Melatonin enhances the expression of β-endorphin in hypothalamic arcuate nucleus of morphine-dependent mice

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Abstract: The study was conducted to investigate the effect of melatonin (MEL) on the expression of β-endorphin (β-EP) in the hypothalamic arcuate nucleus (ARH) of morphine-dependent mice. For a period of 8 consecutive days, male Kunming strain mice were injected subcutaneously (s.c.) with normal saline or increasing doses (10-80 mg/kg) of morphine, and intraperitoneally (i.p.) with MEL (10, 20 or 40 mg/kg) or vehicle (5% ethanol saline) simultaneously. Withdrawal response was induced by naloxone (3 mg/kg, s.c.) at 2 h after final morphine injection on the 8th day. The potency of withdrawal response was evaluated according to the jumping times and the body weight loss. After that, the expressions of β-EP and proopiomelanocortin (POMC) mRNA in ARH were examined by immunohistochemistry and RT-PCR, respectively. The results showed that MEL (i.p., 20 mg/kg) decreased the naloxone-precipitated withdrawal responses in morphine-dependent mice significantly (P<0.05). Meanwhile, MEL increased the intensity of β-EP-like immunoreactivity and enhanced the expression of POMC mRNA in ARH (P<0.05). These results suggest that MEL increases the expression of β-EP in ARH of morphine-dependent mice, which may partly contribute to the action of MEL to inhibit the development of morphine dependence.

Key words: melatonin; morphine dependence; hypothalamic arcuate nucleus; β-endorphin; proopiomelanocortin

褪黑素增加吗啡依赖小鼠下丘脑弓状核 β- 内啡肽的表达

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摘 要: 本文旨在考察褪黑素(melatonin, MEL)对吗啡依赖形成的作用。昆明小鼠皮下注射吗啡的同时腹腔注射MEL，连续8天，形成吗啡依赖，第8天最后一次吗啡注射后2 h，皮下注射纳洛酮催促戒断，采用免疫组织化学和RT-PCR 法，结合计算机图像处理技术，检测MEL对吗啡依赖小鼠下丘脑弓状核(hypothalamic arcuate nucleus, ARH)中β-内啡肽(β-endorphin, β-EP)表达的影响。结果显示，连续8 d 给予MEL(20 mg/kg), 可显著增强ARH中β-EP 表达( P<0.05)，并显著增强ARH中β-EP样免疫阳性反应强度及β-EP 前体物阿黑皮素原(proopiomelanocortin, POMC) mRNA 的表达( P<0.05)。上述结果提示，MEL 可促进ARH中β-EP 的生成，这可能是其抑制小鼠吗啡依赖性形成的机制之一。

关键词: 褪黑素; 吗啡依赖; 下丘脑弓状核; β- 内啡肽; 阿黑皮素原

中图分类号: Q189

Melatonin (N-acetyl-5-methoxytryptamine, MEL), an indoleamine neurohormone, which is synthesized and secreted primarily by the pineal gland in all mammalian species, has shown many effects on a wide range of physiological...
functions\textsuperscript{[1]}. Besides its well established function of regulation of circadian rhythms and seasonal responses, MEL has been involved in some neuropsychopharmacological actions, such as the sedative/hypnotic, anticonvulsant and antinociceptive activity\textsuperscript{[2]}. Recently, several studies suggest that circadian rhythms and MEL are critically involved in cocaine-induced reward\textsuperscript{[3-5]}. It has been reported that alteration in light/dark cycle leads to the impairment of morphine-induced rewarding effect in mice and this effect could be reversed by exogenous administration of MEL\textsuperscript{[6]}. Moreover, Raghavendra \textit{et al.} found that MEL administered intraperitoneally was able to reverse the development of morphine tolerance and dependence in mice\textsuperscript{[7]}. Sirac found in experiments with rats that the administration of MEL prior to repeated cocaine injections prevented the development of cocaine-induced behavioral sensitization\textsuperscript{[8]}. Interestingly, a recent study has established that physiological doses of MEL could reduce anxiety and attenuate craving in heavy smokers during acute withdrawal from nicotine\textsuperscript{[9]}. Those results suggest the possibility of MEL being useful in the treatment of some aspects of drug abuse.

