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Hippocampal HMGB1/TLR4 Pathway Mediates Cognitive Dysfunction in Chronic Stress Mice*

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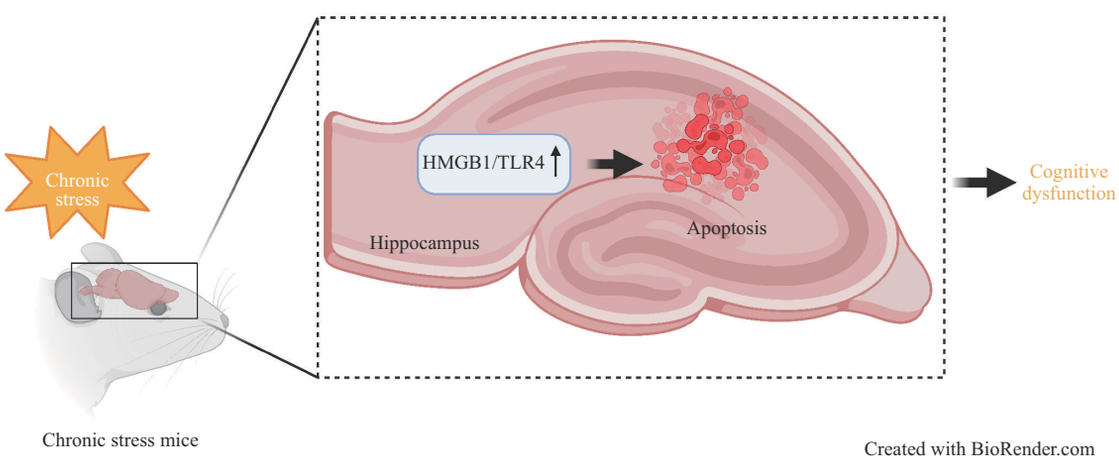
Graphical abstract



Abstract **Objective** Chronic stress can induce cognitive dysfunction, but the underlying mechanisms remain unknown. Studies have confirmed that the high mobility group box 1/Toll-like receptor 4 (HMGB1/TLR4) pathway is closely associated with cognitive impairment. Therefore, this research aimed to explore whether the HMGB1/TLR4 pathway involves in chronic stress-induced cognitive dysfunction. **Methods** The chronic unpredictable mild stress (CUMS) mouse model was established by randomly giving

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different types of stress every day for four consecutive weeks. Cognitive function was detected by novel object recognition test, Y-maze test, and Morris water maze test. The protein expressions of HMGB1, TLR4, B-cell lymphoma 2 (BCL2), and BCL2 associated X (BAX) were determined by Western blot. The damage of neurons in the hippocampal CA1 region was observed by hematoxylin-eosin (HE) staining. **Results** The protein expressions of HMGB1 and TLR4 were significantly increased in the hippocampus of chronic stress mice. Furthermore, inhibition of the HMGB1/TLR4 pathway induced by ethyl pyruvate (EP, a specific inhibitor of HMGB1) and TAK242 (a selective inhibitor of TLR4) treatment attenuated cognitive impairment in chronic stress mice, according to the novel object recognition test, Y-maze test, and Morris water maze test. In addition, administration of EP and TAK242 also mitigated the increase of apoptosis in the hippocampus of chronic stress mice. **Conclusion** These results indicate that the hippocampal HMGB1/TLR4 pathway contributes to chronic stress-induced apoptosis and cognitive dysfunction.

