Effect of melatonin on pro-opiomelanocortin mRNA expression in arcuate nucleus of rat hypothalamus[1]

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KEY WORDS melatonin; pro-opiomelanocortin; messenger RNA; arcuate nucleus; *in situ* hybridization; computer-assisted image processing; analgesia

ABSTRACT

AIM: To observe altered expression of pro-opiomelanocortin (POMC) mRNA localized in neurons in the arcuate nucleus of rat hypothalamus following exogenous administration of melatonin. **METHODS**: The experimental animals were divided into two groups injected ip with melatonin 90 mg·kg^{-1} and vehicle at 12 h interval (9:00 am and 9:00 pm). Twelve hours after the last injection, the rat brains were processed for coronal sections and nonradioactive *in situ* hybridization histochemistry technic was used. The integral optical density (IOD) and mean optical density (OD) of the stained brain sections were measured using the computer-assisted image processing technique. **RESULTS**: The POMC mRNA expression in the arcuate nucleus showed obvious enhancement in the brain sections of rats treated with melatonin. The IOD and OD values in the hypothalamic arcuate nucleus area were increased significantly ($P < 0.01$, $P < 0.05$ respectively) with melatonin treatment. **CONCLUSION**: Melatonin may enhance the POMC mRNA expression in the arcuate nucleus of rat hypothalamus.

INTRODUCTION

Melatonin (MEL, *N*-acetyl-5-methoxytryptamine) is a neurohormone synthesized and secreted mainly by the pineal gland. It has been implicated in some psychopharmacological effects including the sedative/hypnotic, anticonvulsant, and analgesic activity[1,2]. In particular, melatonin has been shown to have an analgesic effect[3-7] which can be antagonized by the administration of naloxone[4-7]. We have also observed that icv naloxone blunted the analgesic effect induced by ip melatonin (CAPS News Commun 1998; 17 suppl 1: 125). The results of these studies indicate that the analgesic effect of melatonin is related to the endogenous opioid system in the brain.

The endogenous opioid peptide, β-endorphin (β-Ep), and its precursor, proopiomelanocortin (POMC), are synthesized in the arcuate nucleus of the hypothalamus and exert numerous effects throughout the brain including the antinociceptive effect[8]. There has been considerable interest in whether melatonin might modify β-endorphin in the brain, thus contributing to mechanism of melatonin analgesia. The present study was to observe the expression of POMC mRNA following exogenous administration of melatonin.

MATERIAL AND METHODS

Chemicals Melatonin was purchased from Sigma Co. It was dissolved in 5 % absolute ethanol saline (v/v) immediately before use. Digoxigenin oligonucleotide tailing labeled kit and Digoxigenin oligonucleotide detected kit were purchased from Boehringer Mannheim Biochemica, Germany. Digoxigenin-labeled POMC cRNA probe (462-mer) was obtained from Department of Neurobiology, Second Military Medical University, China. Specificity of the probe was confirmed by Northern blot analysis and hybridization with sense probe.

Animal and treatment schedule Adult male Sprague-Dawley rats (190 – 210 g, Experimental Animal Center of Shanghai Medical University, Grade Ⅱ, Cer-

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tificate No 02-22-2) were maintained in a temperature-controlled environment under a 12∶12 h light/dark cycle with free access to food and water. Rats were randomly divided into 2 groups∶the rats of group I were injected ip two times (9∶00 am, 9∶00 pm) at 12 h interval with melatonin 90 mg·kg^{-1} body weight, the rats of group II with vehicle (5 % ethanol saline).

Brain section Twelve hours after the last injection, the rats were anesthetized by an ip injection of sodium pentobarbital 40 mg·kg^{-1} body weight, and perfused intracardially with 250 mL of saline followed by 350 mL of fixative containing 4 % paraformaldehyde in phosphate buffer 0.1 mol·L^{-1}(PB, pH 7.4). The brains were removed and immersed in the above fixative at 4 ℃ for 5 h, and then immersed at 4 ℃ sequentially in solutions containing phosphate buffer 0.1 mol·L^{-1} and either 20 % or 30 % sucrose. Coronal brain sections 30 μm were cut frozen with a sliding microtome, collected in 24-well culture dishes, and stored at −20 ℃ in a cryoprotectant solution[9] (phosphate-buffered saline 0.05 mol·L^{-1}, 30 % sucrose, and 30 % ethylene glycol).

