Melatonin prevents sleep deprivation-associated anxiety-like behavior in rats: role of oxidative stress and balance between GABAergic and glutamatergic transmission

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Abstract: Sleep deprivation (SD) has been shown to induce anxiety-like behavior. Melatonin, an endogenous potent antioxidant, protects neurons from oxidative stress in many disease models. Here we investigated the effect of melatonin against SD-induced anxiety-like behavior and attempted to define the possible mechanisms involved. SD was induced in rats using modified multiple platform model. Melatonin (15 mg/kg) was administered to the rats via intraperitoneal injection. The elevated plus maze test, open field test and light-dark exploration were used to evaluate anxiety-like behavior. Serum corticosterone was measured to determine stress level. Malondialdehyde (MDA) level and superoxide dismutase (SOD) enzyme activity of amygdala and serum were performed to determine the level of oxidative stress. Levels of protein were detected by means of Western blot. The results showed that SD induces anxiety-like behavior, while melatonin treatment prevented these changes. Serum corticosterone also increased with SD but its levels were normalized by melatonin. In addition, melatonin reversed SD-induced changes in MDA and SOD in both of amygdala and serum. The results of Western blot showed that melatonin attenuated the up-regulation of NR2B-containing N-methyl-D-aspartate receptors, GluR1 subunit of the alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor as well as phosphorylation of GluR1 at Ser831, and Ca2+/calmodulin-dependent protein kinase II-alpha in SD rats. Meanwhile, melatonin blocked the down-regulation of γ-aminobutyric acid A-alpha-2 receptor. In conclusion, our results suggest that melatonin prevents anxiety-like behavior induced by SD. The possible mechanism may be attributed to its ability to reduce oxidative stress and maintain balance between GABAergic and glutamatergic transmission.

Keywords: Melatonin, sleep deprivation, anxiety-like behavior, oxidative stress, GABAergic/glutamatergic transmission

Introduction

It is well established that sleep plays a vital role in normal body function especially central nervous system restoration and memory consolidation [1]. According to national sleep foundation, 7-8 h sleep is essential for maintenance of good health. However, the normal sleep time everyday has been decreased by 1.5 h and this decrement in sleep time seems to continue to rise [2]. In the United States, at least 100 million people are suffering from sleep related disorders [3]. It is generally accepted that sleep deprivation (SD) is associated with pathological anxiety-like behavior in human beings [4, 5], but mechanistic basis for this relationship remains poorly understood.

Recent studies show that anxiety-like behavior of rats is direct pharmacological induced by oxidative stress [6-8]. Several theories suggest that SD causes oxidative stress which is commonly described as an imbalance between the reactive oxygen species (ROS) generated and clearance by the endogenous antioxidant defense system [9, 10]. In addition, hyperexcitation due to enhanced excitatory transmission or reduced inhibitory transmission can promote
anxiety-like behavior [11]. γ-aminobutyric acid (GABA) is the primary inhibitory neurotransmitter in the adult mammalian brain, including the amygdala [11]. GABA release is important in maintaining inhibitory tone, which also plays an important role in anxiety [12]. GABA receptors, a family of ligand-gated chloride ion channels, mediate the effect of GABA in anxiety-related behavior [13, 14]. Moreover, glutamate mediates activity-dependent processes critical to the mature brain as the major excitatory neurotransmitter. Perturbation of the N-methyl-D-aspartate (NMDA) subtype of glutamate receptor is known to produce impairments in anxiety.

Melatonin (N-acetyl-5-methoxy-tryptamine), a chief secretory product of pineal gland [15], is best known for its antioxidant activities and free radical scavenging ability [16, 17]. Several studies reported that the GABAergic mechanism is involved in the hypnotic action of melatonin [15, 18]. Melatonin increases concentration of GABA in the hypothalamus [19], augments GABA turnover in several brain regions, increases GABA-induced chloride influx in the hypothalamus [20], potentiates GABA receptor mediated current [21] and causes an enhancement of [3H] GABA binding [22]. Electrophysiological experiments in anaesthetized animals show that melatonin exhibits GABA-like effects [23].

