行政院國家科學委員會補助專題研究計畫成果報告

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※ 褪黑激素對睡眠剝奪之鼠松果腺的作用 ※

※Effects of Melatonin on the Pineal Gland of Sleep-deprived Rats※

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計畫類別：✓個別型計畫　□整合型計畫

計畫編號：NSC89－2320－B－040－070－

執行期間：89 年 8 月 1 日至 90 年 7 月 31 日

計畫主持人：藍琴臺

共同主持人：徐至清

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□國際合作研究計畫國外研究報告書一份

執行單位：中山醫學大學解剖學科

中 華 民 國 90 年 10 月 19 日

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一、中英文摘要

經過五天的睡眠剝奪後，在電子顯微鏡下，許多鼠松果腺細胞產生了構造性變化，包括內質網、高爾基氏器與粒線體的擴大，脂肪滴、空泡與密核突觸泡的數目增加。這些現象被認為是松果腺合成與分泌加速的形態學證據。另外，在松果腺細胞與交感神經終末內，也發現到許多被認為是退化性細胞胞器的膜狀物，可能是睡眠缺乏所導致之神經瓦解碎屑。此點可歸因於長期睡眠缺乏的壓力下，松果腺分泌活動的負荷過重，而導致合成分泌功能衰竭與有氧呼吸相關胞器的不可逆退化性變化。在睡眠剝奪的同時連續給予五天的褪黑激素注射後，發現上述松果腺被激活的現象減輕許多，事實上，胞器的退化性傷害更是少見。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傷害的藥物。

關鍵詞：松果腺、松果腺細胞、睡眠剝奪、褪黑激素、電子顯微鏡術

Abstract

The effects of sleep deprivation with or without melatonin treatment on the pineal morphology in rats were studied. Five days after sleep deprivation and by electron microscopy, many of the pinealocytes exhibited structural alterations including dilation of the cisternae of the rough/smooth endoplasmic reticulum, Golgi saccules and mitochondria, and increase in the numbers of lipid droplets, vacuoles and dense-core vesicles. These features were considered as morphological evidence of increased synthesis or secretion by the pineal gland. In addition, numerous membranous profiles, considered to be degraded cellular organelles, were observed in some pinealocytes and sympathetic nerve terminals. It is suggested that the occurrence of degenerating organelles had resulted from the deleterious effect of sleep deprivation. This 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. In sleep-deprived rats receiving a single injection of melatonin (10 mg/kg) for 5 consecutive days, the above features indicative of pinealocytic activation were attenuated. In fact, all signs of degeneration of cellular organelles were rarely found. Present results suggest that the pineal gland is itself a target for exogenously administered melatonin. Thus, melatonin when administered systemically may be used as a potential neuroprotective drug against neuronal damage induced by sleep deprivation.

Keywords: pineal gland; pinealocyte; sleep deprivation; melatonin; electron microscopy

二、計畫緣由與目的

It is well documented that many body functions, e.g. sleep, have circadian rhythms, and that 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 [53]. 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 [43]. The pineal gland is of special importance because it plays a central role in the rhythmic production of melatonin [44], the night signal in all vertebrates, which further modulates the activity of pacemaker cells within the suprachiasmatic nucleus and other hypothalamic structures [26, 33]. 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 [30, 37, 48].

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 [42]. Experimental sleep deprivation could reduce some neurobehavioral functions such as the lower seizure threshold [12], cognitive decline [27], impaired host defense [10, 11] and altered drug-induced behavior [16]. 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 would result in discharge of adrenocorticotropin, glucocorticoids, epinephrine and norepinephrine to adapt the organism to the new conditions by affecting cardiovascular, energy-producing, and immune systems. As mentioned above, the pineal gland of mammals is an end organ of the sympathetic nervous system and hence, stressor like sleep deprivation may affect the pineal gland directly via its sympathetic innervation. Indeed, acute exposure of rats to several types of stressors, such as immobilization, forced swimming, and hypoglycemic shock elevated circulating catecholamines and altered both pineal melatonin content and N-acetyltransferase activity, which is considered to be the rate-limiting enzyme in melatonin synthesis [6, 7, 20]. Morphologically, the pinealocytes of gerbils responded to acute immobilization by forming new concretions [28], which are considered to be associated with the noradrenalin turnover [59] and social stress [15]. Long-term multifactor stress inducement could also elicit the neuroendocrine-like (i.e. pinealocytes with multilocular Golgi apparatus) and ependymal-like (i.e. pinealocytes with abundantly dispersed polysomes, broadened granular reticulum and energized mitochondria) activities in the rat pineal gland, and these features are considered to be evidence of a stimulated, peptidergic-mediated pineal gland activity [29].

Melatonin, with its ability to act as an efficient hydroxyl radical neutralizer [34, 45, 46] or to reduce free radical generation by stimulating glutathione peroxidase [3] and inhibiting nitric oxide (NO) synthase [4, 41], has been shown to exert anticonvulsant, anxiolytic, analgetic, hypnotic, and neuroprotective properties, and share anti-stress and sleep promoting activities [39]. It has been reported that immobilization stress could induce the pineal morphological changes in the rodent [28, 29], but the effects of sleep deprivation have not been evaluated. It also remains to be ascertained if the anti-stress property of melatonin would be sufficient to protect the pineal gland against possible deleterious actions of sleep deprivation. This study sought to clarify if melatonin would have a neuroprotective role for the pineal gland in sleep deprivation.

