Antagonistic effects of melatonin on glutamate release and neurotoxicity in cerebral cortex

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KEY WORDS melatonin; glutamic acid; neurotoxins; synaptosomes; cultured cells; neurons; cerebral cortex; aging

ABSTRACT

AIM: To observe the effects of melatonin (Mel) on glutamate (Glu) release from the cortical synaptosomes in old mice and on neurotoxicity induced by KCl, Glu in cultured cortical cells of fetal rat and to explore the antiaging mechanism of Mel. **METHODS**: Glu release by the synaptosomes in old mouse cerebral cortex was detected in a spectrofluorophotometer. The neuronal viability in primary cultures from rat cerebral cortex was assessed using MTT stain and lactate dehydrogenase (LDH) efflux in the bathing medium. **RESULTS**: Mel inhibited the K^+ (30 $mmol \cdot L^{-1}$)-induced Glu release from synaptosomes either in calcium dependent or independent conditions [control (10.6 ± 1.1), (9.2 ± 0.7) $\mu mol \cdot g^{-1}$(protein); Mel 0.1 $\mu mol \cdot L^{-1}$(6.5 ± 0.9), (7.5 ± 0.6) $\mu mol \cdot g^{-1}$ (protein), respectively, $P < 0.01$ *vs* control group), increased MTT activity (control 0.67 ± 0.04, 0.81 ± 0.03; Mel 0.1 $\mu mol \cdot L^{-1}$ 0.715 ± 0.023, 0.925 ± 0.027, $P < 0.01$ *vs* control group] and decreased LDH efflux (control 0.400 ± 0.016, 0.379 ± 0.016; Mel 0.1 $\mu mol \cdot L^{-1}$ 0.345 ± 0.021, 0.340 ± 0.012, respectively, $P < 0.01$ *vs* control group), therefore, protected the neuronal viability against KCl and Glu-induced injury. **CONCLUSION**: The inhibitory effect of Mel on Glu release from cortical synaptosome and the protective effect of Mel on cortical neurons against neurotoxicity are its antiaging mechanisms.

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INTRODUCTION

Melatonin (Mel) is a hormone secreted mainly by the pineal gland. Many investigators have studied the pineal gland and Mel in the processes of both aging and age-related diseases[1]. The interests are from the importance of MT in a number of biological functions and the fact that Mel production in the organism is gradually lost throughout life[2]. Exogenous Mel could serve to prolong life, postpone aging, and reduce the incidences of age-related diseases[3].

Glu is a major excitatory neurotransmitter in central nervous system. It takes part in normal activity of excitatory neurons, but the excessive release of Glu or inability of uptake will result in very high concentration in central nervous system which induce postsynaptosomal neuronal hyperactivity and death. There is excessive release of Glu and Glu excitotoxicity in cortical neurons in aging and age-related diseases. Glu neurotoxicity (GNT) participates in the neuron loss associated with a number of neuro-degenerative diseases, eg, Alzheimer disease and Huntington disease[4,5]. In this study, we explored the effects of Mel on Glu release from the cortical synaptosome in old mice and on the neurotoxicity induced by KCl and Glu in primary neuronal cultures so as to understand and elucidate the antiaging mechanism of Mel.

MATERIALS AND METHODS

Drugs Mel (synthesized by our institute); Dulbecco's modified Eagles's medium (DMEM), Horse serum donor herd (Gibco BRL, USA); newborn calf serum (Hangzhou Bioengineering Material Institute, China); *l*-glutamic dehydrogenase (*l*-GDH), $NADP^+$ (oxidative coenzyme Ⅱ) (Boehringer Mannheim, Germany); lactase dehydrogenase (LDH)

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kit (Beijing Chemical Factory, China); MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide], poly-*l*-lysine hydrobromide (M_r 150 000 – 300 000), BSA (bovine serum albumin), Glu, egtazic acid (EGTA), TES [*N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid], HEPES, Me_2SO_4 (Sigma, USA); cytarabine (Shanghai № 12 Pharmaceutical Factory, China). Other chemicals were of AR grade. All solutions were prepared with deionized distilled water.

Mice Older Kunming mice (15 months) and fetal Wistar rats (16 – 18 d-old embryos, Grade II) were provided by the Animal Center (Certificate № 01-3001) of Chinese Academy of Medical Sciences.