The amygdala is a key circuit for processing neuronal inputs from other parts of the brain, initiating output signals to responding nuclei and generating various physiological responses related to anxiety [24, 25]. In this study, we investigated the hypothesis that melatonin supplementation prevents SD-induced anxiety-like behavior through anti-oxidative stress properties and maintaining the balance between GABAergic and glutamatergic transmission in amygdala.

Materials and methods

Animals and melatonin treatment

Animal care was in accordance with the principles of medical laboratory animal care issued by the national ministry of health. All experimental procedures were performed under the guidelines of the national ordinances on experimental animals for the ethical use of animals. Adult Sprague-Dawley rats (n = 32, weighing 200-240 g) obtained from the laboratory animal center of the Fourth Military Medical University were used in this study. They were housed 4 rats to a plexiglas cage in a climate-controlled room (22-24°C) on a 12/12-h light-dark schedule (lights on at 7 AM) with a free access to food and water. All rats were allowed to acclimate for 1 week before starting the experiments. Four groups were designated: control group (CG), wide platform group (WPG), sleep deprivation group (SDG) and melatonin group (MTG). Melatonin was dissolved in a small amount of ethanol and diluted with saline to give a final concentration of ethanol less than 5%. Rats in MTG were given intraperitoneal injection of melatonin at a dose of 15 mg/kg/day (body weight) between 7:00-8:00. The melatonin does used in this study was chosen on the basis of previously published experiment [26]. The other groups of rats received the same volume of saline intraperitoneal injection at the same time. All experiments were carried out between 08:00-17:00.

Sleep deprivation

Rats in SDG and MTG were sleep deprived for a 72 h period using the columns-in-water model (modified multiple platform) as described previously [27]. Sleep consists of two main stages non-rapid eye movement (REM) and REM sleep. This method is reported to interfere with both non-REM and REM sleep, but mainly affect REM sleep. Four rats from the same cage were placed in a large aquarium. The aquarium contained 20 columns (platform diameter: 5 cm, with platform 2 cm above the water level), spaced 7 cm apart (edge to edge). The platforms arranged in two rows and rats could move from one platform to another. Food and water were provided ad libitum. Rats were woken up once fall asleep as a result of loss of muscle tone. In addition, to test the effect of stress of the environment, wide platforms (16 cm in diameter) were used in rats of WPG on which rats could sleep without falling into the water and stay for 72 h. Rats in WPG were used in anxiety behavior test and blood test only [28]. Rats in CG were given to no treatment.

Measurement of anxiety behavior

The rats were gently dried with cotton towels after SD and promptly moved to the area of the
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room where anxiety test equipment is located and left to air dry for an hour before the anxiety tests commenced. During this time the rats did not sleep but were observed to be engaged in self-cleaning/grooming.

**Elevated plus maze test**

Elevated plus maze (EPM) was used to evaluate the influence of sleep deprivation on the anxiety behavior in rats as described previously [29]. It was made of two open arms (35 × 5 cm²) perpendicular to two closed arms of the same size with a small central square (5 × 5 cm²) between arms. The maze was elevated 50 cm from the floor in a dim room. Each rat was placed at the center of elevated plus maze with head facing toward the open arm and 5 min free exploration of rats was recorded by a video camera. The total number of entries into the open arm and closed arm, open arm time and closed arm time during the test were evaluated and presented. After each behavior test, the apparatus was cleaned with 70% ethanol, wiped with hand towels and allowed to air dry in between animals.

**Open field test**

The open field test box was a 100 × 100 × 30 cm wooden box used to study the cognitive and emotional reaction by observing the behaviors of animals as described previously [30]. The box was placed in an airtight room with adjustable light intensity of illuminating apparatus. A camera was used to record the path of rats over the box, from which experimental data and image information can be transmitted into computer. During the test, rats were allowed to move freely and to explore the environment around the open field test box for 5 minutes. The box was cleaned with dilute sulphuric acid solution after every test to avoid the left-over effects. Total movement distance (TMD, total running journey of a rat among 5 min in the test) and distance to the center (DTC, average distance of rat movements from the central point) were used as anxiety-like behavior [31].

