Romigi A, Izzi F, et al. (2013) Increased cortical excitability after selective REM sleep deprivation in healthyhumans: a transcranial magnetic stimulation study. Sleep Med 14: 288-292.

46. Ramanathan L, Gulyani S, Nienhuis R, Siegel JM (2002) Sleep deprivation decreases superoxide dismutase activity in rat hip- pocampus and brainstem. Neuroreport 13: 1387-1390.

47. Thakkar M, Mallick BN (1991) Effect of REM sleep deprivation on rat brain acetylcholinesterase. Pharmacol Biochem Behav 39: 211-214.

48. Roman V, Hagewoud R, Luiten PG, Meerlo P (2006) Differential effects of chronic partial sleep deprivation and stress on serotonin-1A and muscarinic acetylcholine receptor sensitivity. J Sleep Res 15: 386-394.