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Improvement of melatonin on learning and memory impairment induced by amyloid β-peptide 25 - 35 in elder rats[1]

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KEY WORDS melatonin; amyloid beta-protein; learning; memory; cerebral cortex; hippocampus

ABSTRACT

AIM: To investigate improvement of melatonin on learning and memory impairment induced by amyloid β-peptide 25 - 35 ($A\beta_{25-35}$) in elder rats. **METHODS**: Step-down type passive avoidance test, shuttle-box test, and Morris water maze were used together to determine effects of $A\beta_{25-35}$ and melatonin on learning and memory. Pathological changes were observed by HE, Congo red, and Ag staining. **RESULTS**: The elder rats were injected bilaterally $A\beta_{25-35}$ 20 μg into the hippocampus to induce learning and memory dysfunction. Melatonin administration (0.1, 1, and 10 mg/kg, ig × 8 d) to the $A\beta_{25-35}$-treated rats prolonged the latency, shortened the total stimulating time, and decreased the number of errors in the step-down test. Suttle-box test showed that melatonin improved amnesic rats' performace at the same doses. Melatonin (0.1, 1, and 10 mg/kg), giving for 10 d, could enhance the spatial resolution of amnesic rats in Morris water maze test. Also in $A\beta_{25-35}$-treated group, a decrease in the number of neurons in cortex and hippocampus, a massive glial reaction, and neurophilic phenomenon were detected by HE staining; the positive vascular amyloidosis by Congo red and fibrils by Ag staining were observed. Melatonin (0.1 and 1 mg/kg) could inhibit above pathological changes in $A\beta_{25-35}$ group. **CONCLUSION**: Melatonin improved the impaired learning and memory induced by $A\beta_{25-35}$ in elder rats.

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INTRODUCTION

Aging and a variety of age-related degenerative conditions in central nervous system (eg, Alzheimer disease, AD) have a common symptom that is learning and memory dysfunction. AD is the most common cause of progressive intellectual failure in aged humans. Major pathological hallmarks of AD are extracellular senile plaques, intracellular neurofibrillary tangles, and drastic neuronal and synaptic degeneration in many areas involved in cognitive function. Amyloid β-peptide (Aβ), spontaneously aggregating peptide of 39 - 43 amino acids, is the primary protein component of senile plaques in AD brains. An increasing amount of experimental as well as genetic evidence supports a causal role for Aβ in the pathogenesis of AD. Briefly, senile plaques containing Aβ as a core protein are often closely associated with degenerating neurons[1]. At present, there is no effective treatment to prevent or ameliorate the progression of AD. A number of studies have shown that high concentrations of Aβ are toxic and damaging biological macromolecules. Its degenerative effects have included alteration of Ca^{2+} homeostasis in cells[2], apoptosis[3], and inhibition of cellular redox activity[4], and these actions are believed to be dependent on the peptide assembly state such as its solution or aggregation[5]. The effects of Aβ are localized to amino acid residues 25 - 35 of the full-length peptide[6]. The Aβ peptide fragment 25 - 35 ($A\beta_{25-35}$) has been shown to be directly toxic to neurons[5] and be able to increase the vulnerability of neurons to other insults[6].

Melatonin, synthesized by the pineal gland of vertebrates including humans during the dark phase of the circadian, has been implicated in the regulation of various neural and endocrine processes that synchronized with the daily change in photoperiod. The original interest in melatonin derives from the suggestion that melatonin may act as a key regulator in aging and senescence[7]. The level of melatonin in the pineal gland declines progressively with age, such that in old animals and

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elderly humans the level of melatonin available to the organism are a fraction of that of young individuals[8]. Recent studies suggest that melatonin exerts a neuroprotective role, and accelerates the early process of neuronal differentiation[9]. These effects are accomplished presumably because of the indole's high lipid solubility which allows free access to the interior of the cell. Support for the neuroprotective effect of melatonin has come, to date, from *in vitro* experiments. However, it is unknown whether melatonin possesses the neuroprotective effect *in vivo*. In certain cases, microinjections of Aβ into cortex or hippocampus produce profound neurodegeneration in rat brain[10-11], whereas no neurotoxic effect was observed by other groups in either rodents[12-13]. The present study was designed to determine whether microinjection of $A\beta_{25-35}$ into rat hippocampus induced the learning and memory dysfunction and whether melatonin had the ability to protect cortical and hipporcampal neurons against $A\beta_{25-35}$ neurotoxicity.

