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Microglial Reactions in the Pineal Gland of Sleep-deprived Rats With or Without Melatonin Treatment

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The effects of sleep deprivation with or without melatonin administration on the microglial reaction in the rat pineal gland were studied by immunohistochemistry and immuno-electron microscopy. Five days after sleep deprivation, pineal microglial cells were hypertrophic and showed an up-regulation of complement type 3 receptors as determined by the antibody OX-42, though the expression of major histocompatibility complex class I and II antigens, and antigen of monocyte/macrophage lineage marked by OX-18, OX-6 and ED1, respectively, were rarely seen. The OX-42 positive cells displayed thiner and longer processes than the control rats, whose processes were short. After a single injection of melatonin (10 mg/kg) for 5 consecutive days, the number of OX-42 labelled microglia in the sleep-deprived rats was comparable to that in the control rats; furthermore, the cells appeared reasonably normal in their external morphology. Quantitative analysis showed that the mean number of OX-42 labelled pineal microglia per area unit (341,914 μm^2) was 52 ± 2 in the control rats. It increased to 75 ± 4 following sleep deprivation but was restored to 54 ± 4 in the sleep-deprived rats treated with melatonin. With the antibodies OX-18, OX-6 and ED1, immunoreactive macrophages/microglia were also undetected in the pineal gland in both control and sleep deprivation coupled with melatonin treatment groups. Immunocytochemical processing of pineal tissues corroborated the above-mentioned findings in that the reactivity of microglial OX-42 was increased after sleep deprivation, but attenuated when combined with melatonin treatment. The induced OX-42 immunoreactivity of microglia suggests the involvement of these cells in the deleterious effect of prolonged sleep deprivation. Since melatonin can suppress microglial activation, it may potentially be used as a neuroprotective drug in the treatment of microglia-related cellular damage induced by sleep deprivation.

Keywords: pineal gland, sleep deprivation, melatonin, electron microscopy, rat

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Introduction

Many body functions, e.g. sleep, have circadian rhythms, and these are entrained by the daily light-dark cycle, acting through the visual pathway and driven by a circadian clock located in the suprachiasmatic nucleus [34]. This master oscillator is connected to the pineal gland by a circuitous route which passes through the hypothalamic paraventricular nucleus, intermediolateral cell column of the spinal cord, superior cervical ganglion, and then to the pineal gland [22]. The pineal gland is of special importance because it plays a central role in the rhythmic production of melatonin [23], the night signal in all vertebrates, which further modulates the activity of pacemaker cells within the suprachiasmatic nucleus and other hypothalamic structures [16,21]. Therefore, the mammalian pineal gland functions not only as a phototransducer, but also as a neuroendocrine organ of multitarget regulative controls, instrumental in the coordination and synchronization of homeostasis and behavior under physiological and stress-inducing influences [19,23,32].

Sleep deprivation, or sleep loss, is extremely stressful. Sleep-deprived rats suffer severe desynchronization of physiological and behavioral rhythms including fatigue, sleepiness, ataxia, stomach ulcers, loss of body weight and even death [27]. Experimental sleep deprivation has been shown to reduce some neurobehavioral functions and bring about such problems as lower seizure threshold [11], cognitive decline [17], impaired host defense [7,8] and altered drug-induced behavior [13]. The body responds to a variety of physical and psychological stressors by increasing activities of the anterior pituitary, adrenal gland and sympathetic nervous system [2]. The increased activities result in the discharge of adrenocorticotropin, glucocorticoids, epinephrine and norepinephrine to help the organism adapt to new conditions b affecting cardiovascular, energy-producing, an immune systems. As mentioned above, the pinea gland of mammals is an end organ in the sym pathetic nervous system and hence, stressor like sleep deprivation may affect the pineal gland directly via its sympathetic innervation. Indeed, our recent study has shown that long-term sleep deprivation can induce ultrastructural abnormalities indicative of neuronal damage in the rat pineal gland [15].