Key words chronic stress, cognitive dysfunction, HMGB1/TLR4 pathway, apoptosis, hippocampus
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The physiological stress response is triggered by fear or a perceived threat. This response leads to the secretion of sympathetic catecholamines and neuroendocrine hormones to promote survival and motivate success[1]. While short-term stress may be adaptive, prolonged or exaggerated stress responses may result in cortisol dysfunction and widespread inflammation[2]. High glucocorticoids (GC) levels due to chronic stress can also impact the function of hippocampus, amygdala[3], and prefrontal cortex[4], leading to cognitive impairment[5]. Chronic stress can cause structural changes in the hippocampus, triggering alterations in hippocampal neurogenesis[6], abnormal neural excitability, and modified neuronal morphology, ultimately leading to neuronal cell death and apoptosis in hippocampal volume[7]. Chronic stress is widely recognized as a major contributor to cognitive impairment and the early onset of dementia[8-9]. Cognitive impairment due to chronic stress imposes a significant burden to society, both directly and indirectly[10]. Therefore, exploring the mechanisms of chronic stress-induced cognitive impairment holds great scientific significance and social value.

Chronic stress has been shown to induce neuroinflammation[11]. Importantly, HMGB1 and TLR4 are key mediators of the pro-inflammatory response[12-13]. Under normal conditions, HMGB1 mainly exists in the cell nucleus and participates in various physiological responses, such as nucleosome stabilization, cell differentiation, DNA repair, and gene transcription[14]. In the event of injury, inflammation, or necrotic cell death, HMGB1 is released into the extracellular space to exert its pro-inflammatory effects[14]. HMGB1 has been shown to activate nuclear factor-κB (NF-κB) *via* TLR4 in inflammatory response, which results in the translocation of p65 subunit of NF-κB into the nucleus and initiates the process of inflammatory cytokine expression and translation[15]. Numerous studies have shown that the HMGB1/TLR4 pathway is significantly upregulated and mediates the pathological process in chronic stress[16-18]. Expression levels of HMGB1 and TLR4 were found to be upregulated in neurodegenerative diseases such as Parkinson's disease[19], Alzheimer's disease[20], and amyotrophic lateral sclerosis[21]. HMGB1/TLR4 pathway also mediates cognitive dysfunction caused by diseases including diabetes[22], Alzheimer's disease[20], and sleep deprivation[23]. Thus, we hypothesized that HMGB1/TLR4 pathway mediates cognitive dysfunction caused by chronic stress. Apoptosis is known to be involved in the pathogenesis of many cognitive dysfunction diseases[24-25]. Interestingly, the HMGB1/TLR4 pathway and apoptosis are also closely linked. For example, the HMGB1/TLR4 pathway promotes hippocampal apoptosis in diabetic patients and apoptosis in human melanoma cells[26]. In addition, inhibition of the HMGB1/TLR4 pathway ameliorated apoptosis induced by myocardial ischemia/reperfusion[27]. Hence, based on these studies, we speculate that the HMGB1/TLR4 pathway also mediates chronic stress-induced apoptosis. The above studies suggest that HMGB1/TLR4 pathway plays an important regulatory role in chronic stress and is intimately associated with cognitive impairment as well as apoptosis. Consequently, we proposed the hypothesis that the hippocampal HMGB1/TLR4 pathway mediates cognitive dysfunction and apoptosis in chronic stress.

Therefore, we aimed to investigate whether the

hippocampal HMGB1/TLR4 pathway mediates cognitive dysfunction and apoptosis in chronic stress mouse model. Our findings revealed that the expressions of HMGB1 and TLR4 were upregulated in the hippocampus of chronic stress-exposed mice, and inhibition of the hippocampal HMGB1/TLR4 pathway induced by ethyl pyruvate (EP, a specific inhibitor of HMGB1) and TAK242 (a selective inhibitor of TLR4) ameliorated cognitive dysfunction in chronic stress-exposed mice. In addition, inhibition of the hippocampal HMGB1/TLR4 pathway attenuated apoptosis in the hippocampus of chronic stress mice. These results suggest that the hippocampal HMGB1/TLR4 pathway mediates the chronic stress-induced apoptosis and cognitive dysfunction. These findings indicate that the HMGB1/TLR4 pathway could provide valuable insights into exploring potential targets for treating cognitive dysfunction and neurodegenerative disorders.