MATERIALS AND METHODS

Animals Sprague-Dawley rats (Grade Ⅱ, Certificate № 005), aged 10 months and weighing 280 – 320 g at the beginning of the experiment, supplied by the Shanghai Experimental Animals Centre of Chinese Academy of Sciences. Animals were housed in plastic cages, with free access to food and water, except during behavioural experiments, and kept in a regulated enviroment (20 ± 1) ℃ under a 12-h light/dark cycle.

Materials $A\beta_{25-35}$ (Sigma Chemical Co, USA) was dissolved in sterile saline at a concentration of 2 g/L and stored at – 20 ℃. To obtain the neurotoxic form of $A\beta_{25-35}$, the peptide solution was placed in an incubator at 37 ℃ for 7 d[4]. Melatonin, purchased from Sigma Chemical Co (USA), was dissolved in 0.9 % sterile saline with absolute ethanol (less than 0.01 %, v/v) and stored at – 20 ℃.

Induction of learning and memory dysfunction by injection of $A\beta_{25-35}$ Injections of $A\beta_{25-35}$ in hippocampus were performed as previously described[14]. Briefly, a microsyringe with a stainless-steel needle was used for all experiments. Rats were anesthetized with sodium pentobarbital (40 mg/kg) and placed in a stereotaxic apparatus. The coordinates for hippocampal injections (AP – 3.5 mm, ML ± 2.0 mm, DV 2.7 mm) were similar to those used by Morimoto[14]. Peptide or vehicle (5 μL) was delivered gradually within 3 min and the syringe was left in the place for 2 min following the injection. Rats were administered melatonin (0.01, 0.1, 1, and 10 mg/kg, ig) 24 h after the injection.

Step-down type passive avoidance test After 8-d injection of $A\beta_{25-35}$ in hippocampus, long-term memory were examined using the step-down type of passive avoidance test. The apparatus consisted of acrylic box with a stainless-steel grid floor. A platform was fixed in the end of the box. Electric shocks (40 V) were delivered to the grid floor for 3 s with an isolated pulse stimulator. At the begining of training, rats were placed on the platform to adapt for 2 min. When the rat steped down and placed all its paws on the grid floor, it would jump to the platform as shock happened to be delivered. Step-down latency (time of staying on the platform), the number of errors, and the total stimulating time were recorded within 5 min and repeated 24 h after training.

Shuttle-box test The apparatus consisted of a standard rectangular shuttle box with plexiglas walls, the floor of which was a stainless-steel grid floor. The box was divided into two compartments with a plate. There is a hole at the bottom of the plate, by which rats can shuttle freely. Two lamps, as a signal in the ceiling of each compartment, were set up an electric circuit alternately for 10 s followed by 20 s electric shocks (40 V) delivered to the grid floor and then 10 s interval every turn. For training, rat was placed in one of the compartments and then it would go to other one when it was shocked by an isolated pulse stimulator. The active escape (to escape following a lamp signal) latency, the accumulated stimulating time, the active escape numbers, and the passive avoidance (to avoid by an electric shock) numbers were recorded automatically during shuttling for 30 times. Rats were used 8 d after injection of $A\beta_{25-35}$.

Morris water maze Rats were used to train in Morris water maze 8 d after injection of $A\beta_{25-35}$. The water maze apparatus consisted of a circular pool, which was filled with water to depth of 30 cm and a circular platform, supported by a base resting on the bottom of the pool, placed 2 cm below the surface of the water. The weter temperature was (21.0 ± 0.5) ℃. The water was made white by milk and rat heads were stained black. For descriptive data collection, the pool was subdivided into four equal quadrants formed by imaging lines. The platform always resided in the center of the southwest quadrant. On the start of a trial, rat was

placed facing the wall. When rats found the platform, they were allowed to remain on it for 15 s. If rats did not locate the platform within 2 min, they were removed from the water and placed on the platform for 15 s. Data collection was automated by an on-line video tracking device designed to track the object in its field with the highest contrast, which was always the black heads of rats on the milk-white background. Tracking was achieved by a system consisting of a video camera mounted over the center of the pool. The tracker's digitized coordinate values were sampled in turn using a computer. Rats were trained for 5 consecutive days, escape latency (time to find the platform), the starting angle, and swimming path were recorded.