三、結果與討論

Results

At lower magnification, the preponderant pinealocytes showed characteristic features uniform throughout the parenchyma in normal rats (Fig. 1). The

polyhedral or elongated pinealocytes were endowed with an abundant cytoplasm with moderate electron density (I in Fig. 1). In general, the nucleus showed a regular outline and contained mostly euchromatin with a few discrete heterochromatin clumps. The cytoplasm contained many slender mitochondria, scattered free and polyribosomes, isolated profiles of cisternae of rough/smooth endoplasmic reticulum (r/s ER), Golgi apparatus and a few lipid droplets (Fig. 1). The terminal buds of pinealocyte processes were filled with clear vesicles admixed with a few vacuoles, mitochondria and microtubules (Fig. 2). Varicose sympathetic nerve endings containing large numbers of clear and dense-core vesicles were distributed in the pericapillary space or near the pinealocytes (Fig. 3).

In rats subjected to a long duration of sleep deprivation, many pinealocytes underwent structural alterations so that the gland was composed of mixture of normal looking cells and those that appeared morphologically activated (II in Fig. 4) or disrupted of their cytoplasmic texture (III in Fig. 4). This along with the grossly dilated intercellular spaces (Fig. 5) gave the pineal gland a rather heterogeneous appearance (Fig. 4). Normal looking pinealocytes resembling those in Group A (I in Fig. 1) were rare. The majority of the pinealocytes in Group B showed morphological changes indicative of increased synthesis and secretion (II in Fig. 4). The electron density of cytoplasm was moderately increased (Fig. 5). The nucleus was more or less indented with margination of heterochromatin (Fig. 5). A salient feature was the increase in the number and size of lipid droplets, many of which appeared to be released into the extracellular spaces (Fig. 4). In some cells, the cytoplasm was filled with vesicles and vacuoles containing flocculent materials (Fig. 5). Not infrequently, the cytoplasm contained large membrane bound vacuoles with vesicular profiles (Fig. 6). The Golgi saccules appeared dilated (Figs. 4 and 6); numerous coated vesicles appeared to bud off from the dilated saccules (Fig. 7). Well-developed parallel oriented cisternae of sER were present in some pinealocytic terminal buds (Fig. 8). Intracristal space-widened mitochondria resembling the energized or "tigroid-like" mitochondria as described previously by Milin et al. [29] and Karasek et al. [19] were common in the pinealocytes (Figs. 5 and 6) and their terminal buds (Figs. 8 and 9). Besides numerous clear vesicles seen in the terminal buds of pinealocyte processes, lipid droplets (Fig. 8), vacuoles (Fig. 10) and dense-core vesicles (Fig. 10) were noted more frequently than in controls (Fig. 2).

While the majority of the pinealocytes in sleep-deprived rats displayed the above features, some cells were characterized by a thin rim of cytoplasm surrounding a deeply infolded nucleus with patched heterochromatin clumps (III in Fig. 4). A common feature was the occurrence of many membranous bodies, myelin-like figures or membranous whorls in the cell body (Fig. 11) and terminal buds (Figs.12 and 13) of pinealocytes. In some areas, the membranous irregular bodies resembled distorted cisternae of rER or collapsed lipid droplets (Fig. 11). Some of the membranous whorls appeared to be derived from the break down of mitochondria (Fig.13). The sympathetic nerve endings also displayed numerous electron-dense small membranous whorls (Fig. 14).

The pinealocytes of the sleep-deprived rats treated with melatonin (Fig. 15) shared features of those of Groups A (Fig. 1) and B (Fig. 4) rats. The morphological changes indicative of pinealocytic activation (II in Fig. 15) were less evident when compared with Group B (Fig. 4). Increase in lipid droplets (Fig. 15) and well-developed concentric lamellae of sER with short tubules (Fig. 16) were observed. However, membranous profiles or debris were rarely seen. Occasional darkened mitochondria were distributed in the sympathetic nerve profiles (Fig. 17).

Discussion

Present results have shown that long-term sleep deprivation induces ultrastructural changes in the rat pineal gland. The pinealocytes showed features characteristic of cells involved in increase in synthesis and secretion, and this points to the stimulation of the pineal gland activity in sleep-deprived rats. Some of the changes included dilatation of r/s ER, Golgi saccules and mitochondria as well as increase in numbers of lipid droplets, dense-core vesicles and vacuoles of various types. Romijn [47] had hypothesized that the vesicular and tubular sER (resembling the concentric lamellate and parallel oriented cisternae of sER, here described) in the pinealocytes of rabbits played a role in indoleamine synthesis. It has been suggested that serotonin or melatonin is stored in the dense-core vesicles [8, 17, 54], while proteinaceous materials are stored in vacuoles filled with flocculent materials [18]. It has been reported that the large single intra-cytoplasmic vacuole in the pinealocytes of hedgehogs was formed by the confluence of small vacuoles [36]. The cytoplasmic vacuoles which appeared in the pinealocytes of the sleep-deprived rats resembled those in the hedgehog pinealocytes [36]. It is suggested that the increased secretions of the pinealocytes in sleep deprivation may be stored in the newly formed intra-cytoplasmic vacuole. There is evidence that enhanced activity of pinealocytes is accompanied by an increase in lipid droplets [19, 29]. In the present study, there was also marked increase in numbers and size of lipid droplets. The most striking feature was the massive release of lipid from the pinealocytes into the extracellular spaces. It is speculated that excessively synthesized compounds such as melatonin induced by sleep deprivation may also be stored in the lipid droplets and discharged simultaneously.