Along with the above, it has been reported that sustained sleep deprivation in rats can result in bacteremia and a septicemic death [8], indicating that the body's resistance to infection is suppressed. It has also been reported that 64 hours of sleep deprivation in humans can elicit an increase in leukocytosis and natural killer cell activity in parallel with neurobehavioral fatigue [7]. Since microglial cells are known to respond to the CNS damage [35], the first objective of this study was to determine if these cells in the pineal gland would react to long-term sleep deprivation.

Melatonin, with its ability to act as an efficient hydroxyl radical neutralizer [22,30,31] or its ability to reduce free radical generation by stimulating glutathione peroxidase [3] and inhibiting nitric oxide (NO) synthase [4,26], has been shown to exert anticonvulsant, anxiolytic, analgetic, hypnotic, and neuroprotective properties, and share anti-stress and sleep promoting activities [24]. In our recent study [15], the efficacy of melatonin in alleviating stress-induced pineal damage in sleep deprivation was shown by the attenuation of pinealocytic activation and concurrent diminution of organelle degeneration. The second objective of the present study therefore is to clarify whether the anti-stress property of melatonin would be sufficient to counteract the pineal microglial reaction induced by sleep deprivation.

Materials and Methods

A total of 45 Sprague-Dawley rats of both sexes, 10-12 weeks of age and weighing 320-380 g were used. They were divided equally into three groups. Group A served as the control group. While the rats in both Group B and Group C were subjected to sleep deprivation for 5 days, the rats in the latter group were given a simultaneous daily injection of melatonin. All animals were housed under natural conditions of day length (i.e. a 12 h light/12 h dark cycle from 06.00 to 18.00 h) at a constant temperature of 20 ±1℃ and with food and tap water *ad libitum*. The procedures of sleep deprivation and administration of melatonin followed those described recently [15]. In the handling of all animals, the NIH regulations along with the Guiding Principles of the Committee for Animal Research of Chung-Shan Medical University were followed.

Immunohistochemistry

Thirty rats (10 in each animal group) were used for OX-42, OX-18, OX-6 and ED1 immunostains. They were sacrificed by transcardiac perfusion under deep anaesthesia with pentobarbital (40 mg/kg, i.p.). Following thoracotomy, a heparin (1000 unit/kg) and sodium nitrite (20 mg/kg) mixture was quickly given through the left ventricle with a 1-ml syringe. This was followed immediately by the perfusion-fixation which began with a prewash of 300 ml of Ringer's solution and then with a 500 ml fixative of periodate-lysine-paraformaldehyde with a concentration of 2% paraformaldehyde according to the method used by McLean and Nakane [18]. The entire perfusion-fixation lasted 30 min. After perfusion, the pineal gland was removed, kept in 0.1M phosphate buffer (PB, pH7.4) containing 30% sucrose, and stored in the refrigerator overnight at 4℃. The pineal gland was cut at 30 μm thickness on the following day with a freezing microtome. Alternate sections of the pineal gland were incubated with one of the following monoclonal antibodies: OX-42 (Serotec MCA275R), OX-18 (Serotec MCA51R), OX-6 (Serotec MCA 46R) and ED1 (Serotec MCA341R) diluted 1:100 with phosphate buffer saline. These antibodies mark the CR3 receptors, MHC I, MHC II and cells of macrophage/monocyte lineage, respectively. Incubation time with the above antibodies was 24h at 4℃. Subsequent antibody detection was carried out through the steps of Avidin-biotin-horseradish peroxidase complex (ABC) method (ABC from Vector Labs Peroxidase kit PK 4000) and was intensified with nickel ammonium sulphate. Finally, all reacted sections were mounted, dehydrated, coverslipped and examined under bright-field illumination. The OX-42 labelled microglia in every area unit (341,914 μm^2) were identified and enumerated at a magnification of ×50. The mean number and standard derivation of the labelled cells per area unit in each animal group were then obtained.