Histology Two weeks after injection, rats administered with saline or aged $A\beta_{25-35}$ were used for histological studies. Brain tissues were fixed with 10 % neutral formalin solution. The sections were stained with haematoxylin-eosin (HE). The amyloid deposits were detected using Congo red and silver.

Statistical analysis Statistical analysis of the data for multiple comparisons was performed by ANOVA followed by Dunnett's test. For single comparisons, the significance of differences between means was determined by t-test.

RESULTS

Effects of melatonin on learning and memory dysfunction induced by $A\beta_{25-35}$ Sprague-Dawley rats, ten months of age, were injected bilaterally $A\beta_{25-35}$ 20 μg or vehicle in the hippocampus to induce learning and memory dysfunction model. Melatonin was administered (ig) with four different doses (0.01, 0.1, 1, and 10 mg/kg) once a day 24 h after the injection of $A\beta_{25-35}$ for 7 – 10 d. In $A\beta_{25-35}$-treated rats, the latencies shortened, the total stimulating time prolonged, and the number of errors increased determined by the step-down test (Tab 1). The shuttle-box test showed that $A\beta_{25-35}$ reduced the latencies, prolonged the stimulating time (Tab 2, 3), and increased the numbers of passive avoidance (Tab 3). Melatonin (0.1, 1, and 10 mg/kg), given for 7 d, improved amnesic rats' performace to some degree.

In Morris water maze test, the mean latencies, the swimming path (distance), and the starting angles

Tab 1. Improvement of melatonin on the learning and memory dysfunction in rats treated with $A\beta_{25-35}$ (20 μg) on the second day of training by step-down test. $n = 8 - 10$. $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* $A\beta_{25-35}$ group. Melatonin was given for 7 d (ig) d 2 after the injection of $A\beta_{25-35}$.

Group	Dose/mg·kg^{-1}	Latency/s	Stimulating time/s	Error numbers
Control	–	276 ± 28^c	21 ± 25^c	4 ± 3^c
$A\beta_{25-35}$	20 μg	198 ± 58	102 ± 58	13 ± 4
Melatonin	0.01	213 ± 48	94 ± 39	10 ± 4
	0.1	258 ± 17^c	42 ± 17^c	7 ± 3^c
	1	238 ± 26^b	62 ± 26^b	9 ± 4^b
	10	240 ± 27^b	60 ± 27^b	10 ± 6

Tab 2. Improvement of melatonin on the learning and memory dysfunction in rats treated with $A\beta_{25-35}$ (20 μg) on the second day of training by suttle-box test. $n = 8 - 10$. $\bar{x} \pm s$. $^bP < 0.05$ *vs* $A\beta_{25-35}$ group. Melatonin was given ig to $A\beta_{25-35}$-treated rats d 2 after the injection of $A\beta_{25-35}$ for 7 d.

Group	Dose/mg·kg^{-1}	Latency/s	Stimulating time /s	Numbers of escape	Numbers of avoidance
Control	–	4.1 ± 2.0^b	4.4 ± 2.5^b	1.1 ± 1.2	13.6 ± 3.2
$A\beta_{25-35}$	20 μg	2.3 ± 1.7	7.8 ± 2.4	1.3 ± 1.2	14.3 ± 1.9
Melatonin	0.01	2.4 ± 1.4	8.1 ± 1.3	0.8 ± 0.9	15.1 ± 3.3
	0.1	2.9 ± 1.1	6.9 ± 1.0	1.4 ± 1.0	13.2 ± 2.2
	1	2.9 ± 2.0	7.8 ± 1.5	1.3 ± 2.5	13.2 ± 4.1
	10	3.3 ± 1.9	5.6 ± 2.5^b	1.5 ± 1.2	14.5 ± 3.1

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Tab 3. Improvement of melatonin on the learning and memory dysfunction in rats treated with $A\beta_{25-35}$ on the third day of training by suttle-box test. $n = 8 - 10$. $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* $A\beta_{25-35}$ group. Melatonin was given ig to $A\beta_{25-35}$-treated rats d 2 after injection of $A\beta_{25-35}$ for 7 d.

