Possible involvement of GABAergic mechanism in protective effect of melatonin against sleep deprivation–induced behavior modification and oxidative damage in mice

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Received 18 May 2010; revised 19 November 2010

Sleep deprivation for 72 h caused anxiety like behavior, weight loss, impaired locomotor activity and oxidative damage as indicated by increase in lipid peroxidation, nitrite level and depletion of reduced glutathione and catalase activity in sleep deprived mice brain. Treatment with melatonin (5 and 10 mg/kg, ip) significantly improved locomotor activity, weight loss and antianxiety effect as compared to control (sleep deprived). Biochemically, melatonin treatment significantly restored depleted reduced glutathione, catalase activity, attenuated lipid peroxidation and nitrite level as compared to control (72 h sleep-deprived) animals. A combination of flumazenil (0.5 mg/kg, ip) and picrotoxin (0.5 mg/kg, ip) with lower dose of melatonin (5 mg/kg, ip) significantly antagonized the protective effect of melatonin. However, combination of muscimol (0.05 mg/kg, ip) with melatonin (5 mg/kg, ip) potentiated protective effect of melatonin as compared to their effect per se. The results suggest that melatonin may produce its protective effect by involving GABAergic system against sleep deprivation-induced anxiety like behavior and related oxidative damage.

Keywords: Anxiety, Locomotor activity, Melatonin, Oxidative stress, Sleep deprivation

Adequate sleep or good quality of sleep is one of the vital physiological functions for the maintenance of health1. It is believed that sleep is essential in the recovery from illness, and that sleep deprivation (SD) is detrimental to multiple physiological processes. Problem of SD is increasing in modern society due to rapid change in life style. SD is a health risk factor associated with several disease processes leading to behavioural2,3, hormonal4 and neurochemical alterations. SD aggravates health risk factors such as depression, anxiety, mood disorders, psychosis that appear to compound disease processes. SD causes oxidative damage5 and decrease strength of antioxidative defense mechanism of the body. Brain tissues are rich in polyunsaturated fatty acids and involve metabolic active system. The antioxidative levels of brain tissues are low as compared to other tissues of the body i.e. it has low levels of the catalase enzyme activity.

Gama-aminobutyric acid (GABA) is an important inhibitory neurotransmitter in CNS. GABAergic system has been involved in the pathophysiology of several nervous disorders such as anxiety, epilepsy, sleep disorders and convulsive disorders. SD alters the content of GABA neurotransmitter in mice suggesting role of GABAergic mechanism in SD induced changes in behaviour alterations and oxidative damage in the animals9.

Melatonin (N-acetyl-5-methoxy-tryptamine), a hormone of the pineal gland has several important functions, such as circadian rhythm regulation, immune enhancement, sleep induction, seasonal reproductive regulation, and light-dark signal transduction10. Melatonin has mild to moderate hypnotic action in animals11. Melatonin is also an effective free radical scavenger and antioxidant12. It has been shown to be highly useful in preventing neurodegenerative changes. Accumulating evidence indicates that melatonin acts by involving GABAergic system in CNS. For example, melatonin increases concentrations of GABA in the hypothalamus13, augments GABA turnover in several brain regions, increases GABA-induced chloride influx in the hypothalamus, potentiates GABA_A receptor-mediated current and causes an enhancement of [^3]H] GABA

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binding. Electrophysiological experiments in anaesthetized animals show that melatonin exhibits GABA-like effects and potentiates the effect of GABA in neuronal activity. In addition, the melatonin-induced decrease in locomotor, anxiolytic, anticonvulsive and analgesic activities are blocked by flumazenil, an antagonist of the benzodiazepine recognition site on the GABA<sub>A</sub> receptor. However, there is no direct evidence to indicate whether the GABA<sub>A</sub> receptor is involved in the activity of melatonin against SD-induced alterations of body functioning. Despite extensive research on the beneficial effects of melatonin in various disease conditions, there are limited studies that explored possible potential in the treatment of sleep deprivation induced anxiety and oxidative damage and its possible mechanism of action. Therefore, aim of the present study to investigate the possible role of GABAergic system in protective effects of melatonin against 72 h sleep-deprived induced behavioural alterations and oxidative damage in mice.

**Materials and Methods**

**Animals**—Male albino mice of 25-30 g body weight bred in Central Animal House of the Panjab University were used. The animals were kept under standard laboratory conditions, maintained on 12 h light/dark cycle, had free access to food and water. Animals were acclimatized to laboratory conditions before the test. Each animal was used once in the experiments. All the experiments were performed between 0900 and 1700 hrs. The experimental protocols were approved by Institutional Animal Ethics Committee and were conducted according to the Indian National Science Academy Guidelines for the use and care of experimental animals.