Electron Microscopy

The remaining 15 rats (5 in each group) were used for OX-42 immunocytochemistry. All rats were sacrificed using transcardiac perfusion as described above. After perfusion, the pineal gland was removed, kept in the same fixative for 2 h and then transferred to 0.1 M PB containing 10% sucrose and stored in the refrigerator overnight at 4℃. The pineal gland was cut serially at 60 μm thickness on the following day with a vibratome. All collected tissue sections were processed with the above-mentioned OX-42 immunostain. They were then postfixed for 1 h in 1% OsO_4 in 0.1 M PB before dehydration through an ascending series of ethanol and embedding in Epon-Araldite. Ultrathin sections were examined under a HITACHI H-7500 electron microscope.

Results

The sleep-deprived rats physically showed yellow ungroomed fur, varying degrees of dermatosis, swollen paws, fatigue, sleepiness, irritable mood, disorientation, severe motor weakness, and loss of body weight (Table 1), although food intake was still normal.

OX-42 immunohistochemistry revealed that microglial cells were widely distributed in the pineal gland in the control group (Fig. 1A). The majority of OX-42 positive cells were oval or elongated bearing branching processes (Fig. 1B, Table 1). After long-term sleep deprivation, the number of OX-42 labelled cells was markedly increased (Fig. 2A) compared with the control (Fig. 1A). The reactive microglia exhibited an intensely stained cell body bearing hypertrophied processes (Fig. 2B, Table 1). In sleep-deprived rats treated with melatonin, the number of OX-42 labelled microglia (Fig. 3A) was comparable to that found in the control rats (Fig. 1A); furthermore, the cells appeared reasonably normal in their external morphology (Fig. 3B, Table 1). Quantitative analysis showed that the mean number of OX-42 labelled pineal microglia per area unit (341,914 μm^2) was 52 ± 2 in the control rats (Table 1). It increased to 75 ± 4 following sleep deprivation but was restored to 54 ± 4 in the sleep-deprived rats treated with melatonin (Table 1). With the antibodies OX-18, OX-6 and ED1, labelled cells were undetected in the pineal gland of control and sleep-deprived rats with or without melatonin treatment.