Group	Dose/mg·kg^{-1}	Latency/s	Stimulating time /s	Numbers of escape	Numbers of avoidance
Control	–	3.7±1.5^b	5.1±2.7^b	1.0±0.9	10.3±3.5^b
$A\beta_{25-35}$	20 μg	1.9±1.4	7.4±1.5	1.1±1.0	15.9±1.7
Melatonin	0.01	2.7±1.7	6.9±1.7	0.8±1.2	14.0±2.1
	0.1	3.6±1.4^b	5.3±2.2^b	0.9±1.2	13.7±2.3
	1	3.6±1.8^b	4.8±2.6^c	0.7±1.3	13.0±2.2^c
	10	3.5±1.9^b	6.0±1.8	1.0±1.3	14.4±3.0

increased (Tab 4, 5, and 6), this means that the $A\beta_{25-35}$-treated rats took longer time to find the platform than the control did. Melatonin (0.1, 1, and 10 mg/kg), given for 10 d, could enhance the spatial resolution of amnesic rats in Morris water maze test.

Effect of melatonin on the pathological changes of the $A\beta_{25-35}$-treated rats In $A\beta_{25-35}$-treated group, a decrease in the number of neurons in cortex and hippocampus, a massive glial reaction (Fig 1), and neurophilic phenomenon were detected by HE staining; the positive vascular amyloidosis by Congo red and fibrils by Ag staining were also observed (Fig 2). Melatonin (1 mg/kg), given for 10 d, increased the number of neurons and reduced glial reaction and the fibrils in $A\beta_{25-35}$-treated group.

Tab 4. Effects of melatonin on the escape latencies of the amnesic rats treated with $A\beta_{25-35}$ in Morris water maze test. $n = 8 - 10$. $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* $A\beta_{25-35}$ group. Melatonin was given ig to $A\beta_{25-35}$-treated rats d 2 after injection of $A\beta_{25-35}$ for 10 d.

Group	Dose/mg·kg^{-1}	Latency/s d 3	d 4	d 5
Control	–	52±13^b	48±13^b	34±9
$A\beta_{25-35}$	20 μg	70±15	68±12	48±15
Melatonin	0.01	58±15	62±13	23±9^c
	0.1	61±13	57±15	29±9^c
	1	53±12^c	55±9^b	29±8
	10	51±13^c	56±10^b	42±11

Tab 5. Effect of melatonin on the distance of the amnesic rats treated with $A\beta_{25-35}$ in Morris water maze test. $n = 10$. $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* $A\beta_{25-35}$ group. Melatonin was given ig to $A\beta_{25-35}$-treated rats d 2 after injection of $A\beta_{25-35}$ for 10 d.

Group	Dose/mg·kg^{-1}	Distance/cm d 3	d 4	d 5
Control		981±204^b	631±124^b	616±145^b
$A\beta_{25-35}$	20 μg	1285±350	794±255	841±341
Melatonin	0.01	1057±229^b	519±114^c	507±164^c
	0.1	880±139^c	565±127^c	432±144^c
	1	987±241^c	586±154^b	518±173^c
	10	1005±178^c	776±240	753±196

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Tab 6. Effects of melatonin on the starting angle of the amnesic rats treated with $A\beta_{25-35}$ in Morris water maze test. $n = 10$. $\bar{x} \pm s$. $^bP < 0.05$, $^cP < 0.01$ *vs* $A\beta_{25-35}$ group. Melatonin was given ig to $A\beta_{25-35}$-treated rats d 2 after injection of $A\beta_{25-35}$ for 10 d.

Group	Dose/mg·kg^{-1}	Starting angle/degrees d 3	d 4	d 5
Control	–	51 ± 13^c	48 ± 15^c	31 ± 8
$A\beta_{25-35}$	20 μg	89 ± 14	81 ± 15	66 ± 6
Melatonin	0.01	62 ± 15^b	60 ± 13^b	52 ± 16^b
	0.1	52 ± 13^c	52 ± 19^c	46 ± 7^c
	1	49 ± 11^c	57 ± 14^b	35 ± 11^c
	10	68 ± 16	73 ± 43	52 ± 20^b