Despite a great deal of researches have been done, the mechanisms involved in the development of opioid dependence remain to be clarified. A lot of neurotransmitters or neuromodulators play roles in the development of opioid dependence, such as serotonin, norepinephrine, dopamine, acetylcholine, oxytocin and vasopressin\textsuperscript{[10]}. Among the several possible mechanisms, the role of \(\beta\)-endorphin (\(\beta\)-EP) and its precursor–proopiomelanocortin (POMC) has received considerable attention in recent years. Some researches showed that the level of \(\beta\)-EP in the brain of opium dependent animals was decreased, and the expression of POMC mRNA was also decreased\textsuperscript{[11-13]}. Recently, we have found that single MEL (80 mg/kg, i.p., prior to the naloxone precipitated withdrawal) injection increased the content of \(\beta\)-EP in the periaqueductal gray (PAG) of midbrain, and decreased the content of \(\beta\)-EP in hypothalamic arcuate nucleus (ARH) in morphine withdrawal mice\textsuperscript{[14]}, and administration of MEL (i.p., 20 mg/kg) consecutively for 8 d following morphine injections increased the \(\beta\)-EP contents in PAG\textsuperscript{[15]}. It is definite that the PAG implicates in these sets of the somatic symptoms of morphine withdrawal, while the \(\beta\)-EP neurons in the central nervous system localize on ARH mainly. The efferent fibers that origin from ARH project to PAG, and release \(\beta\)-EP at termination. Therefore, one may surmise that whether consecutive MEL might affect \(\beta\)-EP level in ARH, thus contributing to mechanism of MEL’s anti-withdrawal response. In the present study, we further investigated whether MEL could inhibit the development of morphine dependence in mice, and then observed the content of \(\beta\)-EP and the expression of POMC mRNA in ARH following exogenous administration of MEL.

1 MATERIALS AND METHODS

1.1 Chemicals

MEL was purchased from Sigma Co. and was dissolved in 5% absolute ethanol saline immediately before use. Morphine hydrochloride injection (10 mg/mL) was purchased from the first drug manufactory of Shenyang (Batch No. 011105). Naloxone hydrochloride powder was purchased from Sigma Co. The rabbit anti-mouse \(\beta\)-EP antibody was obtained from Department of Neurobiology, the Second Military Medical University, China. Histostain\textsuperscript{TM}–Plus Kit was obtained from Zhongshan Co., Beijing, China. Titan one tube RT-PCR system (Cat. No. 1888382) was from Roche Molecular Biochemical, Germany.

1.2 Induction of morphine dependence

According to the method of Suzuki \textit{et al.}\textsuperscript{[16]}, adjusted slightly, mice were injected subcutaneously with morphine hydrochloride daily at 09:00 and 19:00. The morphine dose was increased progressively from 10 to 80 mg/kg over a period of 8 d, i.e., the mice were received 10, 20, 30, 40, 50, 60, 70 mg/kg morphine at 09:00 and 19:00 in consecutive 7 days respectively, and 80 mg/kg at 09:00 only on the 8th day.

1.3 Morphine withdrawal

Withdrawal syndrome was precipitated by injecting naloxone (3 mg/kg, s.c.) 2 h after the final morphine injection. After the naloxone challenge, mice were immediately placed on a glass beaker of 5 000 mL. The number of jumps was recorded over a period of 15 min; the body weight was measured initially and 60 min after the naloxone injection to calculate the weight loss.

1.4 Animal and treatment schedule

Male Kunming strain mice (18-20 g, from the Experimental Animal Center of Fujian Medical University) were maintained in a temperature-controlled environment under a 12 h:12 h light/dark cycle with free access to food and water. All experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals. Mice were randomly divided into 6 groups. The mice in control group were injected subcutaneously with normal saline, and intraperitoneally with vehicle (5% ethanol saline) simultaneously; The mice in MEL group were injected intraperitoneally with MEL (40 mg/kg)
and vehicle (5% ethanol saline) simultaneously; The mice in morphine dependence group (Mor group) were injected subcutaneously with morphine hydrochloride (see in 1.2) and intraperitoneally with (5% ethanol saline) simultaneously; The morphine dependent mice treated with MEL (MEL$_{10}+$Mor, MEL$_{20}+$Mor and MEL$_{40}+$Mor groups) were injected subcutaneously with morphine hydrochloride, and intraperitoneally with MEL (10, 20, 40 mg/kg, respectively) simultaneously for 8 d. The time of all administration was identical to morphine hydrochloride.

