

Inhibitory effects of melatonin on free intracellular calcium in mouse brain cells

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KEY WORDS melatonin; calcium; aging; brain; synaptosomes; cerebral cortex; neurons; Bay-K-8644; potassium chloride; sodium glutamate

ABSTRACT

AIM: To study the effects of melatonin (Mel) on cortical intrasynaptosomal calcium concentration in old mice and on $[Ca^{2+}]_i$ elevation induced by Bay-K-8644, KCl, and sodium *l*-glutamate in isolated brain cells of neonatal mouse, and to determine the antiaging mechanism of Mel.

METHODS: $[Ca^{2+}]_i$ was measured in an RF-5000 recording spectrofluorophotometer by preloading the synaptosomes or cells with Fura 2-AM. **RESULTS:** Long term of administrating Mel inhibited the overload of $[Ca^{2+}]_i$ in old mouse cerebral cortex. The $[Ca^{2+}]_i$ in both high (20 mg·L⁻¹) and low dose (1 mg·L⁻¹) of Mel groups was reduced from (434 ± 32) nmol·L⁻¹ (the older control group) to (330 ± 41) and (313 ± 56) nmol·L⁻¹, respectively, $P < 0.01$. Mel 0.01, 0.1, 1, and 3 μmol·L⁻¹ remarkably reduced $[Ca^{2+}]_i$ elevations in isolated newborn mouse brain cells induced by Bay-K-8644, KCl, and Glu. **CONCLUSION:** The inhibitory effect of Mel on neuronal $[Ca^{2+}]_i$ overload is involved in its antiaging effect.

INTRODUCTION

Melatonin (Mel, *N*-acetyl-5-methoxytrypt-

amine) is a hormone secreted mainly by the pineal body. Many investigators have studied the pineal body and Mel in the processes of both aging and age-related diseases^[1]. The interests are from the importance of Mel in a number of biological functions and the fact that Mel production in the organism is gradually lost throughout life^[2]. Exogenously administering Mel could prolong life, postpone aging, and reduce the age-related diseases^[3]. $[Ca^{2+}]_i$ overload would result in irreversible injury and is a common pathway for cell death, particularly in the central neurons. The rise of $[Ca^{2+}]_i$ increases the free radical production, and the accumulation of free radicals causes the increase of $[Ca^{2+}]_i$. A prominent theory of aging is that the rate of aging attributes to accumulated free radical damage^[4]. The damages may be more obvious in the central nervous system, which is highly susceptible to oxygen-based radicals, and may be especially important in aging. The purpose of this study was to explore the antiaging mechanism of Mel.

MATERIALS AND METHODS

Mel (C₁₃H₁₆N₂O₂, FW 232.3, purity > 99%, light sensitive desiccated, stored at < 0 °C) was synthesized by Institute of Materia Medica, Chinese Academy of Medical Sciences. RF-5000 recording spectrofluorophotometer (Shimadzu Co, Japan); XL-90 ultracentrifugator (Beckman, Germany); SCR20BA-centrifugator (Hitachi Koki Co, Japan); Fura 2-AM, Triton X-100, egtazic acid, Me₂SO, and sodium *l*-glutamate (Glu, Sigma, USA); HEPES and

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Bay-K-8644 (Boehringer, Germany); DMEM medium (Gibco-BRL, USA). Other chemicals were of AR.

Old (15 months) and newborn (3 - 5 d) Kunming mice were provided by the Animal Center of Chinese Academy of Medical Sciences (Grade II, Certificate No 01-3001).

Artificial cerebro-spinal fluid (ACSF); NaCl 132, KCl 3, MgSO₄·7H₂O 1, NaH₂PO₄ 1, glucose 10, HEPES 10, and CaCl₂ 1 mmol·L⁻¹, pH 7.4.

MgSO₄- and NaH₂PO₄-free ACSF; NaCl 132, KCl 3, glucose 10, HEPES 10, and CaCl₂ 1 mmol·L⁻¹, pH 7.4.

Hanks' solution; NaCl 137, KCl 5, CaCl₂ 1.3, MgSO₄·7H₂O 0.8, Na₂HPO₄ 0.6, KH₂PO₄ 0.4, NaHCO₃ 3, glucose 5.6, and HEPES 2 mmol·L⁻¹, pH 7.4.