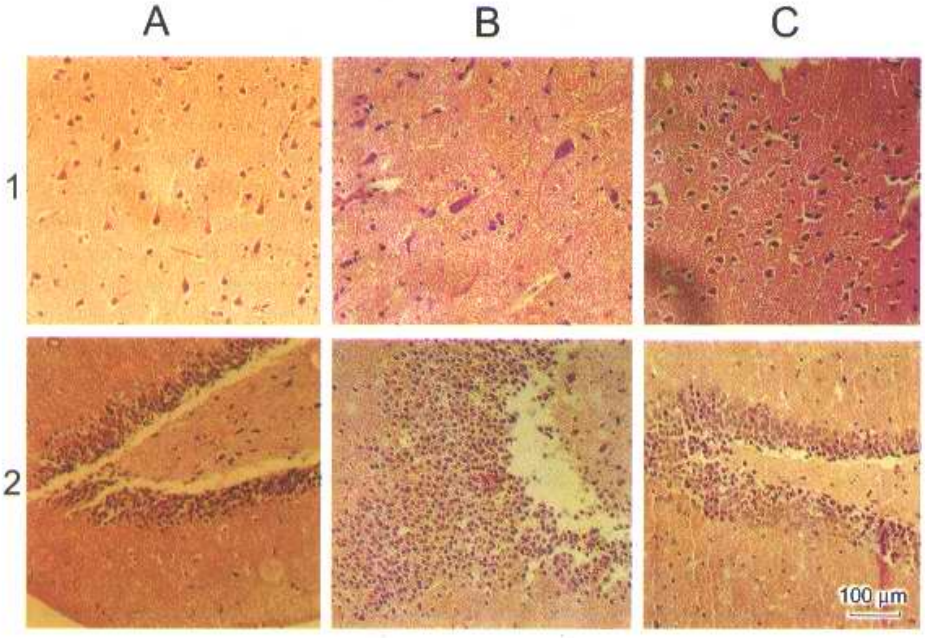

Fig 1. Effect of melatonin on cortical morphology (1) and hippocampal gliosis (2) of rats treated with $A\beta_{25-35}$ (20 μg). A: Control; B: $A\beta_{25-35}$ model; C: Melatonin 1 mg/kg. × 100.

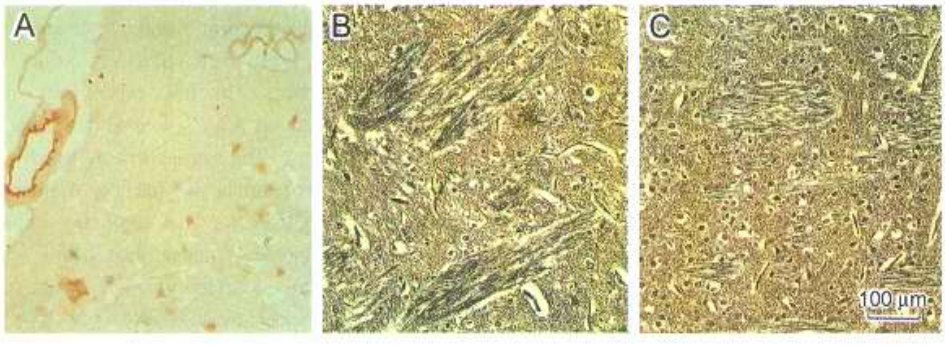

Fig 2. Effect of melatonin on the fibrils in the brain tissue of rats treated with $A\beta_{25-35}$ (20 μg) injection into hippocampus. A: Positive vascular amyloidosis stained by Congo red in $A\beta_{25-35}$ group; B: Fibrils stained by Ag in $A\beta_{25-35}$ group; C: Fibrils stained by Ag in melatonin-treated (1 mg/kg) group. × 100.

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DISCUSSION

The present data demonstrate that a single acute injection of an aged preparation of $A\beta_{25-35}$ in hippocampus induced marked amnesic effects in rats. The step-down and shuttle-box test were used to examine the long-term memory. The results showed that the alternation behaviour was decreased. The amnesic effect of aged $A\beta_{25-35}$ was also proved using the repetitive training procedure of place learning in a Morris water maze, an index of spatial working memory. These results indicated that $A\beta_{25-35}$ was able to induce impairment of learning and memory.

A previous report showed that chronic icv infusion of the $A\beta_{1-40}$ peptide 0.3 nmol/d in rats, delivered using a mini-osmotic pump during 14 d, induced an impairment of learning, based on a reduced performance in the Morris water maze[15]. Acute injection of $A\beta_{1-40}$ peptide into rat brains was also shown to impair memory consolidation[16]. On the other hand, Winkler *et al*[17] observed no significant neuropathological or behavioural effects 15 months after the icv administration of $A\beta_{1-40}$ peptide into rats brains. These results, together with ours, show that β-amyloid related peptides induce learning impairment and anterograde amnesia. To our knowledge, the present study is the first to describe, using three different behavioural paradigms, distinct amnesic profiles for the $A\beta_{25-35}$.