1.5 Immunohistochemistry
Immediately after behavioral testing, the mice were deeply anaesthetized with sodium pentobarbital (40 mg/kg, i.p.). Based on the brain atlas of mouse$^{[17]}$, the brain tissues containing ARH (from bregma -1.22 mm to -1.58 mm) were picked out, fixed in 4% formaldehyde for 4-6 h at room temperature. After dehydration with graded ethanol and xylene, the brain tissues were paraffin-embedded. The serial coronal sections were cut into 4 μm and mounted on the glass slides. The serial sections with an interval of 5 sections were chosen for β-EP immunohistochemistry staining. After deparaffin and dehydration, the sections were incubated in 3% hydrogen dioxide for 10 min to deactivate the peroxidase, and rinsed in 0.01 mol/L PBS for 5 min. Then, antigen recovery was performed by high pressure treating the sections in 10 mmol/L citrate buffer for 5 min, and the nonspecific binding was blocked by 10% goat serum (in PBS) for 15 min. The sections were incubated with monoclonal rabbit anti-β-EP primary antibody (1:1 250) at 37 oC for 2 h, and then washed with PBS at room temperature; followed by biotinylated anti-rabbit IgG secondary antibody at 37 °C for 15 min, then incubated with streptavidin-biotin-peroxidase complex at 37 °C for 15 min. The reaction was terminated by 0.01 mol/L PBS. After that, the sections were stained with diaminobenzidine with 0.6 mg/mL and counterstained with hematoxylin for 30 s, then differentiated by 1% hydrochloric acid ethanol for 1 s, air-dried and mounted with gum.

Photos were taken with light microscope. At least 20 pictures (×400) were selected from each sample. The immunoreactive area (μm$^2$, positive correlation to the expression of β-EP) and the mean gray value/0.1 μm$^2$ (negative correlation to the expression of β-EP) of β-EP immunoreactive cells were analyzed by Video Pro32 image analysis software (Australia).

1.6 RT-PCR for the relative quantities of POMC mRNA in ARH
Total RNA was isolated using TRIzol reagent. Total RNA content was determined by measuring the optical absorbance ratio at 260/280 nm after the sample was dissolved in DEPC-treated water. The RNA concentration of each sample was diluted to 0.2 μg/μL, and then stored at -70 °C. RT-PCR procedure was performed to determine the relative quantities of POMC mRNA in ARH, while GAPDH was used as an internal control. The upstream and downstream primers for POMC mRNA were 5'-GTCTTGAAAC-TCGACCTCTCG-3' and 5'-CATGAAGCCACCG-TAACGC-3' respectively, yielding a 482-bp product, whereas those for GAPDH were 5'-CAAACGGGTCT-ATCACTCCG-3' and 5'-CATGGATGACCTTGCCAG-3' respectively, yielding a 148-bp product. Equal amounts (2 μL) of each total RNA sample were added in a 25 μL reaction mixture exerting one-step amplification with Titan One Tube RT-PCR system. The reaction mixture was incubated at 50 °C for 30 min to reverse transcription, then, went into cycle. The cycle conditions were set to: initial denaturation for 2 min at 94 °C, 35 cycles at 94 °C for 30 s, 56 °C for 30 s, 72 °C for 45 s, and final elongation at 72 °C for 7 min. The PCR products were separated on 2% agarose gel containing ethidium bromide. Densitometry quantization analysis software (Syngene Co., USA). The relative abundance of POMC was expressed as the ratio of POMC to GAPDH product.

1.7 Statistical analysis
Values were expressed as mean±SD. According to the experimental design, statistical analysis was performed using one-way ANOVA followed by multiple comparison methods by Scheffe or Student’s t test. P<0.05 was considered to be statistically significant.

2 RESULTS

2.1 MEL inhibited the development of morphine dependence in mice
In MEL group, the withdrawal jumping times (0±0) and weight loss [(0.41±0.14) g] precipitated by naloxone (3 mg/kg, s.c.), were not significantly different from those in control group (P>0.05), suggesting that the mice in MEL group did not develop MEL dependence. In Mor group, the withdrawal jumping times (11.2±10.9) and weight loss [(1.67±0.58) g] were significantly increased, compared with those in control group (P<0.05), suggesting that the mice in Mor group exhibited chronic morphine dependence. In MEL$_{10}+$Mor, MEL$_{20}+$Mor and MEL$_{40}+$Mor groups, the
withdrawal jumping times (5.40±9.70, 4.20±4.50 and 3.00±2.80, respectively) and weight loss [(1.01±0.23) g, (0.82±0.21) g and (0.55±0.16) g, respectively] were significantly decreased, compared with those in Mor group (P<0.05) (Table 1). It is indicated that coadministration of MEL and morphine could dose dependently prevent the development of chronic physical dependence to morphine.

