Melatonin ameliorates amygdala-dependent emotional memory deficits in Tg2576 mice by up-regulating the CREB/c-Fos pathway

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HIGHLIGHTS

• Melatonin improved amygdala-dependent emotional memory in Tg2576 mice.
• Melatonin increased the expression of c-fos protein levels in basolateral amygdala.
• Melatonin increased the expression of pCREB/CREB ratio in basolateral amygdala.
• CREB/c-Fos pathway play critical role for the efficacy of melatonin in Tg2576 mice.

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ABSTRACT

The effects of melatonin on spatial memory deficits in Alzheimer’s disease (AD) have been thoroughly investigated. Our previous study demonstrated that melatonin rescues hippocampus-dependent spatial memory deficits by arresting hippocampal pathological progression in an animal model of AD, which occurs via the inhibition of GSK3β and an increase in c-Fos. Based on the interaction between the amygdala and hippocampus, it is important to determine whether melatonin also improves amygdala-dependent emotional memory to understand the mechanism of melatonin amelioration of memory deficits in AD. The basolateral amygdala (BLA) is essential for the processing of emotions, including cued fear conditioning and anxiety. In the present study, we intraperitoneally injected Tg2576 mice with melatonin for 4 months and measured amygdala-dependent emotional memory using cued fear conditioning and a step-down passive avoidance test; the expression of c-Fos, Arc, phosphorylated CREB (pCREB) and other related genes were subsequently measured using Real-time polymerase chain reaction (RT-PCR) and Western blot in BLA. Our findings suggest that melatonin ameliorates amygdala-dependent emotional memory in AD via up-regulation of the pCREB/c-Fos pathway.

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1. Introduction

Alzheimer’s disease (AD) is a neurodegenerative disorder characterized by severe deficits in cognitive function. Abnormal hyperphosphorylation of microtubule associated protein tau (p-tau) and overproduction of β-amyloid (Aβ) are the 2 hallmarks in AD brains [1]. Disruptions in synaptic plasticity are associated with multiple neurodegenerative diseases, such as AD [2]. Synaptic plasticity and synaptic-related proteins comprise the major mechanisms that underlie learning and memory [3,4]. Many immediate early genes (IEGs) are linked to synaptic plasticity [5]. c-fos, which represents the first identified IEG [6], is indispensable for the maintenance of normal synaptic plasticity. Mice that lack c-Fos in the brain exhibit impairments in spatial reference and contextual learning, as well as decreased long-term potentiation (LTP) of synaptic transmission at CA3-to-CA1 synapses.
is intraperitoneally. Moreover, (2.1.) 

Melatonin (N-acetyl-5-methoxytryptamine), a tryptophan metabolite and neurohormone that are primarily secreted by the pineal gland, performs many physiological functions, such as circadian rhythm regulation, free radical clearance, improvements in immunity, and inhibition of biomolecule oxidation (17,18). Previous studies have demonstrated that melatonin administration may attenuate Alzheimer-like tau hyperphosphorylation, Aβ overproduction, and behavioral deficits in cells and rats (19,20). Moreover, evidence suggests that melatonin modulates the rhythmic output of the hypothalamic suprachiasmatic nucleus (SCN) potentially by increasing c-Fos expression (21). We have also previously demonstrated that the properly timed administration of melatonin ameliorates hippocampus-dependent spatial memory deficits in Tg2576 likely by increasing c-Fos expression in the hippocampus (22). Tg2576 is a transgenic mouse model in which a human Aβ precursor protein (APP) with Swedish double mutations is expressed.

The structure of the amygdala is essential for the processing of emotions, including cued fear conditioning and anxiety (23,24). The amygdala is a critical site for the development and storage of fear memory (25). The amygdala is a complex group of nuclei implicated in the integration and generation of emotional behavioral responses (26). It consists of a group of anatomically and functionally distinct nuclei, which includes the lateral amygdala (LA), the basolateral amygdale (BLA), the central amygdale (CeA), the central amygdaloid nucleus (CeM), the centro-lateral (Cel), the centro-central (CeC) amygdale and the medial amygdale (MeA) (23,27). Studies have demonstrated that LA, BLA and CeA are necessary and sufficient for the modulation of fear memories (28–30). Following chronic stress, the hippocampus and a specific amygdala subregion, the basolateral amygdala (BLA), exhibit marked reductions in impaired plasticity (31,32). Abundant evidence suggests that these BLA inputs have a major impact on hippocampal function (31). Thus, we hypothesize that melatonin plays an ameliorative role in both the BLA and hippocampus in an AD mouse model via the up-regulation of c-Fos.