**Sleep deprivation**—Animals were sleep deprived for 72 h by placing them on the grid suspended over water method as described by Shinomiya et al. Animals were placed on a grid floor (29x15x7 cm) inside the plastic cage filled with water to 1 cm below the grid surface for 72 h. 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.

**Drugs and treatment schedule**—Animals were divided into ten groups (6-8 animals in each). First and second group were treated as naïve (vehicle) and control (72 h sleep deprived) respectively. Melatonin (5 and 10 mg/kg, ip), flumazenil (0.5 mg/kg, ip), picrotoxin (0.5 mg/kg, ip) and muscimol (0.05 mg/kg, ip) were administered to mice of group 3-7 respectively. Combination of flumazenil (0.5 mg/kg) or picrotoxin (0.5 mg/kg) and or muscimol (0.05 mg/kg, ip) with melatonin (5 mg/kg ip) were administered in group 8-10 respectively. All the drugs were administered intraperitoneally for five days starting two days before 72 h sleep deprivation. Melatonin (5, 10 mg/kg), picrotoxin (0.5 mg/kg) and muscimol (0.05 mg/kg) were dissolved in dimethylsulfoxide (DMSO) and then made-up with water.

**Elevated plus maze test**—Elevated plus maze developed by Kulkarni et al. is a novel test for testing selective anxiogenic and anxiolytic drugs effect in rodents. The plus maze apparatus for mice consist of two open (16 x 5 cm) and two closed arm (16 x 5 x 12 cm) and placed at height of 25 cm. The animals are placed individually at the center of the elevated plus maze with their head facing toward an open arm. During the 5 min test, the number of entries into the open and closed arm and time spent in each arm of the maze was recorded.

**Zero maze test**—The zero maze described by Shepherd et al. is a modification of elevated plus maze model of anxiety in rodents. The maze comprised of black perspex annular platform (105 cm diameter, 10 cm width) elevated to 65 cm above ground level, divided equally into four quadrants. Two opposite quadrants were enclosed by black Perspex walls (27 cm height) on both the inner and outer edges of the platform; the remaining two quadrants were surrounded by Perspex lip (1 cm in height). Animals were placed in the closed quadrant. During the 5 min test, the number of entries into the open and closed quadrants and time spent in each quadrant of the maze were recorded.

**Mirror chamber test**—The mirror chamber consisted of a wooden chamber having a mirror chamber enclosed within it. During the 5 min test session, following parameters were noted : (a) latency to enter the mirror chamber, (b) total time spent in mirror chamber, and (c) number of entries in mirror chamber. Animals were placed individually at the distal corner of the mirror chamber at the beginning of the test. An anxiogenic response was defined as decreased number of entries and time spent in the mirror chamber.

**Measurement of ambulatory activity**—
ambulatory activity was recorded by using actophotometer (IMCORP, Ambala, India). Before locomotor task, animals were placed individually in the activity meter for 3 min. The locomotor activity was recorded using actophotometer for 5 min. Ambulatory activity was recorded and expressed in terms of total photo beam counts for 5 min for each animal.

Biochemical tests

Dissection and homogenization—On day 6, after behavioural quantification day of drug treatments, the animals were sacrificed by decapitation immediately after behavioural assessments. The whole brains were removed and 10% (w/v) tissue homogenate was prepared in 0.1 M phosphate buffer (pH 7.4). Homogenate was centrifuged for 20 min at 15000 rpm and supernatant was used for estimation of lipid peroxidation and reduced glutathione levels. The post nuclear fractions were obtained by centrifugation at 1000 g for 20 min, at 4°C for catalase assay and for other enzyme assays centrifuged at 12,000 g for 60 min at 4°C.

Lipid peroxidation assay—The quantitative measurement of lipid peroxidation was performed according to the method of Wills. The amount of malondialdehyde (MDA), a measure of lipid peroxidation was measured by reaction with thiobarbituric acid (532 nm) using Shimadzu spectrophotometer. The values were calculated using molar extinction coefficient of chromophore (1.56 ×10³ M⁻¹ cm⁻¹) and expressed as percentage of control.