1 Materials and methods

1.1 Animals

All animal protocols in this experiment were approved by the Animal Experimentation Ethics Committee of the University of South China (Permit Number: XYXK20190507) and strictly accorded with the guidelines launched by China Council on Animal Care. Healthy male BALB/c mice, weighing 18−22 g (6−8 weeks of age), were taken from the Hunan SJA Laboratory Animal Center (Changsha, Hunan, China). Mice were housed individually in cages and were given free access to food and water in a specific pathogen-free(SPF) environment. Mice were maintained on a 12-h light/dark cycle (lights on 07: 00−19: 00) and under constant temperature ((23±1)℃) and humidity ((60±5)%). The bedding in the cage was changed once or twice a week to ensure a comfortable environment for the mice. Prior to the experiment, all the mice adapted to the new environment for a week.

1.2 Reagents

TAK242, pentobarbital Na, paraformaldehyde, and Oil Red staining solution were purchased from Sigma (St. Louis, MO, United States). EP was purchased from MedChemExpress (MO, United States). Anti-HMGB1 antibody and anti-TLR4 antibody were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-BAX antibody, anti-BCL2 antibody, and GAPDH antibody were purchased from Proteintech (Proteintech Group Inc., USA). Hematoxylin-eosin (HE) staining solution was purchased from Servicebio (Wuhan, Hubei, China).

1.3 Chronic unpredictable mild stress（CUMS）model and grouping

CUMS is a well-established animal model used to reproduce cognitive deficits in rodents[28]. This model serves as a valuable tool for investigating the underlying mechanisms of stress and unraveling the development of neurodegenerative diseases induced by stress[29-30].

After one week of acclimatization, 40 mice were randomly grouped into control group and CUMS group. Mice in the CUMS groups were randomly exposed to three of nine stressors, including fasting (24 h), water deprivation (12 h), cold water bath (5℃, 5 min), moist bedding (200 ml/100 g, 12 h), tail pinching (1 min), tail suspension (5 min), plantar shock (0.4 mA current intensity, 0.5 s/time, 10 times), day/night reversal (24 h), and stroboscopic illumination (120 times/min, 2 h) for 28 d. The stressors were presented in a randomized order, with a minimum interval of 2 h between two stressors. The same stressor was not repeated consecutively to prevent mice from anticipating the stress onset, and each stressor was administered at least twice. The unpredictability and variability of the stressors in the CUMS model are crucial for the success of the modeling[31].

Subsequently, the mice were randomly divided into four groups: control group, CUMS group, CUMS+EP group, and CUMS+TAK242 group. The mice in the control group were placed in the animal room alone and were given free access to food and water. The control mice were not given any stressors and were intraperitoneally injected with PBS (lysate control, 0.1 ml/d) for 4 consecutive weeks. The mice in the CUMS groups were randomly exposed to 3 of 9 stressors for 4 weeks as described above and intraperitoneally injected with PBS (lysate control, 0.1 ml/d) for 4 consecutive weeks. The mice in CUMS+EP and CUMS+TAK242 group were intraperitoneally injected with EP (50 mg/kg, once daily), or TAK242 (0.3 mg/kg, twice weekly) for four consecutive weeks[32-34], in conjunction with the administration of CUMS treatment. After the end of

drug injection, the mice were subjected to the novel object recognition (NOR) test, Y-maze test, and Morris water maze (MWM) test, with a one-day interval between each experiment. At the end of the MWM test, some mice in each group were extracted from the hippocampus for Western blot (WB) detection, and some mice were perfused to take the brain for HE staining. The flowchart of the experiments was shown in Figure 1.

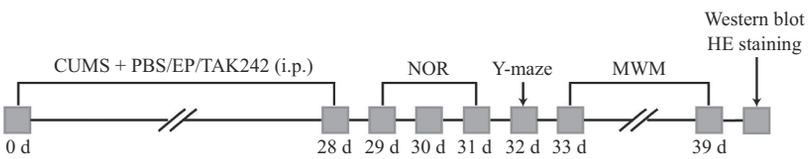

Fig. 1 Experimental procedure

i.p: intraperitoneal; NOR: novel object recognition; MWM: Morris water maze.