In the current study, we investigated the potential amenderatory effect of melatonin on amygdala-dependent emotional memory using cued fear conditioning and the step-down passive avoidance test, as well as explored the mechanism that underlies melatonin-mediated neuroprotection. Our data suggested that melatonin ameliorates amygdala-dependent emotional memory in Tg2576 mice likely via the up-regulation of the pCREB/c-Fos pathway in the BLA.

2. Materials and methods

2.1. Animals and treatments

Tg2576 mice harboring human APP 695 with the Swedish double mutation (HuAPP695; K670N/M671L, Jackson Lab) were intraperitoneally injected with 10 mg/kg melatonin (Sigma, St. Louis, MO, USA) in 0.9% saline or an equal volume of dimethylsulfoxide (DMSO) in 0.9% saline as a vehicle control once every other day for 4 months from 8 months old (22) (n = 20 per group, equal numbers of males and females). The body weight was monitored weekly. The genotypes of the mice were assessed via polymerase chain reaction (PCR) using tail clip digestions (33). All mice were maintained at 24 ± 2 °C on a daily 12 h light-dark cycle with ad libitum access to food and water; all behavioral procedures were performed during the light cycle. The mice were sacrificed under deep anesthesia following the behavioral experiments. The tissues were collected for WB and RT-PCR analyses. The animal experiments were conducted according to the “Policies on the Use of Animals and Humans in Neuroscience Research” approved by the Society for Neuroscience in 1995 and were supervised by the Animal Administration and Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology (22).

2.2. Cued fear conditioning and fear expression

To determine the effect of amygdala-dependent emotional memory, cued fear conditioning (27) was implemented. The mice underwent cued fear conditioning and were tested for fear expression (4,29,34,35). The mice were trained and tested in a sound attenuating chamber with a gridding floor that could administer an electric shock, an overhead control apparatus that consisted of a video camera, and an acoustic generator in one corner of the chamber. Freezing was defined as a complete absence of movement. Movement was recorded by the camera. In the cued fear conditioning training phase, the mice were placed in the chamber with no interference for 120 s; a 30-s 80-dB white noise (conditioned stimulus CS) was subsequently presented, and a 0.5 mA foot shock (unconditioned stimulus US) was administered for 2 s prior to the end of the noise. The CS-US pair was presented three times, with a 1-min inter-trial interval after each stimulus was completed. The cued test was performed in a different context: the mice were placed in the chamber and heard an auditory CS for 30 s after a 3-min undisturbed period without a stimulus; the freezing time was then recorded continuously for 3 min. The chamber included a Plexiglas front and gray side and back walls, and the chamber floors consisted of 26 stainless steel rods. The rods were connected to a shock generator via a cable harness.

2.3. Step-down passive avoidance test

The inhibitory avoidance apparatus was a 50 × 25 × 25 cm³ acrylic box with a floor that consisted of a grid of parallel stainless steel bars (1 mm diameter) spaced 1 cm apart. A platform that was 10 cm² and 2 cm high was placed in the center of the floor (Institute of Materia Medica). An electric current (40 V) was delivered to the grid floor using an isolated stimulator. In the training session, immediately after the mouse stepped down on the grid, it received a scrambled foot shock (0.3 mA, 2 s). During the test session, each mouse was placed on the platform, and its latency to step down (step-down latency, SDL) on the grid with all four paws was measured with an automatic device; the number of errors in 3 min (CE) was also measured. A retention test was conducted 24 h after the first training session. The SDL and CE were recorded to measure long-term memory. The cut-off time was 180 s (36,37).