Estimation of reduced glutathione—Reduced glutathione in brain was estimated according to the method described by Ellman. Supernatant (1 ml) was precipitated with 1 ml of 4% sulfosalicylic acid and cold digested at 4°C for 1 hr. The sample was centrifuged at 1200 g for 15 min at 4°C. To 1 ml of this supernatant, 2.7 ml of phosphate buffer (0.1 M, pH 8) and 0.2 ml of 3,5-dithiobis (2-nitrobenzoic acid) (DTNB) were added. The yellow colour developed was read immediately at 412 nm using Shimadzu spectrophotometer. Results were calculated using molar extinction coefficient of chromophore (1.36 ×10⁴ M⁻¹ cm⁻¹) and expressed as percentage of control.

Estimation of nitrite—The accumulation of nitrite in the supernatant, an indicator of the production of nitric oxide (NO), was determined with a colorimetric assay with Greiss reagent (0.1% N- (1-naphyl) ethylenediamine dihydrochloride, 1% sulfanilamide and 2.5% phosphoric acid) as per Green et al. Equal volumes of supernatant and Greiss reagent were mixed, the mixture was incubated for 10 min at room temperature and the absorbance was measured at 540 nm using Shimadzu spectrophotometer. The concentration of nitrite in the supernatant was determined from a standard curve and expressed as percentage of control.

Protein estimation—The protein content was measured according to the method of Lowry et al. using bovine serum albumin as standard.

Estimation of catalase—Catalase activity was assayed by method of Liu, wherein the breakdown of H₂O₂ was measured at 240 nm. Briefly, the assay mixture consisted of 3 ml of H₂O₂ phosphate buffer (1.25 ×10⁻² M H₂O₂) and 0.05 ml of supernatant of the brain homogenate (10%), and the change in absorbance was recorded at 240 nm using the Shimadzu Spectrophotometer. Enzyme activity was calculated using the millimolar extinction coefficient of hydrogen peroxide (0.07). The result was expressed as micromoles of H₂O₂ decomposed / min/mg protein.

Statistical analysis—All the values are expressed as mean ± SE. The data were analyzed using analysis of variance (ANOVA) followed by Tukey test. In all the test criterion for statistical significance was P<0.05.

Results

Effects of melatonin on anxiety and its modification by GABA modulators in sleep-deprived mice—Plus maze: Sleep deprivation for 72 h significantly increased number of entries, duration in closed arm and decreased number of entries as well as duration in open arm of plus maze performance task as compared to naïve group (placed on sawdust). Pretreatment with melatonin (5 and 10 mg/kg, ip) significantly increased number of entries, duration in open arm as well as decreased the number of entries and duration in closed arm as compared to control (sleep deprived) (Table 1). Combination of melatonin (5 mg/kg) with flumazenil (0.5 mg/kg), and or picrotoxin (0.5 mg/kg) decreased number of entries, duration in open arm as well as increased number of entries and duration in closed arm as compared to their effect per se (Table 1). However, the combination of melatonin (5 mg/kg) with muscimol (0.05 mg/kg), GABA_A agonist...
Effects of melatonin (MEL) and its modification by flumazenil (FLU), picrotoxin (PTX) and or muscimol (MUS) on plus maze performance task

Table 1— Effects of melatonin (MEL) and its modification by flumazenil (FLU), picrotoxin (PTX) and or muscimol (MUS) on plus maze performance task

<table>
<thead>
<tr>
<th>Treatment group (mg/kg, ip)</th>
<th>Open arm</th>
<th>Closed arm</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naïve (vehicle)</td>
<td>5.4 ± 0.4</td>
<td>75.5 ± 2.6</td>
</tr>
<tr>
<td>Control (SD)</td>
<td>1.7 ± 0.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>26.5 ± 1.2&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>MEL (5)</td>
<td>4.1±0.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>44.3±1.9&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>MEL (10)</td>
<td>4.6±0.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>56.5±2.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>FLU (0.5)</td>
<td>1.5±0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20.5±1.1&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>MEL (50)+ FLU(0.5)</td>
<td>2.2±0.3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>23.5±1.7&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>PTX(0.5)</td>
<td>1.2±0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>21.9±1.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>MEL(50)+ PTX (0.5)</td>
<td>2.6±0.2&lt;sup&gt;c&lt;/sup&gt;</td>
<td>30.5±1.6&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>MUS(0.05)</td>
<td>3.5±0.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>36.3±1.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>MEL(50)+ MUS(0.05)</td>
<td>5.3±0.3&lt;sup&gt;c,d&lt;/sup&gt;</td>
<td>53.2±2.3&lt;sup&gt;c,d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values are mean ± SE from 6 animals in each group