1.4 Y−maze test

Y-maze test is used to assess hippocampal-dependent working memory by evaluating the spontaneous alternation behavior[35]. The experimental setup for the labyrinth consists of two parts: one part with three arms of 90 cm×90 cm×70 cm, and the other part with a camera lens unit. The angle between the arms was 120°. Prior to the start of the experiment, the arms were labeled and the camera lens unit was positioned on top of the Y-maze experimental setup to record the mice's movements within the arms.

At the beginning of each trial, the mice were placed at the intersection of the three arms and allowed to freely explore for 5 min. After each trial, the maze is wiped clean using an alcohol spray bottle to eliminate any residual odor that could affect subsequent experimental results. Correct alternation is recognized when the mice sequentially enter three different arms in the following sequences: A-B-C, B-A-C, C-A-B, B-C-A, C-B-A, A-C-B; conversely, two consecutive entries into the same arm, such as A-A-A, C-C-C, B-B-B, A-C-A, A-A-C, A-B-A, C-A-C, B-C-B, B-A-B, C- B-C, A-B-A, A-A-B, A-A-C, B-B-C, B-B-A, C-C, C-B-C, A-B-B, A-C-C, B-A-A, B-C-C, C-A-A, C-B-B, were considered incorrect alternation. The number of correct alternations within 5 min was recorded for each group of mice, and the rate of correct alternations was calculated and statistically analyzed. The rate of correct alternations is determined using the formula: (number of correct alternations/total number of arm entries) ×100%, which was used to determine the spatial discrimination ability of the mice. In addition, the total number of arm entries is measured to represent the activity capacity of the mice.

1.5 Novel object recognition（NOR）test

NOR test, a well-established method for assessing cognitive function in rodent, was conducted with minor modifications. The NOR experimental system setup consisted of a video capture system and two identical opaque black plastic boxes. The experimental procedure was divided into 3 parts: acclimatization, training, and testing. (1) Acclimatization. This phase lasted for 2 d. Each day, the mice were placed in the experimental box from the same location and allowed to freely explore the box for 5 min to familiarize themselves with the new environment. (2) Training. During this phase, two identical objects were placed in the experimental box and the mice were given 5 min to freely explore the objects. (3) Testing. After completing for 1 h, the objects in the training part were randomly replaced with another new object, and then the mice were placed in the training part for 1 h. Another new object will be used to replace the object inside the training part randomly, and then the mice will be put inside the box, so that the mice can explore freely inside the box for 5 min. During the experiment, the experimenter recorded the exploration time of the mice on both the new and old objects in sequential order, calculated the total exploration time of the mice on the new and old objects in the detection part of the experiment, and analyzed the discrimination index, which will be calculated by the formula of the discrimination index: (time to explore the new object−time to explore the old object)/(time to explore the new object+time to explore the old object)×100%. Before each experimental shift to another group of mice, the

experimental box should be wiped with 50% alcohol to remove the odor left by the previous group of mice inside the experimental box, so as not to affect the mice in the back.