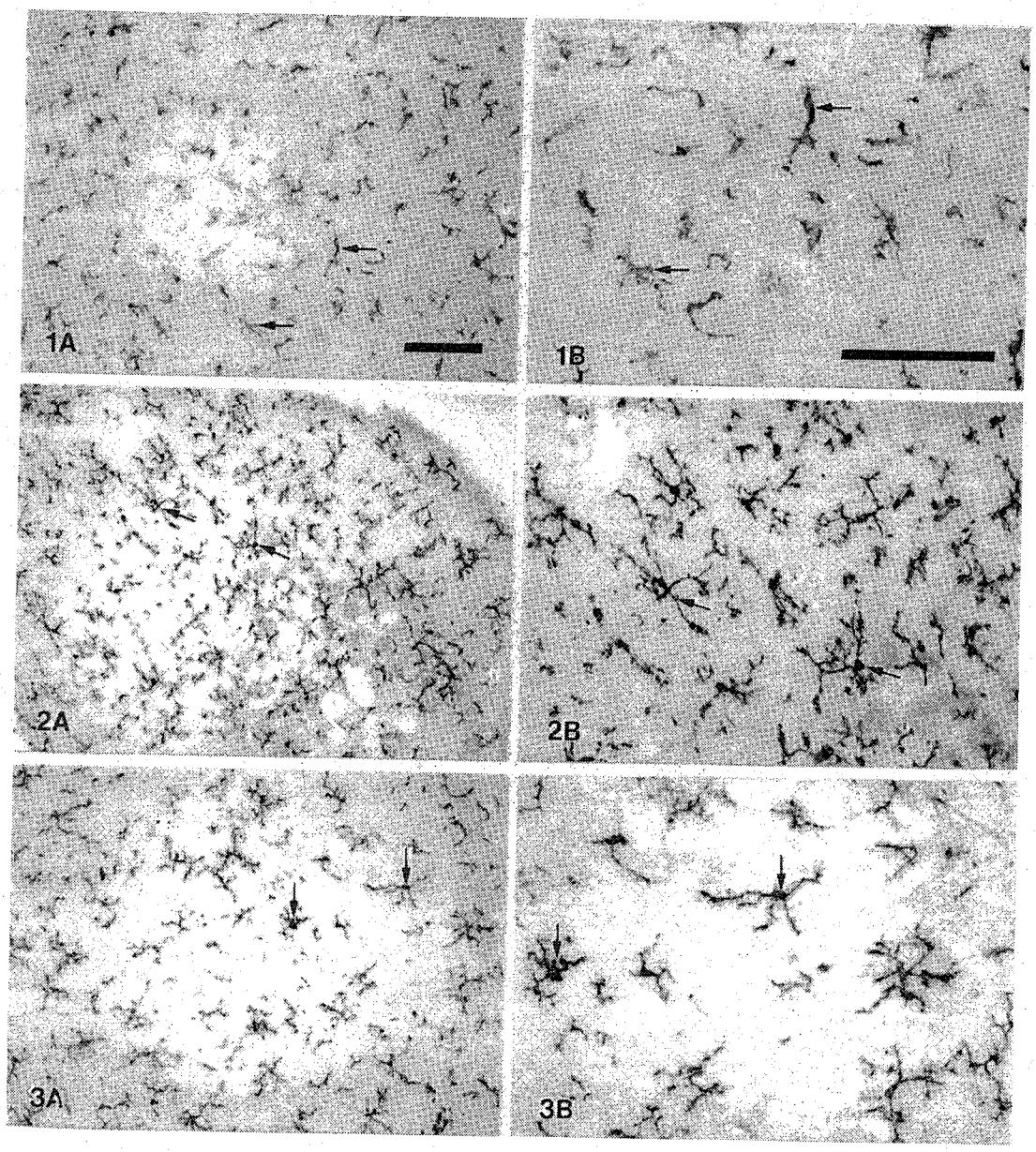
Compared with the control (Fig. 4), the immunoreactivity of OX-42 labelled microglia, as determined by the immuno-electron microscopy, was also intensified following sleep deprivation (Fig. 5, Table 1). This was manifested by a greater concentration of reaction products outlining the microglial cell membrane and uneven filling of the cytoplasm (Fig. 5). The OX-42 labelled pineal microglia in the sleep-deprived rats treated with melatonin (Fig. 6) shared morphological features of those of control (Fig. 4) and untreated sleep-deprived (Fig. 5) rats. Microglial OX-42 immunoreactivity in the sleep-deprived rats (Fig. 5, Table 1) appeared to be suppressed by the melatonin treatment (Fig. 6, Table 1).

Discussion

Earlier studies by Everson et al. [9] and Cirelli et al. [6] did not report any noticing structural alterations or histopathological changes

Table 1 Comparison of the mean body weight (MBW) and the mean number per area unit (MN/341,914 μm^2), immunoreactivity and morphology of the OX-42 positive pineal microglia in the normal (N), sleep-deprived (SD), sleep-deprived plus melatonin-treated (SD+M) groups. –, negative.

	N	SD	SD+M
MBW	356.6±16.9	319.1±27.6	342±20.8
MN/341,914 μm^2 (OX-42)	52±2	75±4	54±4
Immunoreactivity (OX-42)	Moderate	Increased	Between the N and SD groups
Morphology (OX-42)	Oval or elongated cell body bearing few branching processes	Intensely stained cell body bearing hypertrophied processes	Between the N and SD groups
OX-18 Immunostain	–	–	–
OX-6 Immunostain	–	–	–
ED1 Immunostain	–	–	–



Figs. 1A-B. OX-42 immunostained microglia in the pineal gland of a control rat (Fig. 1A). At higher magnification, most of the labelled microglia are ramified bearing a few branching processes (Fig. 1B). Arrows indicate the typical microglia in the control group. Figures 1A, 2A and 3A are of same magnification, scale bar = 100 μ m. Figures 1B, 2B and 3B are of same magnification, scale bar = 100 μ m.