Effects of melatonin on ambulatory movements and its modification by GABA modulators in sleep-deprived mice—Sleep deprivation for 72 h significantly reduced ambulatory activity as compared to naïve mice (placed on sawdust). Treatment with melatonin (5 and 10 mg/kg, ip) for 5 days significantly increased ambulatory movements as compared to control (72 h sleep deprived) (Table 2). However, combination of melatonin (5 mg/kg) with muscimol (0.05 mg/kg) significantly reduced latency to enter in mirror chamber, increased number of entries and duration in mirror chamber as compared to their effect per se (Table 3). However, combination of melatonin (5 mg/kg) with muscimol (0.05 mg/kg) significantly reduced latency to enter in mirror chamber, increased number of entries and duration in mirror chamber as compared to their effect per se (Table 3). Combination of melatonin (5 mg/kg) with flumazenil (0.5 mg/kg), and or picrotoxin (0.5 mg/kg) significantly increased time latency to enter in mirror chamber, decreased number of entries and duration in mirror chamber as compared to their effect per se (Table 3).
Effects of melatonin on biochemical parameters and its modification by flumazenil (FLU), picrotoxin (PTZ) and or muscimol (MUS) on locomotor activity and body weight of sleep-deprived animals

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Latency to enter mirror chamber (sec)</th>
<th>No. of entries in mirror chamber</th>
<th>Time spent in mirror chamber (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naïve (vehicle)</td>
<td>32.1±1.4</td>
<td>5.5±0.7</td>
<td>97.5±1.4</td>
</tr>
<tr>
<td>Control (sleep deprived)</td>
<td>112.3±2.5b</td>
<td>1.2±0.2a</td>
<td>18.6±0.5a</td>
</tr>
<tr>
<td>MEL (5)</td>
<td>77.5±3.1b</td>
<td>2.7±0.3b</td>
<td>34.3±1.1b</td>
</tr>
<tr>
<td>MEL (10)</td>
<td>53.4±2.9c</td>
<td>4.2±0.3c</td>
<td>42.1±1.5c</td>
</tr>
<tr>
<td>FLU (0.5)</td>
<td>119.3±3.5</td>
<td>1.1±0.1</td>
<td>36.3±1.0</td>
</tr>
<tr>
<td>MEL (50)+ FLU (0.5)</td>
<td>91.4±3.5c</td>
<td>2.3±0.2c</td>
<td>21.2±1.9c</td>
</tr>
<tr>
<td>PTX (0.5)</td>
<td>121.5±3.8</td>
<td>0.9±0.2</td>
<td>14.9±1.5</td>
</tr>
<tr>
<td>MEL(50)+ PTX (0.5)</td>
<td>94.5±3.1c</td>
<td>1.7±0.3c</td>
<td>20.5±1.7</td>
</tr>
<tr>
<td>MUS (0.05)</td>
<td>89.3±3.4b</td>
<td>2.3±0.2b</td>
<td>31.9±1.3</td>
</tr>
<tr>
<td>MEL(50)+ MUS (0.05)</td>
<td>56.2±3.2d</td>
<td>3.9±0.3c,d</td>
<td>40.2±1.8</td>
</tr>
</tbody>
</table>

*p<0.05 as compared to a naïve, b control (72 h SD), c MEL (5 mg/kg), d MUS (0.05 mg/kg).

Effects of lipid peroxidation and nitrite activity in sleep-deprived mice—Sleep deprivation (72 h) significantly increased lipid peroxidation and nitrite levels as compared to naïve (vehicle) mice. Treatment with melatonin (5 and 10 mg/kg, ip) significantly attenuated elevated lipid peroxidation (Fig. 1c) and nitrite activity (Fig. 1d) as compared to control (72 h sleep deprived). A combination of flumazenil (0.5 mg/kg) and or picrotoxin (0.5 mg/kg) with melatonin (5 mg/kg ip) did not influence significantly the lipid peroxidation (Fig. 1c) and nitrite activity (Fig. 1d) as compared to control (72 h sleep deprived). Further, combination of muscimol (0.05 mg/kg, ip) with melatonin (5 mg/kg, ip) caused significant reduction in lipid peroxidation level (Fig. 1c) and nitrite activity (Fig. 1d), which was significant compared to their effect per se.

Discussion

Sleep deprivation is considered as a risk factor for
development of several neurological and neuropsychiatric diseases. The behavioural and biochemical changes produced by sleep deprivation that results in health consequences, however, are largely unknown. Sleep deprivation caused various behavioural disturbances involving motor activity, anxiety level, memory and metabolic functions related to anabolic hormones, body weight etc. Sleep deprivation has recently been reported to cause oxidative stress. Ramanathan et al. also reported a significant decrease in superoxide dismutase activity in the hippocampus and brain stem in sleep-deprived rats. SD has been reported to influence motor activity, anxiety and produces oxidative stress.