1.6 Morris water maze（MWM）test

The MWM test is widely employed to assess spatial learning and memory function. The experimental setup for the MWM comprises three parts: the analysis software system, the image acquisition system, and the water maze experimental setup. The experiment was divided into 3 phases. (1) Acquisitive training phase. In this phase, the pool was divided into four quadrants as per theoretical considerations, and the platform was placed in the center of any quadrant. The mice were placed in the water with their heads facing the wall of the pool, and their swimming length, speed and distance to reach the platform were recorded. If the mice did not find the platform within 120 s, they were guided to the platform and allowed to stay on the platform for 20 s. Following training, the mice were wiped clean and returned to their cages. Mice will be trained 4 times/d, with each time staring from different quadrants and in a different order each day. The interval between training sessions was strictly maintained at 15−20 min, and the training phase for 5−7 d. (2) Exploration phase. The exploration phase was conducted on the second day after the completion of the acquisitive training phase. During this phase, the escape platform was removed and the mice were placed in the water on the opposite side of the quadrant from the original platform. The percentage of time in the observation area (the middle ring of the target quadrant) and the number of times that the mice crossed the platform were recorded. (3) The visual platform test was used to exclude interference with the experimental data caused by visual and motor differences in the animals. At the end of the detection phase, the escape platform was placed on the opposite side of the target quadrant, 2 cm above the water surface. Mice were placed in the pool from the opposite quadrant of the platform. The swimming speed and escape latency were also recorded in the visible platform test.

1.7 Western blot （WB）

The protein expressions of HMGB1, TLR4, BAX, and BCL2 in the hippocampus were detected using WB. Equal amounts of total protein (25 μg) were separated on 10%−12% SDS-PAGE and electro-transferred to PVDF membranes (IPVH00010, Merck Millipore, Birrika, MA, USA). After blocking with Tris buffered saline Tween (TBST, 50 mmol/L Tris-HCL, pH 7.5, 150 mmol/L NaCl, 0.05% Tween-20) containing 5% skim milk for 2 h at room temperature, the PVDF membranes were incubated overnight with the primary antibodies against HMGB1 (1∶1 000 dilution), TLR4 (1∶1 000 dilution), BAX (1∶1 000 dilution), BCL2 (1∶1 000 dilution), and GAPDH (1∶2 000 dilution) on shaker at 4℃. Afterward, the membranes was washed with TBST three times and incubated with a secondary antibody (1∶5 000) diluted in 5% skim milk for 2 h at room temperature, and the PVDF membrane was washed with TBST twice again. Finally, the membranes were visualized under the gel imaging system (Tanon-5600, Shanghai, China). The integrated optical density of the protein band was calculated by Image-J software.

1.8 Hematoxylin−eosin（HE）staining

The pathological changes in brain tissues were assessed using HE staining. Fresh brain samples were fixed overnight in 4% formaldehyde and embedded in paraffin. Then, HE staining was performed according to the instruction manual of HE staining kit. The observations were made under the microscope.

1.9 Statistical analysis

The statistical analyses were analyzed by SPSS 20.0 software, and the data were expressed as mean± SEM. Differences between groups were determined by the least significant difference test (LSD-test) in one-way ANOVA. $P<0.05$ was used to determine the differences between groups.

2 Results

2.1 The HMGB1/TLR4 pathway is enhanced in the hippocampus of mice exposed to chronic stress

To determine the role of HMGB1/TLR4 pathway in cognitive dysfunction during stress, the expressions of HMGB1 and TLR4 in the hippocampus were detected. WB results revealed that the protein expressions of HMGB1 and TLR4 in the hippocampus of chronic stress-exposed mice were significantly upregulated compared with the control mice. Furthermore, both EP (a specific inhibitor of HMGB1) and TAK242 (a selective inhibitor of TLR4) downregulated the protein expressions of HMGB1 and TLR4 in the hippocampus of chronic stress mice

(Figure 2a, b). These results indicated that the HMGB1/TLR4 pathway is promoted in the hippocampus of chronic stress-exposed mice.