Incubation solution NaCl 122, KCl 3.1, KH_2PO_4 0.4, $NaHCO_3$ 5.0, TES 20, $MgSO_4$ 1.2, glucose 10 ($mmol \cdot L^{-1}$), BSA 16 $\mu mol \cdot L^{-1}$; pH 7.4. It was freshly prepared before use.

Preparation of cerebral cortex synaptosome[6,7] Cortical synaptosomes were isolated from old mice (15 months, weighing 44 g ± *s* 4 g). Animals were decapitated in a Petri dish on ice. Bilateral cerebram cortexes were placed in ice-cold sucrose 0.32 $mol \cdot L^{-1}$. The homogenate was spun at 4 ℃, 1000 × *g* for 10 min (SCR20BA-centrifugator, Hitachi Koki Co Ltd, Japan). The supernatant was placed in the gradient density solutions consisting of sucrose 0.8 and 1.2 $mol \cdot L^{-1}$ and spun at 4 ℃, 150 000 × *g* for 50 min (XL-90 ultracentrifugator, Beckman, German). The suspension layer between sucrose 1.2 and 0.8 $mol \cdot L^{-1}$ solutions was collected and spun at 4 ℃, 20 000 × *g* for 30 min. The pellets were resuspended in sucrose 0.32 $mol \cdot L^{-1}$. The content of protein in 0.2 mL of suspension was determined[8] (752-Z UV/VIS spectrophotometer, Beijing Optical Instruments Factory, China). The content of protein was 1 $g \cdot L^{-1}$. The suspension was spun at 4 ℃, 9500 × *g* for 10 min. The sediments were resuspended in incubation solution, and stored at 0 – 4 ℃.

The procedures of measurement[9] The prepared synaptosomes were diluted to the final concentration of 0.67 g protein $\cdot L^{-1}$ in incubation solution, and incubated at 37 ℃ for 30 min. Synaptosomal suspension 1.9 mL incubated was moved into the cell with stir apparatus. Then, $NADP^+$ 0.5 $mmol \cdot L^{-1}$, Ca^{2+} 1.3 $mmol \cdot L^{-1}$ (or EGTA 1.3 $mmol \cdot L^{-1}$), and *l*-GDH 25 u were added to the sample in sequence. The total reactive volume was 2 mL. By addition of KCl 30 $mmol \cdot L^{-1}$ (final concentration), 5 min later, the changes of fluorescent intensity were continually measured in Ca^{2+}, Ca^{2+}-free conditions (RF-5000 recording spectro-fluorophotometer, Shimadzu Co, Japan).

The observations of Mel action Two min before the addition of KCl 30 $mmol \cdot L^{-1}$, four final concentrations (0.01, 0.1, 1, and 3 $\mu mol \cdot L^{-1}$) of Mel were added respectively, to observe the effect of Mel on the Glu release from synaptosome.

Complete medium ($g \cdot L^{-1}$) DMEM (net Wt) 13.4, newborn calf serum 100 mL, horse serum 100 mL, $NaHCO_3$ 3.7, HEPES 2.383, benzylpenicillin 100 000 IU, streptomycin 0.1; pH 7.2 – 7.4.

Primary cortical cell culture The mixed cortical neurons were prepared from 16 – 18 d-old Wistar rat fetuses using Choi's technique with minor modification[10] (clean working platform, № 1 Semiconductor Apparatus Factory of Beijing, China). The cerebral cortex was dissected and gently triturated with a polished pipette. The cortical cells were filtered through nylon mesh (200 mesh, hole size 95 μm) to 3 × 10^{-6} cells $\cdot L^{-1}$ with heated-inactivated complete medium. Cell suspension 1 mL of was taken, seed onto 24-well plates (24-well cell culture cluster, Costar, USA) coated with *l*-poly-lysine and incubated at 37 ℃ in 5 % CO_2 atmosphere (CO_2 humidified incubator, Heraeus, Germany).

The medium containing unattached cells was removed and fresh complete medium was added 24 h later. After 3 – 4 d in culture, non-neuronal cell division was halted by 1 – 2 d of exposure to cytosine arabinoside (final concentration 10 $\mu mol \cdot L^{-1}$). The culture medium was renewed every 3 – 4 d.