2.2 MEL increased the immunoreactivity of β-EP in ARH

After behavioral observation, β-EP immunoreactive neurons in ARH were observed in mice of control, Mor and MEL20+Mor groups. The positive staining was predominantly restricted to neuronal cytoplasmic perinuclear regions, principal dendrites and some axons. With light microscope observation, it was revealed that a smaller proportion of β-EP immunoreactive neurons and less intense immunostaining of the positive neurons in sections treated with morphine than in those treated with vehicle, but after treatment with MEL (20 mg/kg, for 8 d), the β-EP immunoreactive activity was increased (Fig. 1). The analysis of the stained sections by the computer-assisted image processing and analysis system showed that, compared with control group, in Mor group the immunoreactive area was smaller and the mean gray value/0.1 µm² was increased (P<0.05), but in MEL20+Mor group, the immunoreactive area was larger and the mean gray value/0.1 µm² was decreased compared with those in Mor group (P<0.05) (Table 2). It is indicated that morphine could decrease the content of β-EP in ARH, while MEL could increase it significantly.

2.3 MEL enhanced the expression of POMC mRNA in ARH

POMC is the precursor of β-EP, and associated with the synthesis of β-EP, so we examined the expression of POMC mRNA. In the ARH of mice treated with morphine (Mor group), the expression of POMC mRNA was at low level. Compared with the Mor group, the POMC mRNA expression in the ARH of mice treated with MEL (MEL20+Mor group) was obviously increased (Fig. 2). The

<table>
<thead>
<tr>
<th>Group</th>
<th>Dose of MEL (mg/kg)</th>
<th>Times of jumping</th>
<th>Weight loss (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>0±0</td>
<td>0.48±0.23</td>
</tr>
<tr>
<td>MEL</td>
<td>40</td>
<td>0±0</td>
<td>0.41±0.14</td>
</tr>
<tr>
<td>Mor</td>
<td>0</td>
<td>11.20±10.90*</td>
<td>1.67±0.58*</td>
</tr>
<tr>
<td>MEL10+Mor</td>
<td>10</td>
<td>5.40±9.70*</td>
<td>1.01±0.23*</td>
</tr>
<tr>
<td>MEL20+Mor</td>
<td>20</td>
<td>4.20±4.50*</td>
<td>0.82±0.21*</td>
</tr>
<tr>
<td>MEL40+Mor</td>
<td>40</td>
<td>3.00±2.80*</td>
<td>0.55±0.16*</td>
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Control group: The mice treated with normal saline (s.c.) and vehicle (5% ethanol saline, i.p.) simultaneously for 8 d. MEL group: The mice treated with MEL (40 mg/kg, i.p.) and vehicle (5% ethanol saline, i.p.) simultaneously for 8 d. Mor group: The mice treated with morphine (s.c.) and vehicle (5% ethanol saline, i.p.) simultaneously for 8 d. MEL10+Mor, MEL20+Mor, MEL40+Mor groups: The mice treated with morphine (s.c.) and with 10, 20 or 40 mg/kg MEL (i.p.) simultaneously for 8 d. On the test day, the animals were precipitated by administration of naloxone (3 mg/kg, s.c.), 2 h after the final morphine injection. The number of jumping was tested for 15 min and the weight loss was calculated 60 min after the naloxone injection. Values represent mean±SD from 10 mice (n=10). *P<0.05 vs control, "P<0.05 vs Mor.

<table>
<thead>
<tr>
<th>Group</th>
<th>Immunoreactive area (µm²)</th>
<th>Mean gray value/0.1 µm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>18.05±1.60</td>
<td>81.15±3.73</td>
</tr>
<tr>
<td>Mor</td>
<td>15.10±1.32*</td>
<td>111.00±2.64*</td>
</tr>
<tr>
<td>MEL20+Mor</td>
<td>17.71±0.56*</td>
<td>90.23±4.71*</td>
</tr>
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The immunoreactive area (µm², positive correlation to the expression of β-EP) and the mean gray value/0.1 µm² (negative correlation to the expression of β-EP) of β-EP immunopositive cells were analyzed by Video Pro32 image analysis software. Values represent mean±SD from 10 mice (n=10). *P<0.05 vs control, "P<0.05 vs Mor.
analysis by the computer-assisted image processing and analysis system revealed that the \( \frac{OD_{POMC}}{OD_{GAPDH}} \) value in Mor group was decreased compared with that in control group, but increased significantly in MEL\(_{20}\)+Mor group compared with that in Mor group (\( P<0.05 \)) (Fig. 3).