***In situ* hybridization histochemistry** A free-floating technique was employed to perform a non-radioactive *in situ* hybridization histochemistry. Prior to hybridization with the probe, the sections were fixed in 4 % paraformaldehyde for 5 min, washed in PBS 0.1 mol·L^{-1}(phosphate-buffered saline, pH 7.4) four times for 15 min each, pretreated with proteinase K (1.5 mg·L^{-1}) in Tris-HCl buffer 0.01 mol·L^{-1}(pH 8.0) with EDTA 1 mmol·L^{-1} at 37 ℃ for 30 min, rinsed in PBS 0.1 mol·L^{-1} two times for 15 min each, followed by acetylation with 0.25 % (v/v) acetic anhydride in triethanolamine 0.1 mol·L^{-1} for 10 min, and treated with 50 % formamide in 4 × SSC (saline sodium citrate) for 20 min. Then, the sections were incubated in hybridization solution at 42 ℃ for 20 h. Hybridization solution contained the digoxigenin-labeled POMC cRNA probe (1 mg·L^{-1}), 50 % formamide, 5 × SSC, 0.02 % SDS, and 2 % blocking reagent. Following hybridization, the sections were rinsed sequentially in 2 × SSC twice for 15 min each, 2 × SSC containing RNase 20 mg·L^{-1} at 37 ℃ for 30 min and 0.1 × SSC twice at 42 ℃ for 15 min each. After the post-hybridization wash, the signals of POMC mRNA in neurons were detected with color reaction by Digoxigenin oligonucleotide detection kit. The sections were incubated in alkaline phosphatase-labeled anti-digoxigenin antibody (diluted 1∶3000) at 37 ℃ for 2 h and treated with alkaline phosphatase substrate solution, containing nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate in dark for 7 h to develop color.

For identifying the specificity of hybridized signals, brain sections were treated with A) RNase to digest the POMC mRNA followed by incubation in probe-containing hybridization buffer, B) incubation in hybridization buffer without the probes, and C) incubation without the above antibody.

Data analysis Specific hybridization was quantified using a computer-assisted image processing and analysis system (Leica Q500IW, Germany). The integral optical density (IOD) and mean optical density (OD) were measured in an area of 85011.5 μm^2 in the arcuate nucleus of the hypothalamus. Measurements were performed in four fields (two brain sections, bilateral) per rat at the arcuate nucleus of the hypothalamus. Values after subtracting background density[10] were averaged to give an IOD value or an OD value for each animal that corresponds to POMC mRNA level per animal. Brain structures were identified by referring to the rat atlas[11]. Date were expressed as $\bar{x} \pm s$ and analyzed by two-tailed t-test.

RESULTS

In sections treated with RNase or incubated without the probe or without the antibody, no signals were found.

In the brain sections of rats treated with vehicle, the POMC mRNA positive neurons were found in the arcuate nucleus of hypothalamus and the expression of POMC mRNA was at low level (Fig 1). Compared with the vehicle-treated group, the POMC mRNA expression in the brain sections of rats treated with melatonin showed obvious enhancement with light microscopic observation (Fig 1). By analysing the hybridized brain sections using the computer-assisted image processing and analysis system, it was revealed that the IOD values and OD values were increased significantly ($P < 0.01$, $P < 0.05$ respectively) with melatonin treatment (Tab 1).

DISCUSSION

In the present experiment, we have observed for the first time that the exogenous administration of melatonin could enhance the POMC mRNA expression in the arcuate nucleus of the rat hypothalamus. Regarding melatonin

Tab 1. Effect of melatonin on the expression of POMC mRNA in the arcuate nucleus of the rat hypothalamus. $n = 5$ rats. $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* vehicle-treated group.