Preparation of old mouse cerebral cortex synaptosome Old mice (15 months) of either sex weighing 44 g ± s 4 g were randomly divided into the low dose treated (*n* = 10), high dose treated (*n* = 10), and the control (*n* = 10) groups. Treated group: after the addition of Mel 1 mg·L⁻¹ (low dose) and 20 mg·L⁻¹ (high dose) to the drinking water of mice on a nightly basis for 2 months, they were injected ip Mel 0.5 mg·kg⁻¹ and 2 mg·kg⁻¹ for 1 month. Control group received the same amount of vehicle (NS).

Cortical synaptosomes were isolated from old mice^[5,6] with some modifications. The homogenate was centrifuged at 4 °C, 1000 × *g* for 10 min. Supernatant was placed in the gradient density solution consisting of sucrose solution 0.8 and 1.2 mol·L⁻¹ and ultracentrifuged at 4 °C, 150 000 × *g* for 50 min. The suspension layer between sucrose solution 1.2 and 0.8 mol·L⁻¹ was collected and centrifuged at 4 °C, 20 000 × *g* for 30 min. The pellets were resuspended in ACSF. The suspension was centrifuged at 4 °C, 9500 × *g* for 10 min. The sediments were resuspended in 5 mL of ACSF. The samples

were stored at 0 - 4 °C. The determination of protein was performed^[7]. The content of protein was 1 g·L⁻¹.

Preparation of neonatal mouse brain cells

Brain cells were prepared according to the method^[8,9] with some modifications. Newborn (3 - 5 d) Kunming mouse brain cells were filtered through nylon mesh (200 mesh, hole size 95 μm) and collected in a flask. Cells were centrifuged at 300 × *g* for 5 min twice. The supernatant was decanted and the cells were resuspended in warm Hanks' solution. An aliquot of cell suspension was taken, trypan blue staining consistently showed 90 % - 95 % cellular viability rate. The suspension was diluted to 2 × 10⁶ cells·L⁻¹ with Hanks' solution and shaken at 37 °C for 5 min.

The experiment was divided into 2 groups. Control group: 5 min after the resting [Ca²⁺]_i was determined, final concentrations of KCl 50 mmol·L⁻¹, Glu 10 mmol·L⁻¹, and Bay-K-8644 0.1 mmol·L⁻¹ were added, and 5 min later, [Ca²⁺]_i was determined. Treated group: The resting [Ca²⁺]_i was determined. After 5 min Mel (final concentrations 0.01, 0.1, 1, and 3 μmol·L⁻¹) were given to observe the changes of [Ca²⁺]_i. Five minutes after Mel administration, the above calcium agonists were added to observe the elevation of [Ca²⁺]_i.

Fura 2-AM-loaded samples Fura 2-AM (a Ca²⁺ fluorescent indicator) 5 μmol·L⁻¹ in Me₂SO was added to an aliquot of suspension. The suspension was shaken at 37 °C for 45 min, then centrifuged at 13 000 × *g* (synaptosomes) or 300 × *g* (cells) for 5 min twice. The pellets or cells were resuspended in MgSO₄- and NaH₂PO₄-free ACSF (synaptosomes) or warm Hanks' solution (cells), respectively for detection.

Measurement of [Ca²⁺]_i An RF-5000 recording spectrofluorophotometer was used for fluorescence determination. The condition of

measurement; alternate λ time scan; λ_{ex} A = 340 nm, λ_{ex} B = 380 nm, λ_{em} A = 500 nm, λ_{em} B = 500 nm; bandwidth (Ex) 5 nm, bandwidth (Em) 10 nm; time interval; 2 s; response: 0.02 s; sensitivity: high; temperature; (37 ± 1) °C.

After Fura-2 loading, the peak of excitation spectrum in samples was shifted from 380 nm to 340 nm. When addition of Triton X-100 (final concentration 0.1 %) to lyse the plasmatic membranes, the spectrum peak in 340 nm increased. By addition of egtazic acid (final concentration 5 mmol·L⁻¹, pH > 8.5), the spectrum of Fura-2 released a peak at 370 - 380 nm.

Calculation of [Ca²⁺]_i [Ca²⁺]_i = $K_d \times (R - R_{min}) / (R_{max} - R) \times (sf_2/sb_2)$. K_d was the dissociation constant of Fura-2 for Ca²⁺ and assumed to be 224 nmol·L⁻¹ at 37 °C. R was the ratio of corrected fluorescence signals at 340 and 380 nm. R_{max} (maximal fluorescence) was the ratio obtained by final concentration of 0.1 % Triton X-100 added. R_{min} (minimal fluorescence) was the ratio of the corrected signals obtained by using final concentration of egtazic acid 5 mmol·L⁻¹ (pH > 8.5) to deplete calcium. sf_2 and sb_2 represented the emission intensities at 380 nm excitation in saturated and calcium-free conditions, respectively.