The use of aged peptide results in a more marked amnesic effect, based on the observations that, when the $A\beta_{25-35}$ was not aged, the impairment of alternation was reduced and the decrease in passive avoidance was not significant. Incubation of Aβ in water, for several days, produces a conformational transformation from random coil to β-sheet coinciding with an increase in peptide neurotoxic potency[18]. Indeed, solubilisation of $A\beta_{1-40}$ peptide in solvents that prevent aggregation and/or the use of the non-aggregated $A\beta_{1-40}$ peptid results in amnesia but only after chronic icv infusion during 14 d[15]. The aggregated form of the peptide is not necessary to induce amnesia, but markedly facilitates its appearance.

In present studies, a decrease in the number of neurons in cortex and hippocampus, a massive glial reaction, and neurophilic phenomenon were detected by HE staining in $A\beta_{25-35}$-treated group; the positive vascular amyloidosis by Congo red and fibrils by Ag staining were also observed, as supported by previous observations[19]. It appears from our studies that administration of 20 μg of aged $A\beta_{25-35}$ produced moderate but significant neuronal degeneration and loss at the hippocampal formation and cortical layer. A previous study by Rush *et al*[10] suggested that the neuronal loss induced by central injection of β-amyloid might result from the aggregation of peptide and the subsequent displacement tissue and not to any direct neurotoxic mechanism. It has also been suggested that neuronal atrophy, and not cell death, is the main hallmark of AD[19]. A potential induction of apoptosis-mediated degeneration of neurons by $A\beta_{25-35}$ *in vivo*, as observed with *in vitro* models of β-amyloid-mediated neurotoxicity, may initially induce amnesia before the appearance of significant neuronal damage.

Numerous studies have demonstrated a significant corelation between the number of senile plaques and the degree of cognitive deficits in AD brains. In rodent models of AD, the number of amyloid plaques may not accurately indicate the extent of β-amyloid deposition and/or the degree of dementia. It is clear that the present model does not encompass all of the neuropathological effects observed in AD, such as neurofibrillary tangle formation as well as increased $A\beta_{25-35}$ rather than $A\beta_{1-40}$ deposition. However, neurofibrillary tangle formation is generally not observed in rodent brains, it does however possess similar neurotoxic and amyloidogenic activity as the complete $A\beta_{1-40}$ peptide, thereby making it biologically relevant to the etiology of AD.

The treatment of melatonin resulted in a signifcant improvement on rat learning and memory dysfunction induced by $A\beta_{25-35}$, this effect was bell shaped, did not appear in a dose-dependent manner. It suggested that melatonin may exert high effective action with an appropriate amount. In this experiment, melatonin exerted more effect on $A\beta_{25-35}$-treated rats at the doses of 0.1 and 1 mg/kg. The mechanisms by which melatonin improved the rats learning and memory dysfunction may be related to antioxidation and regulating inflammatory and immune responses (unpublished data). Whether melatonin mediates its effects through classic membrane receptors is still under investigation.

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褪黑激素改善淀粉样β多肽25-35片段诱导的老年大鼠学习记忆功能障碍[1]

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关键词 褪黑激素; 淀粉样β蛋白; 学习; 记忆; 大脑皮质; 海马

目的: 观察褪黑激素对淀粉样β多肽25-35片段($A\beta_{25-35}$)诱导的大鼠学习记忆功能障碍的改善作用. **方法**: 学习记忆功能检测采用大鼠跳台法、穿梭法和Moriss水迷宫法; 病理组织观察采用HE染色、刚果红染色和银染色. **结果**: 大鼠双侧海马内注射$A\beta_{25-35}$(20 μg)可引起大鼠学习记忆功能障碍, 主要表现为在跳台作业中潜伏期缩短, 累计刺激时间延长, 累计错误次数增多; 穿梭实验中, 主动回避潜伏期缩短, 主动回避次数减少, 被动回避次数增加; Moriss水迷宫同样也证实大鼠出现空间学习记忆障碍; HE染色发现皮质和海马神经细胞数量减少, 胶质细胞呈反应性增生, 嗜神经现象增多; 刚果红染色血管出现阳性; 银染色提示有纤维蛋白丝状物. 褪黑激素(0.1, 1, 10 mg/kg)连续给予(ig) 7-10 d分别对上述改变有不同程度的抑制作用. **结论**: 褪黑激素能改善$A\beta_{25-35}$诱导的大鼠学习记忆功能障碍.

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