Treatment	IOD/10^4 μm^2 (area)	OD
Melatonin	66 ± 23^c	0.092 ± 0.013^b
Vehicle	17 ± 7	0.070 ± 0.011

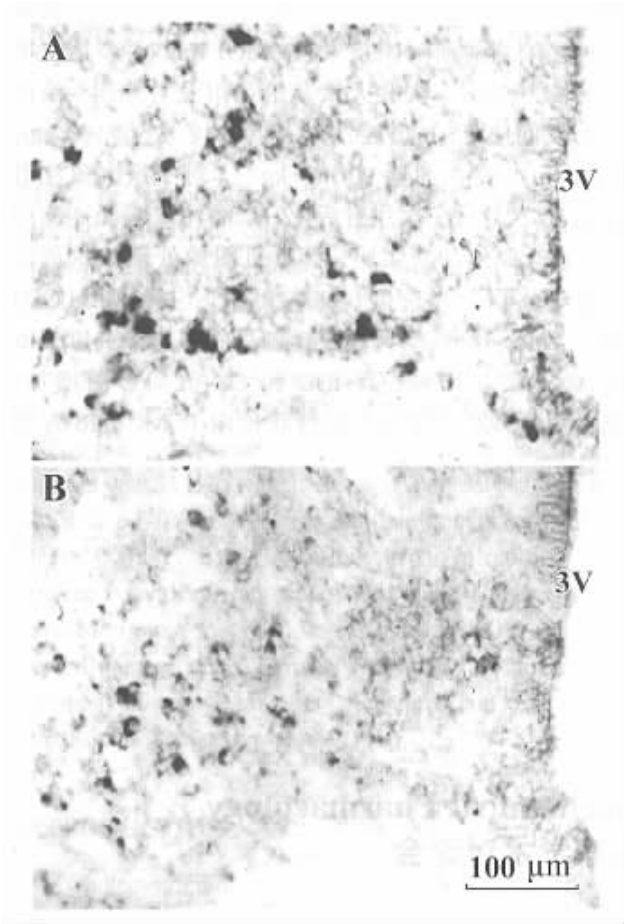

Fig 1. POMC mRNA expression enhanced by melatonin. Photographs show stained coronal sections through the left arcuate nucleus of the rat hypothalamus after *in situ* hybridization analysis. A: rat injected with melatonin; B: rat injected with vehicle. 3V, third ventricle.

and the release of β-Ep in the brain, in a recent study[12], we have shown that melatonin may promote the release of β-Ep from the periaqueductal gray in rats. Lincoln *et al*[13] have reported that the placement of bilateral microimplants of melatonin in the mediobasal hypothalamus resulted in an increase in the plasma concentration of β-Ep in rams. It has also been reported[14] that the β-Ep concentration in the mouse hypothalamus significantly decreased 30 min after melatonin was administered intraperitoneally. These studies[12-14] suggest that melatonin may promote the release of β-Ep in the brain. The increase in β-Ep release brings about an increase in the synthesis of β-Ep, and its precursor, POMC, which is associated with an enhancement of POMC mRNA expression. Therefore, it is logical to assume that the increase in β-Ep release may be one of reasons resulting in the up-regulation of POMC mRNA expression. Thus the results in the present paper suggest that melatonin might promote the release of β-Ep in the brain, and this may be related to the analgesic effect of melatonin.

The POMC mRNA expression does not seem to be an immediate but rather a long-term effect of melatonin. Wajs *et al*[15] have reported that melatonin can stimulate the expression of POMC gene in the lymph nodes and bone marrow in rats. In the present paper, it has been demonstrated that melatonin promoted the POMC mRNA expression in the arcuate nucleus. Thus it seems that melatonin may play a role in mediating the POMC gene expression. However, its biological significance needs to be further elucidated.

In conclusion, it has been observed that melatonin enhances the POMC mRNA expression in the arcuate nucleus of rat hypothalamus.

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褪黑素对大鼠下丘脑弓状核前阿黑皮素 mRNA 表达的影响[1]

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关键词　褪黑激素；前阿片黑素细胞皮质激素；信使 RNA；弓状核；原位杂交；计算机辅助图像处理；镇痛

目的：观察外源性给予褪黑素对大鼠下丘脑弓状核内神经细胞的前阿黑皮素（POMC）mRNA 表达水平的影响. 方法：实验大鼠分给药组及对照组，给药组大鼠间隔 12 h 腹腔注射褪黑素 2 次（9：00 am，9：00 pm），每次剂量 90 mg·kg^{-1}，对照组大鼠注射配药液. 最后一次注射后 12 h，灌注取脑、冰冻切片，进行原位杂交实验，计算机图像处理技术测定染色脑片积分光密度（IOD）值、平均光密度（OD）值. 结果：给药组大鼠下丘脑弓状核内 POMC mRNA 表达明显增强；其 IOD 和 OD 值均显著增加. 结论：褪黑素可加强大鼠弓状核内 POMC mRNA 的表达.

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