**Light-dark exploration**

The rats were subjected to light-dark exploration test. Rodents were nocturnal and preferred darker areas, and a decrease in the exploratory activity in a lighted area was believed to be indicative of increased anxiety-like behavior [32, 33] and the time spent in the light was considered as a measure of anxiety-like behavior. The light-dark box consisted of a light compartment (27 × 27 × 27 cm) and a dark compartment (black colored surrounding walls and floor, 27 × 18 × 27 cm) separated by a partition with a single opening (7 × 7 cm) for passage from one compartment to the other as previously described. The apparatus was situated within a screen enclosed area of the behavior core facility room with only one experimenter/observer present in the room at the time of experiment under standard lighting conditions of approximately 700 lx as previously described. Time was recorded by manual scoring as previously described. The total time spent in the illuminated part was recorded for 5 min by an observer blinded to treatment as described [32, 33]. A rat was defined to have entered the light or dark box when both front paws and shoulders were inside the respective compartment.

**Serum corticosterone and oxidative stress measurement**

Animals were anesthetized with CO₂ and two 2 ml blood samples were taken by thoracotomy from heart: one was mixed well by centrifugation at 3000 r/min for 10 min and kept at 4°C, which was ready for detecting superoxide dismutase (SOD) activity and malondialdehyde (MDA) level; the other was added with EDTA and retardant peptidase for separation of plasma and kept at -80°C in a refrigerator, which was ready for detecting corticosterone. Samples were blindly analyzed using an EIA based kit specific for rats (DRG International Inc., USA).

**Estimation of amygdala oxidative stress**

Animals were sacrificed after blood samples were taken. The amygdala was separated on an ice-cold surface. Tissue homogenates were prepared as described [34]. 10% wet weight per volume amygdala tissue was prepared to determine the oxidative stress. The SOD enzyme activity was determined colorimetrically according to the kit manufacturer’s instruction (Jiancheng Biological Engineering Research Institute, Nanjing, China). Quantitative measurement of MDA was performed by assay kits (Jiancheng Biological Engineering Research Institute, Nanjing, China) according to the manufacturer’s protocol.
Western blot analysis

The Western blot analysis was performed as described previously [35]. Briefly, tissue samples from the amygdala were removed from brain under an anatomical microscope at the end of treatment, and total protein was extracted by using RIPA lysis buffer and protease inhibitors were added immediately before use. Samples from eight rats were analyzed by Western blot and each analysis repeated three times. Then equal amounts of protein (30 µg) for GABA<sub>A</sub>-α-2 receptor, N-methyl-D-aspartate receptor 2A (NR2A), NR2B, glutamate receptor 1 (GluR1), GluR1 phosphorylation at Ser831, glutamate decarboxylase 67 (GAD67), Ca<sup>2+</sup>/calmodulin-dependent protein kinase II-alpha (CaMKIIα) and β-actin as the loading control were separated by SDS-PAGE. After electrophoresis, the proteins were electrotransferred onto PVDF membranes. The membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (anti-rabbit/anti-IgG for the primary antibodies). The densitometric analysis of the Western-blot was conducted using a ChemiDoc XRS according to the instructions. As to data quantification, band intensity of each blot was calculated as ratio relative to β-actin. We set the ratio of control group as 100%, and other treatment groups were expressed as percentage to the CG.

Statistical analysis

All data are expressed as the mean ± SEM. The statistical significance of differences between groups (SPSS version 19.0) was performed using one-way analysis of variance (ANOVA) followed by LSD-t post-hoc test. A value of \( P < 0.05 \) was chosen as statistically significant.