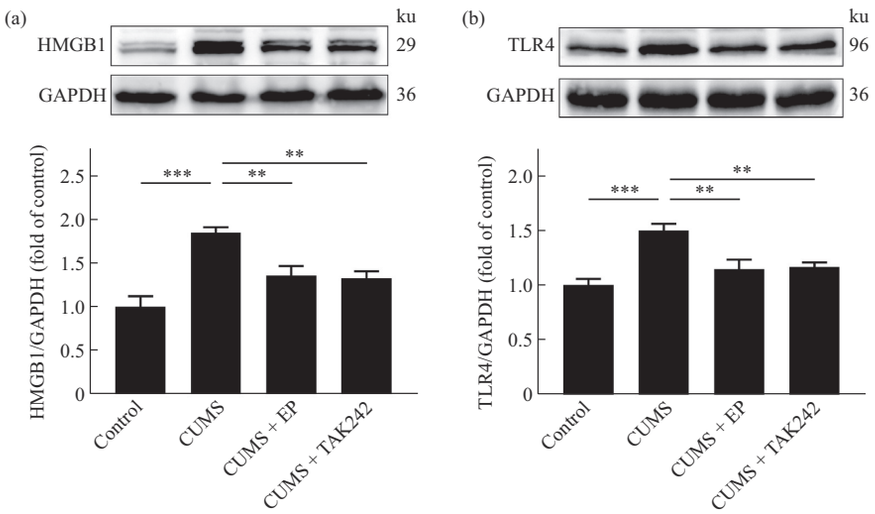

Fig. 2 Effects of CUMS on the protein expressions of HMGB1 and TLR4 in the hippocampus of chronic stress mice

In conjunction with the administration of chronic stress treatment, mice were injected intraperitoneally with PBS (lysate control, 0.1 ml/d), EP (50 mg/kg, once a day), or TAK242 (0.3 mg/kg, twice a week) for 4 consecutive weeks. The protein expressions of HMGB1 (a) and TLR4 (b) in the hippocampus were detected by Western blot. Values are mean±SEM (n=5). $^{**}P$<0.01, $^{***}P$<0.001.

2.2 Inhibition of the HMGB1/TLR4 pathway reverses the decline in spatial working memory of chronic stress−exposed mice in the Y−maze test

In order to preliminarily explore the role of the HMGB1/TLR4 pathway in cognitive dysfunction in chronic stress mice, the effects of EP (a specific inhibitor of HMGB1) or TAK242 (a selective inhibitor of TLR4), which successfully inhibited the HMGB1/TLR4 pathway in the hippocampus of chronic stress-exposed mice, on memory was detected by the Y-maze test (Figure 3a). As shown in Figure 3b, the correct alternation rate in chronic stress mice was significantly lower than that in the control mice, while both EP and TAK242 treatment significantly increased the correct alternation rate of chronic stress mice. The total number of shuttles in the three arms did not differ significantly among the four groups (Figure 3c). These results indicated that the

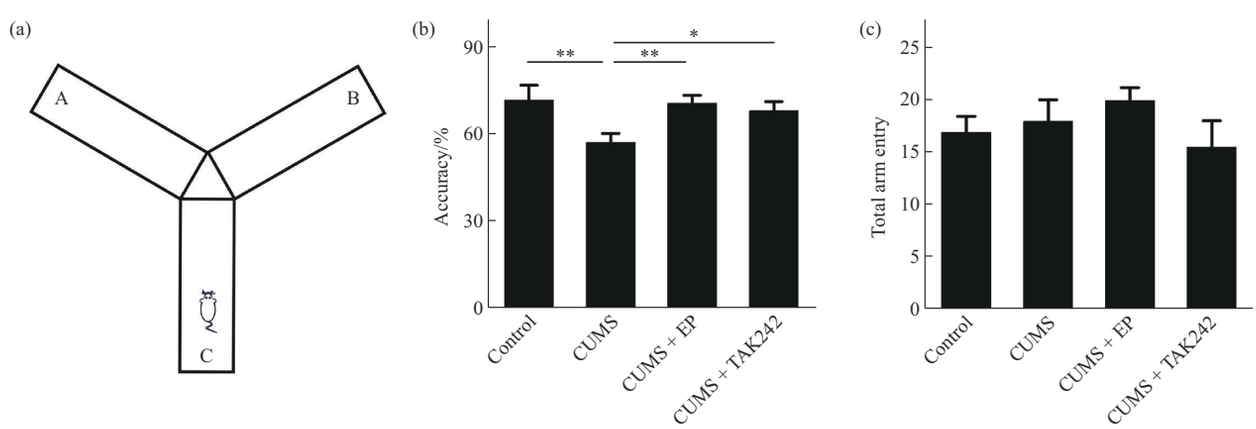

Fig. 3 Effects of EP and TAK242 on the cognitive function of chronic stress−exposed mice in the Y−maze