Present observations of the pinealocytic activation correlate an earlier finding of elevation of the plasma melatonin level and an impaired rhythmic secretion of the pineal melatonin after 72-h sleep deprivation in male rats [35]. It is known that pinealocytic activity, both peptidergic and indolergic, is initiated by the binding of norepinephrine to adrenergic β1-receptors, enhanced by α1-adrenoceptors' binding and acting through cAMP and cGMP cascades with an amplifying assistance of Ca^{+2}, protein kinase C and arachiodonic acids [57]. Present results suggest that the sleep deprivation stress could have activated the superior cervical ganglia whose postganglionic axon terminals impinge directly on the pinealocytes [23]. Stimulation and activation of pinealocytes would hence result in increase in protein or indoleamine synthesis. However, over-stimulation of nerve cells may lead to irreversible excitotoxicity and cell death due to oxidative stress [26, 33]. There is evidence to suggest that norepinephrine-induced cGMP accumulation involves stimulation of a Ca^{+2}/calmodulin-sensitive form of NO-synthase, resulting in the enhanced formation of NO from L-arginine in the pineal gland [13, 22, 51, 52, 56]. NADPH-diaphorase histochemical and NO-synthase immunohistochemical techniques have localized stained nerve fibers, endothelium and/or pinealocytes in the pineal gland of the rat [24, 55, 58], sheep [25] and frog [50]. The presence of the two enzymes in the pinealocytes of the rat suggests a possible involvement of norepinephrine in NO synthesis in response to sleep deprivation. NO can interfere with vital cellular processes, including mitochondria oxidative phosphorylation and ribonucleotide reductase [1, 31]. NO is also able to react rapidly with superoxide anion to yield the peroxynitrite anion, which decomposes to hydroxyl radical, the most reactive species. The latter can attach proteins, deoxynucleic acids and lipid membranes, thereby disrupting cellular functions and membrane integrity [1, 9]. As a result of sleep deprivation, the activated pinealocytes and sympathetic nerve endings may produce massive quantities of the free radical NO, which in turn may cause cellular damage leading to the accumulation of degenerating products in the cytoplasm as

shown in the present study. Oxidative stress therefore may be the causative, or at least an ancillary, factor in the organelle degradation in the pineal gland of the sleep-deprived rats.

Melatonin has been shown to increase the efficiency of oxidative phosphorylation and electron transport [1] and to also scavenge NO [34, 45, 46]. The efficacy of melatonin in alleviating stress-induced pineal damage in sleep deprivation is shown by the attenuation of pinealocytic activation and concurrent diminution of organelle degeneration. Present results therefore suggest that administration of exogenous melatonin may counteract the pineal damage induced by sleep deprivation. Cagnacci et al. [5] have reported that administration of melatonin could reduce the stimulated norepinephrine. There is evidence to support that melatonin is the most powerful and effective endogenous hydroxyl radical scavenger detected to date and it provides on-site protection to all biomolecules due to its lipophilic nature [39, 45]. In the light of our study as well as others, it is speculated that excessive activation of β1-adrenoceptors and oxidative stress may be involved in the pineal damage in the present study. In this regard, melatonin seems to have the opposing roles by protecting the pineal gland against over-stimulation and subsequent oxidative damage in 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 [32, 49], in those with a low intake of tryptophan [60] and in individuals suffering from insomnia [14] and fatal familial insomnia [40]. Hence, adequate melatonin levels and well-developed diurnal rhythms in circulating melatonin with nocturnal levels would be vital. Present results have demonstrated that melatonin may help attenuate the ultrastructural damage in pineal gland associated with sleep disorders.

四、計畫成果自評

本計畫如期完成，並在計畫執行中已將研究發現之重要部分撰文發表，刊載於 Brain Research 910 (2001) 1-11，頗受好評。現階段著手將次要部分整理出來，準備刊登於本校醫學雜誌。本研究突破了以往睡眠剝奪對神經系統傷害的莫衷一是，提出確切的解剖學證據，證明睡眠缺乏的確會造成松果腺的嚴重傷害。而松果腺及其分泌物褪黑激素乃控管日夜睡眠周期之鑰，故無怪乎睡眠缺乏將引起一連串生理時鐘的失調。藉此研究，吾等有機會明白松果腺的作用機制外，更進一步略窺了其他生理時鐘的相關神經核區，可做為往後深入探討失眠症神經迴路的基石。

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