Statistical analysis Data were expressed as $\bar{x} \pm s$ and analyzed by t test.

RESULTS

Resting [Ca²⁺]_i of cortical intrasynaptosomes in old mice Cortical intrasynaptosomal free calcium concentration in old mice [15 months, (434 ± 32) nmol·L⁻¹] was higher than that in adult mice [3 - 6 months, (265 ± 59) nmol·L⁻¹]. Compared with the older control group, the [Ca²⁺]_i in both high and low dose of Mel groups reduced obviously [(330 ± 41) and (313 ± 56) nmol·L⁻¹, $P < 0.01$], but was still higher than that in adult mice ($P <$

0.05). There was no significant difference in [Ca²⁺]_i between 2 treated groups ($P > 0.05$).

Inhibitory effects of Mel on [Ca²⁺]_i elevation by KCl, Glu, and Bay-K-8644

Mel did not cause a significant change in resting [Ca²⁺]_i ($P > 0.05$). In comparison with the control, Mel 0.01, 0.1, 1, 3 μmol·L⁻¹ remarkably reduced [Ca²⁺]_i elevations induced by KCl, Glu, and Bay-K-8644 ($P < 0.01$) (Tab 1).

Tab 1. Effects of melatonin on 3 calcium agonists-induced [Ca²⁺]_i (nmol·L⁻¹) in isolated brain cells of neonatal mice. $n = 5$ cell suspensions (each suspension was pooled from 6 newborn mice). $\bar{x} \pm s$. ^a $P > 0.05$, ^c $P < 0.01$ vs resting [Ca²⁺]_i. ^f $P < 0.01$ vs Bay-K-8644 (0.1 mmol·L⁻¹) group. ⁱ $P < 0.01$ vs KCl (50 mmol·L⁻¹) group. ^l $P < 0.01$ vs Glu (1 mmol·L⁻¹) group.

Group	Control	Melatonin/μmol·L ⁻¹		
		0.01	0.1	1
Resting	204 ± 36	210 ± 37 ^a	211 ± 36 ^a	211 ± 39 ^a
Bay-K-8644	441 ± 40 ^c	360 ± 32 ^f	318 ± 35 ^f	312 ± 40 ^f
KCl	334 ± 37 ^c	259 ± 29 ⁱ	243 ± 25 ⁱ	233 ± 24 ⁱ
Glu	279 ± 23 ^c	222 ± 22 ^l	218 ± 22 ^l	213 ± 23 ^l

DISCUSSION

The reduction in Mel with age may be contributory to aging and the onset of age-related diseases. Mel has beneficial effects in terms of aging and age-related diseases, may be an antiaging hormone. The view is based on the recent observations that Mel is the most potent hydroxyl radical scavenger thus far discovered^[10]. Besides its ability to directly scavenge the highly toxic hydroxyl radical, Mel also promotes the activity of the antioxidative enzyme glutathione peroxidase, thereby further reducing oxidative damage. Thus, Mel preferentially affords antioxidant protection to the brain^[11]. In some studies where animals have been supplemented with exogenous Mel

throughout life, life span has been increased by 25 %^[12]. Using several different mouse strains, giving Mel in the drinking water nightly prolongs life, postpones aging, and maintains the animals in a more youthful state^[13]. The gonadotropin-releasing hormone (GnRH) stimulated LH release by increasing $[Ca^{2+}]_i$ in neonatal rat pituitary cells. Mel inhibited the GnRH-induced elevation of $[Ca^{2+}]_i$ in a dose-dependent manner. Mel 100 nmol·L⁻¹ inhibited GnRH-induced increase of $[Ca^{2+}]_i$ by 40 %. It is concluded that Mel may act by inhibiting Ca²⁺ influx. Mel may inhibit the GnRH-induced increase in $[Ca^{2+}]_i$ through a mechanism involving a pertussis toxin-sensitive G-protein^[14]. Mel also averted the amyloid β protein (A β)-induced increases in $[Ca^{2+}]_i$ and lipid peroxidation. Mel was remarkably effective in preventing death of cultured neuroblastoma cells as well as oxidative damage and $[Ca^{2+}]_i$ increases induced by cytotoxic fragment of A β . The importance of this finding was that, in contrast to conventional antioxidants, Mel had a proposed physiological role in the aging process^[15].