Results

The effect of melatonin and/or sleep deprivation on anxiety-like behavior

The results of EPM, open field and light-dark test showed that there were no significant differences of the anxiety-like behaviors of animals in CG and those in the WPG (\( P > 0.05 \), Figures 1, 2). But the anxiety-like behaviors of
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SDG rats were significant different from these of animals in CG. This ability was improved by melatonin (P < 0.05, Figures 1, 2).

The effect of melatonin and/or sleep deprivation on serum corticosterone

Serum corticosterone level was shown in Figure 3. The serum corticosterone level of the rats in SDG was significant improved compared to that in the CG (P < 0.05, Figure 3). There was no significant difference between CG and WPGs (P > 0.05, Figure 3). With melatonin treatment, serum corticosterone level of rats in MTG decreased significant than that of SDG (P < 0.05, Figure 3).

The effect of melatonin and/or sleep deprivation on SOD enzyme activity and MDA level of serum and amygdala

After SD, a significant increase in the MDA level and a decrease in the SOD enzyme activity in serum and amygdala were observed in SDG rats when compared with those in CG (P < 0.05, Figure 4). MDA of serum and amygdala tissue homogenates in MTG were significantly reduced than those in the SDG by administering melatonin. By contrast, SOD enzyme activity in serum and amygdala was increased (P < 0.05, Figure 4).

The effect of melatonin and/or sleep deprivation on the excitatory and synaptic transmission-related proteins in amygdala

Excitatory synaptic transmission-related proteins were detected in the amygdala of rats.
The levels of GluR1 and phosphorylation of GluR1 at Ser831 (p-GluR1-Ser831) significantly increased after SD (P < 0.05, Figure 5A, 5C, 5E). However, SD had a different effect on the levels of N-methyl-D-aspartate receptor (NMDAR) subtypes in the amygdala. There is a remarkably increased expression of NR2B-containing NMDARs (P < 0.05, Figure 5A, 5D) while no difference of NR2A-containing NMDARs (P > 0.05, Figure 5A, 5B), suggesting the different roles of NMDAR subtypes in amygdala. Another critical excitatory synaptic protein CaMKII-alpha also significantly increased after SD (P < 0.05, Figure 5A, 5F). Treatment with melatonin significantly reversed the alteration of above proteins in the amygdala (Figure 5).

The effect of melatonin and/or sleep deprivation on GABA receptor in amygdala

The levels of GABA\textsubscript{\textalpha}-\textalpha-2 receptor were notably decreased in amygdala after SD (P < 0.05, Figure 6A, 6C). However, no significant difference was observed in the levels of GAD67 (P > 0.05, Figure 6A, 6B). Treatment with melatonin significantly reversed the down-regulation of GABA\textsubscript{\textalpha}-\textalpha-2 receptor expression in the amygdala of SDG rats (P < 0.05, Figure 6A, 6C), but had no effects on the GAD67 expression (P > 0.05, Figure 6A, 6B).

Discussion

The result of the present study provides evidence for the anxiolytic effect of melatonin on SD induced anxiety-like behavior. In addition, melatonin reverses the levels of oxidative stress markers including MDA level and SOD enzyme activity. Moreover, it is demonstrated that melatonin treatment counteract anxiety induced by SD partially through maintaining a balance between GABAergic and glutamatergic transmission in amygdala. The modified multiple platform model is widely used to deprive sleep. It is reported that this model is similar to the method that use electroencephalogram recording to evaluate subjects.
Our results showed that rats in WPG which could sleep on the wide platform in the same aquarium did not show any difference from those in the CG in anxiety-like behavior (Figures 1, 2) and without stress (Figure 3). As a result, SD is considered to be the major factor which causes anxiety-like behavior.

Since anxiety is regarded as an innate fear, the structural basis of anxiety resides in the neural circuitry related to fear response. The amygdala is a key structure for processing neuronal inputs from other parts of the brain, initiating output signals to responding nuclei, and generating various physiological responses, including behavioral, autonomic, and hormonal responses related to anxiety [24]. In both humans and animals, electrical stimulation of the amygdala induces anxiety, whereas lesion of the amygdala impairs the perception of fear [37]. EPM, open field and light-dark test are widely used to evaluate the anxiety-like behaviors.