2.4. RT-PCR

Total RNA was isolated using Trizol reagents according to the instructions (Tiangen Technologies, Beijing, China). Total RNA (3 μg in 25 μl) was reverse transcribed using a commercial kit (Takara, Dalian, China), and the produced cDNA (1 μl) was used to detect the transcripts. The following primers were used: c-fos, 5′-CCGAGGAGCCTAAGAG-3′ (forward primer), 5′-TCTGGAAGCAGGTCAT-3′ (reverse
primer; 5′-GAGACCATCGGAACTG-3′ (forward primer), 5′-CGTATGTCATGCGCCTACG-3′ (reverse primer); and GAPDH, 5′-ATGGTAGGCTGGTGTCG-3′ (forward primer), 5′-TGAAGGGGTCTGATGATG-3′ (reverse primer). PCR amplification consisted of 40 cycles, and each cycle was run with the following program: denaturing at 95 °C, 30 s; annealing and chain extension as one step at 60 °C, 30 s. The PCR products were separated on 1.0% agarose gels, which were stained with GoldView and visualized under UV light.

2.5. Western blot analysis

BLA samples were collected. Briefly, three adjacent coronal brain sections of 500 μm were obtained from the rostral (Bregma = −0.58 mm), medial (Bregma = −1.08 mm) and caudal (Bregma = −1.58 mm) amygdala and were maintained at −80 °C until further analysis [38]. The primary and secondary antibodies employed in the present study are listed in Table 1. Western blot analysis was performed as described [39]. BLA extracts were mixed with sample buffer that contained 50 mM Tris-HCl (pH 7.4), 2% sodium dodecyl sulfate (SDS), 10% glycerol, 10 mM dithiothreitol (DTT), and 0.2% bromophenol blue and were subsequently boiled for 10 min. The proteins were separated via 10% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose/polyvinylidene fluoride (NC/PVDF) membranes. Immunoreactive bands were visualized with an Odyssey chemiluminescent substrate kit and exposed to CL-Xposure film (Pierce, Rockford, IL, USA). The blots were scanned, and the sum optical density was quantitatively analyzed using Kodak Digital Science 1D software (Eastman Kodak Company, New Haven, CT, USA) as previously described [22,40,41]. The immunoreactivities of IEG, synaptic protein, pCREB and CREB were normalized against GAPDH.

2.6. Statistical analyses

The data were analyzed using SPSS 12.0 statistical software (SPSS Inc., Chicago, IL, USA) and are expressed as the mean ± SEM. One or two-way analysis of variance (ANOVA) followed by least significant difference post hoc tests were used to identify significant differences between the group means (p < 0.05).

3. Results

3.1. Melatonin administration ameliorates amygdala-dependent emotional memory

Our previous studies have demonstrated that properly timed administration of melatonin improves hippocampal-dependent spatial learning and memory in Tg2576 mice [22]. In this report, we investigated whether melatonin ameliorates amygdala-dependent emotional memory deficits in Tg2576 mice. Amygdala-dependent emotional learning and memory were measured using cued fear conditioning and the step-down passive avoidance test. As expected, we demonstrated that melatonin administration significantly increased the freezing response during the conditioned stimulus of the Tg2576 mice in the training phase and the retrieval test at 24 h after training (Fig. 1A–C). Moreover, the long-term administration of melatonin significantly ameliorated the SDL and CE in the learning session and the test session in the Tg2576 mice (Fig. 1D–G). These findings suggested that melatonin supplementation improves amygdala-dependent learning and memory in Tg2576 mice.

3.2. Melatonin increases the expression of synapse-associated proteins in BLA

As one hallmark of synapse loss, synapse-associated proteins significantly decrease at an early stage of AD in Tg2576 mice [42]. Synapse-associated proteins are necessary for learning and memory [43]. Previous reports have demonstrated that melatonin prevented the decrease of postsynaptic proteins, including PSD95 and synaptophysin, in the hippocampus [22]. To determine how melatonin improves amygdala-dependent emotional memory in AD, as well as to investigate whether melatonin affects postsynaptic protein expression in the BLA, we measured the levels of PSD95 and synaptophysin via Western blotting. We demonstrated that the levels of these two postsynaptic proteins significantly increased after melatonin treatment (Fig. 2A and B).

3.3. Melatonin reduces the reversal of c-Fos and Arc in the BLA

c-Fos is required for long-term memory consolidation [44]. Our previous study demonstrated that melatonin alleviated hippocampal-dependent memory impairments with associated improvements in hippocampal c-Fos expression [22]. To determine whether melatonin enhances the expression of IEG in the BLA, we assessed the expression of c-Fos, as well as Arc in Tg2576 mice using RT-PCR and Western blotting.