Treatment with KCl, Glu, and Mel Experiments were carried out 12 – 14 d seeding, and divided into three groups. Serum-free control group: The complete medium was replaced with serum-free medium. Toxic control groups: Final concentrations of KCl 20 $mmol \cdot L^{-1}$ and Glu 100 $\mu mol \cdot L^{-1}$ were added to the serum-free medium, respectively. Treated groups: 5 min after the Mel was added in serum-free medium (final concentrations 0.01, 0.1, and 1 $nmol \cdot L^{-1}$, respectively), KCl 20 $mmol \cdot L^{-1}$ or Glu 100 $\mu mol \cdot L^{-1}$ was added into the medium. Then, cell

culture was transferred to CO_2 incubator for 18 – 24 h.

Morphological observation Morphological changes of neurons were observed and photographed with the phase microscope (phase-contrast microscope, Olympus, Japan).

MTT stains Eighteen hour after the cell cultures were treated with KCl, Glu, and Mel, MTT 0.1 mL in PBS (5 g·L^{-1}, pH 7.3) was added to each well of 24-well plates (0.5 g · L^{-1} final concentration) and incubated at 37 ℃ for 4 h. 50 % DMSO and 20 % SDS mixed solution was added to each well overnight to extract the blue formazan. Cell viability and its mitochondrial activity were assessed by measuring the ability of cortical neurons to reduce MTT to a colored formazan[11]. The absorbance of formazan was measured at a wavelength of 570 nm (A_{570}) by 752 Z/VIS spectrophotometer and expressed as the MTT value[12]. The system was calibrated according to the following formula: Inhibitory rate (%) = $A_{Mel} - A_{Glu} / A_{Control} - A_{Glu}$ or: Inhibitory rate (%) = $A_{Mel} - A_{KCl} / A_{Control} - A_{KCl}$.

LDH efflux assay Neurotoxicity was estimated by LDH activity released into media from damaged neurons. The medium and frozen-thawed cell liquid were collected after the cultures were incubated for 24 h. LDH was determined by a spectrophotometer at 440 nm. The ratio of extracellular LDH to total LDH was calculated.

Statistical analysis Data were expressed as $\bar{x} \pm s$. Significance was determined by t test.

RESULTS

Effect of Mel on calcium-dependent or calcium-independent glutamate release In $CaCl_2$ 1.3 mmol · L^{-1} or calcium-free condition (EGTA 1.3 mmol·L^{-1}) conditions, the synaptosomes were depolarized by KCl 30 mmol · L^{-1}, released a large amount of Glu, and the fluorescent intensity increased obviously. Mel not only remarkably inhibited the Ca^{2+}-dependent release of glutamate, but also reduced the Ca^{2+}-independent release of glutamate from synaptosome in old mice induced by KCl 30 mmol ·L^{-1} ($P < 0.01$) (Tab 1).

Tab 1. Effect of Mel on calcium-dependent or independent release of glutamate from synaptosomes in old mice induced by KCl 30 mmol · L^{-1} by continuous fluorometry. $n = 5$ mice. $\bar{x} \pm s$. $^bP < 0.05$ vs Ca^{2+} group. $^cP < 0.01$ vs control group.

MT/μmol·L^{-1}	L-Glu/μmol·g^{-1} (protein)	
	Ca^{2+}	Egtazic acid
0 (control)	10.6 ± 1.1	9.2 ± 0.7^b
0.01	7.1 ± 1.3^c	8.0 ± 1.2^c
0.1	6.5 ± 0.9^c	7.5 ± 0.6^c
1	6.2 ± 1.2^c	7.3 ± 0.9^c
3	3.4 ± 0.6^c	5.7 ± 0.9^c

Prevention of KCl and Glu neurotoxicity by Mel On 14 d in culture, neurons developed an extensive network, showing large and phase-bright cell bodies. After the culture was changed to serum-free medium, the cell bodies became bigger, the neuronal processes shortened and decreased. When cultures were exposed to KCl or Glu, swelling and darkening of neurons were seen, neuritis disappeared, neuron number reduced, even debris occurred in some areas. While sister cultures were exposed to Mel prior to KCl or Glu, compared with the serum withdrawal group, it showed almost without or only a slight neuronal damage (Fig 1).