In the present study, 72 h SD-induced stress significantly impaired ambulatory activity, caused anxiety-like behavior in rats. SD-induced stress activates hypothalamus-pituitary-adre-
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Hypothalamic (HPA) axis and influences several biological effects at both central and peripheral level. Besides, neurotransmitters and neuropeptides also influence HPA axis activity by acting at the hypothalamic or suprachiasmatic level. In the present study, serum corticosterone of SDG rats was significantly increased than that of ones in CG (Figure 3). It is strongly implicated by hyperactivity of central nervous system in the pathophysiology of behavioral alterations. Increased corticosterone level has been linked with anxiety-like behavior and decreased motor behavior response in humans [38, 39] which is also supported by our results (Figures 1-3). SD significantly influence brain functions and causes long-term changes in multiple neural systems. Many of these effect is mediated by stress-induced neurochemical and hormonal abnormalities that are often associated with oxidative stress [40].

Oxidative stress has been implicated in the pathophysiology of many neurological disorders. Experiments indicate the existence of an association between stress and disease in which ROS is involved [41-43]. SD may also cause the formation of oxidants and induce oxidative change to lipids, resulting in alterations in membrane functions, proteins damage and reduction in intracellular antioxidant defenses in different areas of the brains [44]. 72 h SD significantly caused oxidative damage and weakness antioxidant defense system [41-43]. In the present study, 72 h SD significantly increased MDA and reduced SOD enzyme activity suggesting oxidative stress in rat amygdala and serum (Figure 4). Antioxidant defense mechanisms include removal of oxygen, scavenging of reactive oxygen/nitrogen species or their precursors, inhibition of ROS formation, binding of metal ions needed for the catalysis of ROS generation and up-regulation of endogenous antioxidant defenses [42].

Melatonin is the primary secretory product of the pineal gland [15]. The secretion of melatonin with circadian rhythm in the blood of mammals is functionally linked to the adjustment of 24-hour cycles and to the circannual rhythm regulation. Since the circadian change of melatonin is opposite to that of HPA-related hormones, it may suggest a connection between these two factors. It is reported that melatonin could reverse the HPA-axis activation induced by stress which is similar to our results [45]. Besides, melatonin has also been reported to mark inhibitory effect on both spontaneous and stimulated HPA axis activity [46]. Stress activates HPA axis and influences several biological effects at both central and peripheral level. Furthermore, melatonin impaires contextual fear conditioning, a hippocampus-dependent task. On the contrary, melatonin facilitates the extinction of conditional cued fear without affecting its acquisition or expression, and melatonin facilitates cued fear extinction only when it is present during extinction training [47]. SD is showed to promote a dopaminergic influence in the striatal melatonin anxiolytic effect [48]. It is reported that melatonin doses as low as 1 mg/kg affords a significant anxiolytic effect [49]. The present study also reaffirmed the anxiolytic effect of melatonin in a SD model of the anxiety-like behavior (Figures 1, 2).

Melatonin is best known as a potent antioxidant [50, 51] and has been shown to be highly effective in reducing oxidative damage in the central nervous system which is especially sensitive to free radical damage. The central nervous system has high utilization of O₂ and contains large amounts of easily oxidizable fatty acids. Because melatonin is mainly produced in pineal gland and released into the cerebrospinal fluid [52], its concentrations in human serum and cerebrospinal fluid is present in adequate amounts to protect central nervous system [53]. Melatonin is a broad-spectrum antioxidant by scavenging hydroxyl, alkoxyl, peroxyl radicals, NO and °O₂ profile. As an antioxidant, melatonin may be regenerated after radical quenching through different processes [54]. It can also assist in stimulating antioxidant enzymes including SOD, glutathione, peroxidase and so on [55]. Due to oxidative stress plays an important role in the HPA axis and anxiety-like behavior during SD, melatonin may show anxiolytic effect as an antioxidant and free radical scavenger.