Consistent with our previous studies in the hippocampus, we demonstrated that melatonin supplementation significantly increased the mRNA levels of c-Fos, as well as Arc in the BLA (Fig. 3A and B). Furthermore, we demonstrated that melatonin significantly increased the expression of c-Fos, as well as Arc proteins in the BLA (Fig. 3C and D).

3.4. Melatonin increases the expression of CREB and phosphorylated CREB (pCREB) in the BLA

To investigate whether CREB, the upstream regulatory factor of c-fos, is involved in the role of melatonin in amygdala-dependent emotional memory, we determined the CREB and pCREB levels via Western blotting. As expected, melatonin increased the levels of CREB and pCREB, as well as the pCREB/CREB ratio in the BLA (Fig. 4A and B).

However, we did not identify significant differences in the behavioral experiments (Supplementary Fig. 1) or the synaptic protein (Supplementary Fig. 2) or IEG (Supplementary Fig. 3) expressions between the wild-type (WT) mice with melatonin (Mel) and the vehicle control (DMSO).

4. Discussion

Melatonin is a potent endogenous antioxidant that acts directly as a free radical scavenger or indirectly by inducing the expression of antioxidant defense-related genes. It has been reported that melatonin levels significantly decrease in the cortex of aged mice; moreover, the oral administration of melatonin effectively reverses the age-related decrease in mice [45], and retards the imbalance of the cerebral pro-oxidant status [46]. Our previous study demonstrated that treatment with melatonin arrests tau/β-amyloid pathological progression in the hippocampus and improves the hippocampus-dependent spatial memory of Tg2576 mice [22]. The amygdala and hippocampus perform different roles in the regulation of memory; thus, it is critical to determine whether melatonin also ameliorates the impaired amygdala in AD. Amygdala-dependent emotional memory facilitates hippocampus-dependent spatial memory, and the amygdala modulates stress-induced hippocampal memory deficits [31,47]. Fear conditioning is commonly used to detect emo-
Table 1
Antibodies Employed in the Study.

<table>
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<tr>
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WB, Western blotting.

Fig. 1. Supplementation with melatonin improves amygdala-dependent emotional memory deficits in Tg2576 mice. Tg2576 mice were intraperitoneally injected with Mel or a vehicle control (DMSO) for 4 months. Amygdala-dependent emotional memory was measured using cued fear conditioning and the step-down passive avoidance test. The training phase of the fear conditioning and fear expression was assessed 24 h after fear conditioning (A). Freezing response during training (B); retrieval test of the freezing response (C) at 24 h after training. The mice administered melatonin exhibited substantially longer SDLs (D) and fewer errors (E) in the learning session. In the retention test, the mice administered melatonin exhibited substantially longer SDLs (F) and fewer errors (G). The data in B and C are expressed as the mean ± SEM (two-way ANOVA; n = 8–10). The data in D–G are expressed as the mean ± SEM (one-way ANOVA; n = 8–10). *p < 0.05 versus the Tg-DMSO group. Abbreviations: DMSO, dimethylsulfoxide; Mel, melatonin; Tg, transgenic; SDL, step-down latency; CE, count of errors.

Fig. 2. Melatonin increases synapse-associated proteins in Tg2576 mice in the basolateral amygdala. The mice were sacrificed after the behavioral experiments. Basolateral amygdala extracts were prepared for the measurement of PSD95 and synaptophysin via Western blot (A) and quantitative analysis (B). The synapse-associated protein levels were normalized against GAPDH; the data are expressed as the mean ± SEM (one-way ANOVA; n = 5–6). *p < 0.05 versus the DMSO group. Abbreviations: PSD95, postsynaptic density 95; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
Melatonin reverses the reduction in Arc and c-Fos in Tg2576 mice in the basolateral amygdala. Basolateral amygdala homogenates were prepared as described; RT-PCR (A and B) and Western blotting (C and D) were performed to assess the c-Fos and Arc levels. The c-Fos and Arc levels were normalized against GAPDH, and the data are expressed as the mean ± SEM (one-way ANOVA; n=5–6). *p<0.05 versus the DMSO group.