This morphologic change was confirmed by the assay of MTT (Tab 2). KCl or Glu induced a marked decrease in A_{570}, which was nearly abolished by the pretreatment with Mel, indicating that Mel enhanced the cell viability.

KCl and Glu caused an increase of LDH efflux. The LDH efflux was obviously diminished by pretreatment with Mel (Tab 3), suggesting that Mel had a protective effect against GNT.

DISCUSSION

Glu is the main excitatory amino acid (EAA) in the brain, but under certain conditions it can become neurotoxic, for example, induces a receptor-mediated increase of intracellular free Ca^{2+} ($[Ca^{2+}]_i$)[13], which persists a long time, after the removal of the amino acid from the medium[14]. Persistent stimulation of Glu receptors causes GNT and neuronal death. A large body of evidence has proved that delayed Glu-induced neuronal death was associated with sustained increase in

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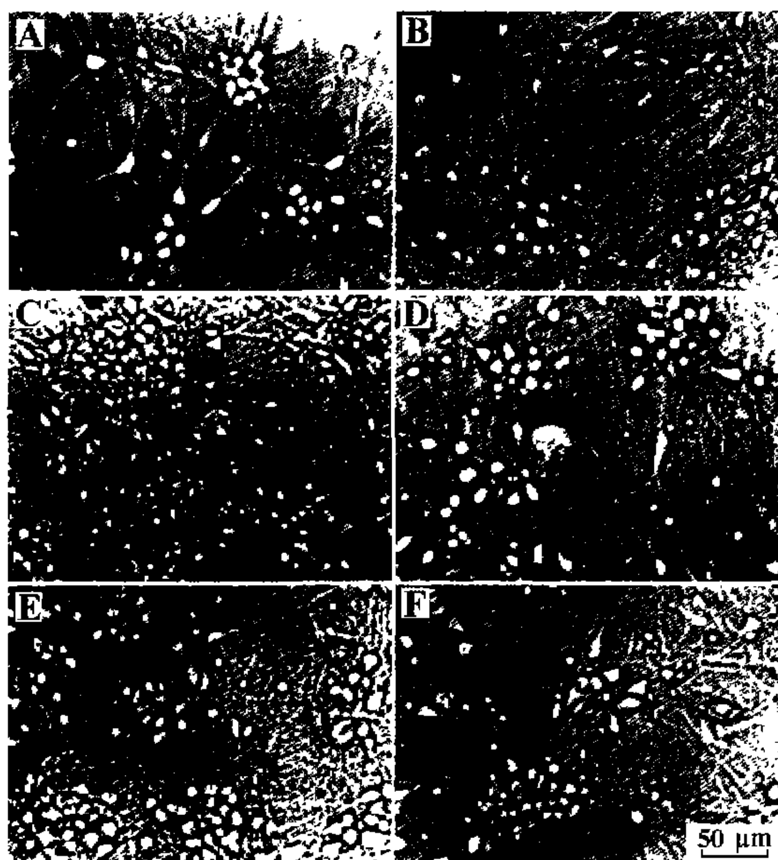

Fig 1. Rat cortical neurons visualized by phase-contrast microscope (×200). Bright-field photomicrographs after exposure for 20 h. A) control 1 (complete medium). B) control 2 (serum-free medium). C) control 3 (Glu 100 μmol·L^{-1}). D) treated 1 (Mel 1 μmol·L^{-1} + Glu). E) control 4 (KCl 20 mmol·L^{-1}). F) treated 2 (Mel 0.01 μmol·L^{-1} + KCl).

Tab 2. Protective effects of Mel (μmol·L^{-1}) on Glu (100 μmol·L^{-1})- and KCl (20 mmol·L^{-1})-induced neurotoxicity in cultured rat cortical cells. n = 6 cells. $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* Glu or KCl toxic control. $^fP < 0.01$ *vs* serum-free control.

Treatment	MTT A_{570}	Increase of A/%	Inhibition by Mel/%
Serum-free control 1	0.79±0.03		
Glu toxic control	0.67±0.04^f		
Glu + Mel 1	0.720±0.025^{cf}	7.5	42.6
0.1	0.715±0.023^{cf}	6.7	38.3
0.01	0.704±0.024^{cf}	5.1	29.0
Serum-free control 2	0.989±0.027		
KCl toxic control	0.812±0.030^f		
KCl + Mel 1	0.932±0.028^{cf}	14.8	67.6
0.1	0.925±0.027^{cf}	13.9	63.8
0.01	0.895±0.028^{cf}	10.2	46.7

$[Ca^{2+}]_i$, and this is a key step in the process of neuronal death[15,16].