Hyperexcitation can promote anxiety-like behavior because of enhanced excitatory transmission or reduced inhibitory transmission [56]. Pathological anxiety is due to the imbalance of excitation and inhibition in neural circuits of the amygdala [57]. It is reported that tonic GABAergic reduction is induced by social isolation stress [58]. It has been reported that
GABA level in the posterior hypothalamus increased during sleep [46]. SD causes a significant increase in GABA contents as well as an elevation of L-glutamic acid decarboxylase activity [59, 60]. In the present study, protein level of excitatory glutamate receptors, including GluR1, NR2B, p-GluR1-Ser831 and CaMKIIα of SD rats amygdala increased, whereas the level of inhibitory GABA_α-2 receptor decreased (Figures 5, 6). However, there was no difference of amygdala protein level of NR2A and GAD67 in rats between CG and SDG. NR2A- and NR2B-containing NMDARs have been linked to different intracellular cascades and have different roles in synaptic plasticity. The alterations of NMDAR subtypes after acute stress suggest differing functions of NMDAR subtypes in the development of anxiety.

Many studies have found that melatonin interacts with GABA [61] to induce tranquilizing actions [49]. For instance, melatonin administration increases GABA level in some brain regions [20]. A great deal of evidences show that melatonin directly influences central GABAergic neurotransmission, since pinealectomy decreases benzodiazepine binding and a chronic treatment with melatonin increases GABA binding. This hormone increases the GABA synthesis and promotes hypnotic actions, which are inhibited by picrotoxin, a blocker of the GABA-activated chloride ionophore [62, 63]. In addition, the anxiolytic and sedative actions of melatonin can also be justified by its metabolite N-acetyl-5-methoxykynuramina, which stimulates GABA-benzodiazepine receptors [64]. Overall, this evidence supports the idea that the behavioural actions of melatoninergic compounds observed here could be caused by their direct or indirect effects on GABAergic transmission. In the present study, melatonin treatment decreased protein level of GluR1, NR2B, p-GluR1-Ser831 and CaMKIIα (Figure 5) as well as increased GABA_α-2 (Figure 6). Therefore, the anxiolytic effect of melatonin was, at least partially, through regulating the GABAergic and glutamatergic transmission in the amygdala of rat.

In conclusion, the present study provides strong evidence that melatonin has the ability to reduce SD-induced anxiety-like behavior. The underlying mechanisms are involved in oxidative stress and imbalance between GABAergic and glutamatergic transmission.

**Acknowledgements**

This work was supported by development projects of science and technology in Shaanxi province (No. 2011K15-06-10).

**Disclosure of conflict of interest**

None.

**Authors’ contribution**

Lei Zhang, Hong-Liang Guo and Fang-E Liu contributed to the experimental design, data acquisition, analysis and interpretation, drafting of the manuscript and revision of the manuscript. Hu-Qin Zhang contributed to the research designing and data interpretation. Tian-Qi Xu, Bing He, Zhen-Hai Wang, Yi-Peng Yang and Xiao-Dan Tang and Peng Zhang contributed to drafting of the manuscript and critical revision of the manuscript. All authors approved for the publication of the article.

**Abbreviations**

SD, sleep deprivation; ROS, reactive oxygen species; GABA, y-aminobutyric acid; NMDA, N-methyl-D-aspartate; CG, control group; WPG, wide platform group; SDG, sleep deprivation group; MTG, melatonin group; REM, rapid eye movement; EPM, elevated plus maze; TMD, total movement distance; DTC, distance to the center; SOD, superoxide dismutase; MDA, malondialdehyde; NR, N-methyl-D-aspartate receptor; GluR, glutamate receptor; GAD, glutamate decarboxylase; CaMKIIα, Ca²⁺/calmodulin-dependent protein kinase II-alpha; HPA, hypothalamus-pituitary-adrenal; NMDAR, N-methyl-D-aspartatereceptor; P-CaMKII, phosphorylated Calcium-calmodulin dependent kinase II.

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