3 DISCUSSION

It is well known that opiate addiction is a major social and medical problem that results in immense harm to both individuals and societies. Despite the size and scope of this
problem, there is little effective treatment for opiate addiction. Some studies have shown that MEL possesses anti-withdrawal action on rats or mice with morphine-dependence\textsuperscript{18-20}. Our recent preliminary work has shown that MEL inhibited the development of morphine dependence and attenuated the withdrawal contraction induced by naloxone in an isolated guinea pig ileum\textsuperscript{21}. It has also been reported that MEL could reverse the development of tolerance and dependence to morphine\textsuperscript{7}, and prevent the development of cocaine-induced behavioral sensitization\textsuperscript{8}. Moreover, our previous findings have shown that MEL has an antinociceptive effect without producing physical dependence in mice\textsuperscript{22}. Sugden has reported that MEL possessed very low acute toxicity when administered to mice and rats by various routes (i.p., p.o., s.c., i.v.)\textsuperscript{23}. These findings appear to suggest that MEL has no addictive characteristics itself, while its analogues may have therapeutic potential in the treatment of opiate addiction. Indeed, this hypothesis needs to be tested in more animal species and in more animal models as well as in human beings with appropriate trials.

However, the mechanism underlying MEL-induced anti-withdrawal is obscure. Some reports have shown that multiple mechanisms may be involved in the action. Previous studies inferred that MEL could affect cholinergic neurotransmission on the nucleus accumbens\textsuperscript{24}, facilitate inhibitory GABAergic neurotransmitter functions\textsuperscript{25,26}, or antagonize 5-HT2A/2C receptor mediated behavioral responses\textsuperscript{27,28}. Moreover, in many instances, modulation of the functions of some other neurotransmitters or neuromodulators may play different roles in the development of dependence on opioids\textsuperscript{29,30}, e.g., ACh, NO and β-EP.

Several observations pointed out a significant interaction between MEL and opioid peptides in the brain \textsuperscript{31,32}, but the mechanism underlying the putative action of MEL on the opioid system remains unclear. Shavali et al.\textsuperscript{33} found that bovine pineal gland possesses δ and μ opioid receptors, but MEL did not interact directly with the opioid receptors, actually, the cultured mouse pituitary AtT-20 cells treated with MEL released higher levels of β-EP, suggesting that MEL-induced analgesic effects could be mediated via enhanced release of β-EP. These results were consistent with our previous reports which demonstrated the release of β-EP by administration of exogenous MEL\textsuperscript{34}, and an increase in pain threshold along with β-EP release in the perfusate from the rat PAG following an intraperitoneal administration of MEL\textsuperscript{35}. Also, Lincoln et al.\textsuperscript{36} reported that the placement of bilateral micro-implants of MEL in the mediobasal hypothalamus resulted in an increase in the plasma concentration of β-EP in rams, and recently, we have found that acute MEL administration could inhibit the withdrawal syndrome induced by naloxone, increase the content of β-EP in PAG, and decrease the content of β-EP in ARH of morphine withdrawal mice\textsuperscript{40}. The ARH is the only site of β-EP synthesis in the forebrain, and ethanol stimulates β-EP release from hypothalamic cultures\textsuperscript{37}, the PAG receives ARH efferent projections. So, we supposed that maybe acute MEL administration could enhance the release of β-EP from ARH to PAG, which resulted in the higher β-EP level in PAG and the lower level in ARH. As we know, β-EP has several physiological and behavioral effects that are similar to morphine, and the higher β-EP level in PAG can competitively activate μ-opioid receptors that blocked by naloxone to attenuate the withdrawal syndrome in morphine-dependent mice. In another research, we found that chronic administration of MEL for 8 d following morphine injections increased the β-EP contents in PAG\textsuperscript{41,42}. As we known, some afferent nerve fibers of PAG derive from ARH. So, in the present study, we utilized the mice model of morphine physiologic dependence to observe whether MEL could affect β-EP in ARH. The results showed that morphine decreased the content of β-EP in ARH of mice, but the intraperitoneal injection of MEL (20 mg/kg) for 8 d increased it significantly. According to the above-mentioned results, we propose that chronic administration of MEL may both enhance the synthesis of β-EP in ARH and promote it to release to PAG, which contributes to MEL-induced anti-withdrawal. However, the mechanism needs further elucidation.

The increase of β-EP content will bring about the increase of 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 increased expression of POMC mRNA induced by MEL may be one of the reasons resulted in the increase of β-EP content. From this point of view, the present study was to observe whether MEL affects the expression of POMC mRNA. We found, that in the ARH of mice treated with morphine, the expression of POMC mRNA decreased, and the intraperitoneal injection of MEL (20 mg/kg) enhanced its expression obviously. However, the detailed biological significance about it still needs to be further elucidated.

In conclusion, MEL may enhance the POMC mRNA expression in the ARH, thus increase the content of β-EP, which may partly contribute to the action of MEL to inhibit the development of morphine dependence in mice.
REFERENCES