Melatonin increases CREB and the pCREB/CREB ratio in Tg2576 mice in the basolateral amygdala. Basolateral amygdala extracts were prepared to measure pCREB, CREB and the pCREB/CREB ratio via Western blot (A and B). The pCREB and CREB levels were normalized against GAPDH, and the data are expressed as the mean ± SEM (one-way ANOVA; n=5–6). *p<0.05; *p<0.01 versus the DMSO group. Abbreviations: pCREB, phosphorylated cAMP response element-binding protein; CREB, cAMP response element-binding protein; DMSO, dimethylsulfoxide; Mel, melatonin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Tg, transgenic.

In this study, we demonstrated for the first time that melatonin administration repairs the BLA damage measured using cued fear conditioning and the step-down passive avoidance test. These findings suggest that melatonin improves not only hippocampus-dependent spatial memory but also amygdala-dependent emotional memory in Tg2576 mice.

AD associated memory deficits result from abnormal synaptic plasticity and memory, which may be evaluated by reduced synapse-associated proteins [42]. In the present report, we confirmed that melatonin supplementation prevents the decline of two synaptic proteins, PSD95 and synaptophysin, in the BLA, which is consistent with our previous study in the hippocampus [22].

C-fos has been demonstrated to participate in memory. Various stimuli, including serum, growth factors, tumor promoters and cytokines, induce c-Fos expression. Consistent with our previous study in the hippocampus [22], the current findings indicate that melatonin improves amygdala-dependent emotional memory via the up-regulation of c-Fos. Furthermore, we verified the involvement of pCREB in melatonin induced amygdala amelioration. C-fos is one of the most important downstream target genes of pCREB [14]; thus, we hypothesized that melatonin treatment arrests emotion memory deficits via pCREB/c-Fos pathway up-regulation in the BLA.

In addition to c-Fos, Arc is a crucial protein involved in essential synaptic remodeling and plasticity. Arc transcription depends on one or several downstream signaling kinases, including PKA and ERK, and a wide variety of extracellular signaling components [10]. We will suggest that Arc is involved in melatonin induced emotion memory amelioration through regulators other than pCREB if future evidence indicates that Arc cannot be activated by pCREB.

However, it is unclear how c-Fos and Arc contribute to synaptic plasticity. Accumulating evidence supports the assumption that c-Fos and Arc contribute to synaptic plasticity by indirectly regulating synaptic proteins.

Previous studies have demonstrated that the melatonin MT1 and MT2 receptors mainly mediate the effects of melatonin via activation of the Akt pathway [48,49]. Disrupted schizophrenia (DISC1) is a risk factor for chronic mental diseases, such as AD. The neurotrophin VGF plays the important roles in mental disease. DISC1 regulates the expression of the neurotrophin VGF through the AKT/CREB pathway [50]. Thus, we presumed that the MT1 and MT2 receptor mediated melatonin exerts these effects via AKT/CREB/c-Fos signaling pathway activation in our study. We plan to investigate the mechanism in future studies.

In the present study, we demonstrated that melatonin significantly decreased the emotion memory deficits in Tg2576 mice. Moreover, we subsequently evaluated the expressions of c-Fos, Arc, pCREB and other related genes using RT-PCR and Western blot in
in the BLA. The previously described discrepancies were not identified in the amygdala via immunohistochemistry. It will be interesting to determine the distribution of these molecules in the amygdala in future studies. In summary, the current findings establish that melanin sup-
plementation ameliorates amygdala-dependent memory deficits in TgS76 mice. Furthermore, our findings suggest that the up-
regulation of the pCREB/c-Fos pathway may contribute to the role of melanin in amygdala-dependent memory improvement. Bio-
logical or pharmacological interventions based on melanin may represent a novel, plausible strategy in delaying memory deficits
associated with the pathological progression of AD.

Disclosure statement

The authors have no conflicts of interest to disclose.

The animal experiments were conducted according to the "Poli-
cies on the Use of Animals and Humans in Neuroscience Research" approved by the Society for Neuroscience in 1995 and were super-
visied by the Animal Administration and Ethics Committee of Tongji
Medical College, Huazhong University of Science and Technology.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in
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