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. Melatonin was recently found to be a free radical scavenger and antioxidant. It has several functions in term of its antioxidative ability, readily scavenges the most highly toxic free radical, the hydroxyl radical[17]. The release of excitatory amino acids such as Glu enhances endogenous hydroxyl radical formation. The activation of central excitatory amino acid receptors suppress melatonin synthesis and is therefore accompanied by a reduced detoxification rate of hydroxyl radicals. Aged animals and humans are Mel-deficient and more

Tab 3. Inhibitory effects of Mel ($\mu mol \cdot L^{-1}$) on KCl (20 $mmol \cdot L^{-1}$)- and Glu (100 $\mu mol \cdot L^{-1}$)-induced intracellular LDH release in cultured primary cortical neurons in rats. The released LDH activity was expressed as the percentage of total LDH activity released from freeze-thawed sister cultures. $n = 6$ wells. $\bar{x} \pm s$. ${}^cP < 0.01$ vs Glu or KCl toxic control. ${}^dP > 0.05$, ${}^fP < 0.01$ vs serum-free control.

	Extracellular LDH/kU·L⁻¹	Total LDH/ kU·L⁻¹	LDH release rate/%
Serum-free control	2.57±0.13	7.58±0.21	0.339±0.012
Glu toxic control	3.50±0.18	8.73±0.16	0.400±0.016^f
Glu + Mel 1	2.56±0.12	7.86±0.14	0.326±0.014^{cd}
0.1	2.78±0.22	8.05±0.25	0.345±0.021^{cd}
0.01	2.91±0.21	8.15±0.20	0.357±0.024^{cd}
KCl toxic control	2.97±0.12	7.85±0.20	0.379±0.016^f
KCl + Mel 1	2.60±0.18	7.50±0.28	0.348±0.009^{cd}
0.1	2.55±0.17	7.49±0.30	0.340±0.012^{cd}
0.01	2.54±0.25	7.60±0.33	0.334±0.015^{cd}

sensitive to oxidative stress[18]. It has been reported that Mel affords protection against kainate-induced *in vitro* and *in vivo* excitotoxicity[19], *in vitro* lipid peroxidation[20], and *in vivo* apoptosis[21] in brain.

The present study showed that Mel could inhibit the Ca^{2+}-dependent and -independent Glu release from cerebral synaptosomes in old mice, and antagonize the excitotoxicity induced by KCl and Glu in primary fetal rat cortical cell cultures, suggesting that the inhibitory effect of Mel on Glu release, the protection of neurons against neurotoxicity, further, prevention of $[Ca^{2+}]_i$ overload and lipid peroxidation in the brain is probably involved in its antiaging effect.

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褪黑激素对大脑皮层谷氨酸释放及其神经毒性的拮抗作用

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关键词 褪黑激素; 谷氨酸; 神经毒素; 突触体; 培养的细胞; 神经元; 大脑皮质; 衰老

目的: 观察褪黑激素(Mel)对老年小鼠大脑皮层突触体谷氨酸(Glu)释放以及 KCl、Glu 在原代培养胎鼠脑细胞诱发的神经毒性的影响, 以探讨 Mel 抗衰老的作用机制. **方法**: 制备老年小鼠大脑皮层突触体, 用 RF-5000 型双波长荧光分光光度计检测谷氨酸释放量. 应用原代培养的大鼠皮层细胞 MTT 染色和乳酸脱氢酶(LDH)测定法评估神经元活性. **结果**: Mel 能够抑制高浓度氯化钾(30 $mmol \cdot L^{-1}$)诱发的老年小鼠大脑皮层突触体钙依赖性及非依赖性谷氨酸释放, 抵抗 KCl 和 Glu 诱发的皮层细胞损伤, 对神经元有保护作用. **结论**: Mel 对大脑皮层突触体谷氨酸释放的抑制作用以及对大脑皮层神经元的保护作用可能是其抗衰老作用机制之一.

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