Figs. 2A-B. At 5 days after sleep deprivation, microglial reaction as manifested by cellular hypertrophy with enhanced OX-42 immunoreactivity is evident in the pineal gland (Fig. 2A). The majority of the reactive microglia show a prominent cell body with thicker processes (Fig. 2B). Arrows indicate the typical microglia in the sleep deprivation group.

Figs. 3A-B. The morphological changes indicative of pineal microglial activation as illustrated in Fig. 2A are attenuated following melatonin administration to the sleep-deprived rats (Fig. 3A). The OX-42 labelled microglia appear reasonably normal (Fig. 3B) when compared with the same cell type of control rats (Fig. 1B). Arrows indicate the typical microglia in the sleep deprivation coupled with melatonin treatment group.

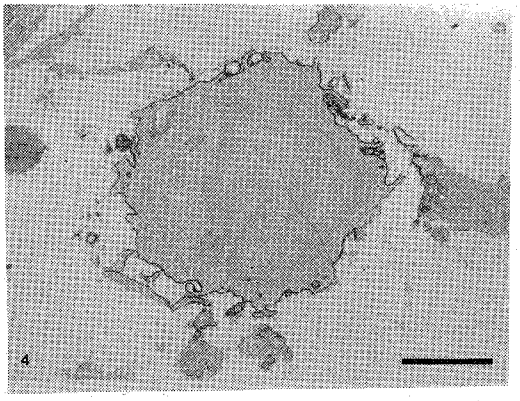


Fig. 4. Electron microscopic view of an OX-42 positive microglial cell in the pineal gland of a control rat. The reaction products outline its cell membrane. From figures 4 to 6, scale bar = 2 μ m.

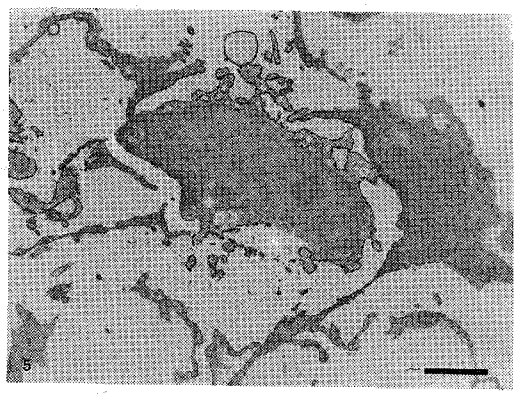


Fig. 5. Compared with the control (Fig. 4), the immunoreactivity of OX-42 labelled microglia is intensified following sleep deprivation (Fig. 5). This is manifested by a greater concentration of reaction products outlining the microglial cell membrane and also uneven filling of the cytoplasm (Fig. 5).

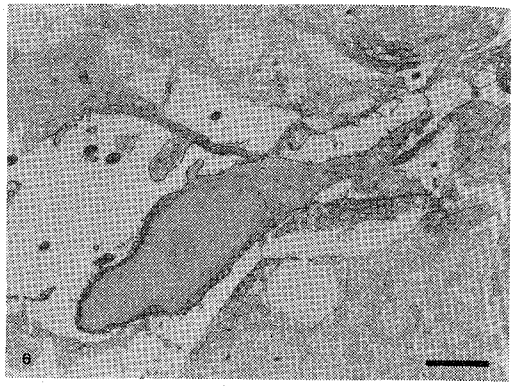


Fig. 6. The OX-42 labelled pineal microglial cell of a sleep-deprived rat treated with melatonin (Fig. 6) shares morphological features of that of control (Fig. 4) and sleep-deprived (Fig. 5) rats. Note the suppression of OX-42 immunoreactivity in the microglial cell following melatonin treatment (Fig. 6) as compared with the untreated sleep-deprived group (Fig. 5).