Our study showed that Mel inhibited $[Ca^{2+}]_i$ elevations induced by Bay-K-8644, KCl, and Glu directly in brain cells of newborn mice and the overload of $[Ca^{2+}]_i$ in old mouse cerebral cortex synaptosomes, suggesting that the reduction of $[Ca^{2+}]_i$ overload may be one of the antiaging mechanisms of Mel. It is possible that Mel reduced $[Ca^{2+}]_i$ overload in old mouse cerebral cortex by inhibiting Ca²⁺ influx.

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REFERENCES

1 Reiter RJ. The pineal gland and melatonin in

- relation to aging: a summary of the theories and of the data. *Exp Gerontol* 1995; 30: 199 - 212.
- 2 Schmid HA, Requentina PJ, Oxenkrug GF, Sturmer W. Calcium, calcification, and melatonin biosynthesis in the human pineal gland: a postmortem study into age-related factors. *J Pineal Res* 1994; 16: 178 - 83.
- 3 Pierpaoli W, Regelson W. Pineal control of aging: effect of melatonin and pineal grafting on aging mice. *Proc Natl Acad Sci* 1994; 91: 787 - 91.
- 4 Sandyk R. Possible role of pineal melatonin in the mechanisms of aging. *Int J Neurosci* 1990; 52: 85 - 92.
- 5 Dodd PR, Hardy JA, Oakley AE, Edwardson JA, Perry EK, Delaunoy JP. A rapid method for preparing synaptosomes: comparison with alternative procedures. *Brain Res* 1981; 226: 107 - 18.
- 6 Gao H, Feng YP. Estimation of free calcium level within synaptosomes by using Fura-2 and the effect of calcium channel agonist and antagonist. *Acta Pharm Sin* 1993; 28: 404 - 9.
- 7 Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951; 193: 265 - 75.
- 8 Dildy OH, Leslie SW. Ethanol inhibits NMDA-induced increase in free intracellular Ca²⁺ in dissociated brain cell. *Brain Res* 1989; 499: 383 - 7.
- 9 Zeng WZ, Liu TP. Effects of tetrandrine on free intracellular Ca²⁺ in isolated rat brain cells. *Acta Pharmacol Sin* 1993; 14: 397 - 400.
- 10 Tan DX, Chen LD, Poeggeler B, Manchester LC, Reiter RJ. Melatonin: a potent, endogenous hydroxyl radical scavenger. *Endocr J* 1993; 1: 57 - 60.
- 11 Poeggeler B, Reiter RJ, Tan DX, Chen LD, Manchester LC. Melatonin, hydroxyl radical-mediated oxidative damage, and aging: a hypothesis. *J Pineal Res* 1993; 14: 151 - 68.
- 12 Maestroni GJM, Conti A, Pierpaoli W. Melatonin, stress and the immune system. *Pineal Res Rev* 1989; 7: 203 - 26.
- 13 Pierpaoli W, Dallara A, Pedrinis E, Regelson W.

The pineal control of aging: the effects of melatonin and pineal grafting on the survival of older mice. *Ann NY Acad Sci* 1991; 621: 291 - 313.

14 Vanecek J, Klein DC. Melatonin inhibits gonadotropin-releasing hormone-induced elevation of intracellular Ca^{2+} in neonatal rat pituitary cells. *Endocrinology* 1992; 130: 701 - 7.

15 Pappolla MA, Sos M, Omar RA, Bick RJ, Hickson-Bick DLM, Reiter RJ, *et al*.

Melatonin prevents death of neuroblastoma cells exposed to the Alzheimer amyloid peptide.

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褪黑激素对小鼠脑细胞内游离钙浓度的抑制作用

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关键词 褪黑激素; 钙; 衰老; 脑; 突触体; 大脑皮质; 神经元; Bay-K-8644; 氯化钾; 谷氨酸钠

目的: 研究褪黑激素(Mel)对老年小鼠大脑皮层突触体内钙含量以及激动剂诱发的新生小鼠脑细胞 $[Ca^{2+}]_i$ 升高的影响, 以探讨 Mel 抗衰老的作用机理. **方法:** 钙离子荧光染料 Fura 2-AM 负载已制备的突触体或细胞, 用 RF-5000 型双波长荧光分光光度计测定 $[Ca^{2+}]_i$. **结果:** 长期使用 Mel 抑制老年小鼠大脑皮层 Ca^{2+} 超负荷, Mel 也降低钙通道激活剂 Bay-K-8644、高浓度氯化钾(KCl)和谷氨酸钠诱发的分离的新生小鼠脑细胞 $[Ca^{2+}]_i$ 升高. **结论:** Mel 对中枢神经元 $[Ca^{2+}]_i$ 超载的抑制作用与其抗衰老作用有关.

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