In the present study, 72 h SD significantly caused anxiety like behaviour impaired motor activity and oxidative damage. Melatonin is well known hypnotic, used clinically in different sleep related problems. Pretreatment with melatonin caused antianxiety effect. Melatonin is also well known antianxiety agent and acts by GABAergic system. Role of GABAergic system in anxiety and related behaviour has also been well reported. Melatonin treatment also improved motor activity and body weight of a sleep deprived mice, suggesting its antidepressant effect. Besides, melatonin is an anabolic hormone that could be the reason of improved motor activity and weight gain.

Sleep deprivation has been reported to cause oxidative damage in discrete areas of brain. Sleep deprivation increases lipid peroxidation, nitrite level and deplete reduced glutathione as well as catalase activity in SD animals. Previous studies report the modulatory link between lipid peroxidation and GABAergic system. Freitas et al. reported the occurrence of lipid peroxidation and nitrite formation during seizure that could be responsible for the GABAergic receptor concentration changes during the establishment of status epilepticus. β-estradiol protects against lipid peroxidation in vulnerable brain areas of ethanol-withdrawn rats, in part through the GABAergic system. Other studies also documented that the effect of GABA derivative on lipid peroxidation. Nitrite estimation is an important parameter to explore the role of nitric oxide and oxidative stress in neurological diseases. Present results also reconfirmed the above findings. Further, melatonin treatment significantly reversed sleep deprivation-induced oxidative damage in mice, suggesting its antioxidative properties. Melatonin is well known antioxidant and act by free radical scavenging action. Melatonin up-regulates several antioxidant enzymes, particularly glutathione peroxidase, glutathione reductase, presumably indirectly via oxidized glutathione pathway. In some tissues Cu, Zn and/or Mn-superoxide dismutases and catalasates have been reported to be unregulated.

GABA is one of the important neurotransmitters mediating inhibitory postsynaptic potentials. Up to 30-50% of all synapses are GABAergic in CNS. Increasing evidence points to an important role for GABA in regulating sleep. It has been reported that GABA levels in the posterior hypothalamus are increased during sleep. Sleep deprivation causes a significant increase in gamma-amino-butryric acid.
(GABA) contents as well as an elevation of L-glutamic acid decarboxylase (GAD) activity. It is well known that the GABA-benzodiazepine receptor is a supramolecular complex together with the central-type benzodiazepine receptor, comprising several recognition sites (such as the benzodiazepine (BZD), picrotoxin and GABA sites). Flumazenil, specific antagonist of the BZD ligand site on the GABA-BZD receptor complex, is a valuable in vivo tool because it can indicate whether a drug is acting at the BZD site to produce a pharmacological response. In the present study, flumazenil blocked the protective effect of melatonin against SD-induced behavioral modifications (such as locomotor activity, anxiety like behavior) body weight. However, it caused oxidative damage comparable to control. The results suggest that melatonin might bind to BZD receptor binding site in modulating its behavioural as well as its effect on oxidative stress. Previous studies also support the present observations indicating that several neuropharmacological effects of melatonin such as antianxiety, antioxidant, sedative and anticonvulsive actions involved by involving benzodiazepine binding site.

The picrotoxin recognition site on the GABA<sub>A</sub> receptor is another important site where action of GABA can be non-competitively inhibited by several convulsants directly by inhibiting the chloride channel. However, when picrotoxin was used in combination with melatonin, blockade of melatonin’s protective effects on behaviour as well as oxidative stress were observed. Therefore, picrotoxin site on the GABA<sub>A</sub> receptor could be another target site.

Muscmol, a selective GABA<sub>A</sub> ligand produced its action through conformation change of the receptor that allows the opening chloride channel. However, when muscmol (0.05 mg/kg) was administered in combination with melatonin, poteniation in the protective action of melatonin was observed. This further supports the hypothesis that melatonin exerts its actions by acting on GABA<sub>A</sub> receptor. Present findings further support the therapeutic potential of melatonin as neuroprotectant in the treatment of sleep disorders and its GABAergic mechanism of action.

In summary, the present results showed that protective effect of melatonin against sleep deprivation-induced behavioural and biochemical alterations were significantly antagonized by flumazenil, picrotoxin and potentiated by muscmol, suggesting that protective effect of melatonin may not be mediated by only one specific binding site, but possibly be mediated through the benzodiazepine site and the picrotoxin site together on the GABA<sub>A</sub> receptor. The GABA binding site on the GABA<sub>A</sub> receptor may partially participate in melatonin-induced sleep.

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