by postmortem examinations of various organs, including the brain, following sleep deprivation. Neither did they observe any microscopic neural degeneration that might account for the death and debilitation of the sleep-deprived animals. However, our recent study has shown that long-term sleep deprivation could induce a pinealocytic activation and the production of numerous membranous profiles, considered to be degraded cellular organelles, in some pinealocytes and sympathetic nerve terminals in the rat pineal gland [15], suggesting that the occurrence of degenerating organelles had resulted from the deleterious effect of sleep deprivation. This degeneration may be attributed to an overload of secretory activity of the pineal gland during stress elicited by the long-term sleep deprivation, leading to functional exhaustion and irreversible damage of the oxidation-related organelles. The present study has provided the first morphological evidence implicating microglial reaction in the rat pineal gland following sleep deprivation. The significance of pineal microglial activation in the long-term sleep deprivation is unknown. It may have certain relationships with the consistent effects of prolonged sleep deprivation, ex. irreversible damage of the oxidation-related organelles in the pineal gland as previously described [15]. Sleep deprivation per se is definitely an insult to the brain. Microglial activation is a step-wise process that depends on the severity of insult [10]. Graded activation of microglia from resting to activated states can be seen in experimental results as well as human diseases [36]. It has been speculated that the kind of microglial activation observed in the

present study may be a response to subtle changes in the microenvironment after sleep deprivation, which include such changes as the pineal neuronal damage [15], the increased levels of cytokines like superfluous interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) necessitated for induction of non-rapid eye movement sleep [14] or for augmenting intracerebral immune responses [5].

Melatonin has been shown to increase the efficiency of oxidative phosphorylation and electron transport [1] and to also scavenge NO [22,30,31]. The efficacy of melatonin in alleviating stress-induced pineal neuronal damage in sleep deprivation in our previous study [15] is shown by the attenuation of microglial activation in the present study. Our present results, therefore, suggest that administration of exogenous melatonin may counteract the microglia-related cellular damage induced by sleep deprivation. Abnormalities of melatonin secretion have been described in several conditions from physiology to pathology. A reduction in circulating levels of melatonin has been found in aged individuals [20,33], in those with a low intake of tryptophan [37] and in individuals suffering from insomnia [12] and fatal familial insomnia [25]. Hence, adequate melatonin levels and well-developed diurnal rhythms in circulating melatonin with nocturnal levels are vital. The present study also demonstrates that melatonin may help suppress the microglial activation associated with the ultrastructural damage in pineal gland after sleep deprivation.

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未經與經過褪黑激素治療後鼠松果腺的微小膠細胞反應

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本研究以免疫組織化學法及免疫電子鏡技術，探討睡眠剝奪對未經與經過褪黑激素治療下的大白鼠，其松果腺內部微小膠細胞反應之影響。經過五天的睡眠剝奪後，松果腺內微小膠細胞增生，並且經由OX-42抗體偵測到其第三型補體受器有向上調升現象；受OX-42標誌到的微小膠細胞發展出長細的突起，有別於正常鼠的短小突起之外觀形態。在睡眠剝奪的同時連續給予五天的褪黑激素注射後，發現上述微小膠細胞被激活的現象減輕許多，數目與外觀形態均回復到正常合理狀態。定量分析顯示，在鼠松果腺的單位面積（341,914 μm^2）內，正常組的微小膠細胞的平均數目為52±2，睡眠剝奪後陡升到75±4，經過褪黑激素治療後回復到54±4。以OX-18、OX-6與ED1抗體來分別檢驗主要組織一致性複合體級一與級二抗原以及單核球／吞噬細胞連結抗原，則發現三動物組的松果腺微小膠細胞均無此種免疫活性存在。利用電子顯微鏡偵測微小膠細胞的OX-42免疫活性，更加證實上述光學顯微鏡的實驗結果，亦即睡眠剝奪後微小膠細胞的OX-42免疫活性劇增，而褪黑激素對此現象則有壓制效果。因此本研究建議褪黑激素或可做為睡眠缺乏時保護神經系統對抗與微小膠細胞相關的神經傷害之藥物。

關鍵詞：松果腺、睡眠剝奪、褪黑激素、電子顯微鏡術、大白鼠

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