In conjunction with the administration of chronic stress treatment, mice were injected intraperitoneally with PBS (lysate control, 0.1 ml/d), EP (50 mg/kg, once a day), or TAK242 (0.3 mg/kg, twice a week) for 4 consecutive weeks. (a) The schematic diagram of Y-maze. (b, c) The accuracy (b) and total arms entry (c) were recorded. Values are mean±SEM (n=6−10). $^{*}P$<0.05, $^{**}P$<0.01.

HMGB1/TLR4 pathway contributes to spatial working memory deficient in chronic stress-exposed mice.

2.3 Inhibition of the HMGB1/TLR4 pathway promotes cognitive memory of chronic stress-exposed mice in the NOR test

We then used the NOR experiment to detect the changes in cognitive memory in each group of mice (Figure 4a). As shown in Figure 4b, compared with the control mice, the NOR experiment discrimination index of chronic stress mice was significantly reduced. However, both EP and TAK242 treatment increased the discrimination index of chronic stress mice. Additionally, there was no difference in the total exploration time among the four groups in the training period (Figure 4c) and testing period (Figure 4d). These results indicated that the HMGB1/TLR4 pathway mediates cognitive dysfunction of chronic stress-exposed mice.

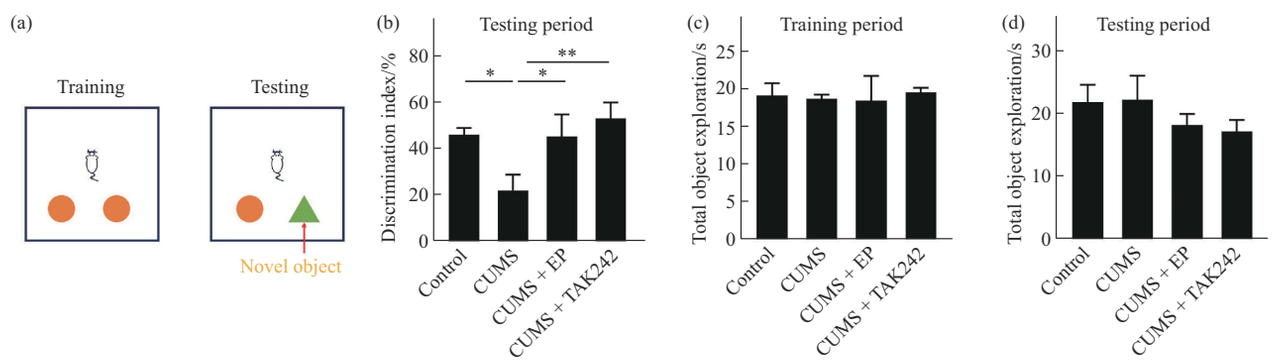

Fig. 4 Effects of EP and TAK242 on the cognitive function of chronic stress-exposed mice in the NOR test

In conjunction with the administration of chronic stress treatment, mice were injected intraperitoneally for four consecutive weeks with PBS (lysate control, 0.1 ml/d), EP (50 mg/kg, once a day), or TAK242 (0.3 mg/kg, twice a week). (a) The schematic diagram of novel object recognition test. (b–d) The discrimination index (b) and the total time spent exploring the object in the training period (c) and testing period (d) were recorded. Values are mean±SEM (n=7–10). $^{*}P$<0.05, $^{**}P$<0.01.

2.4 Inhibition of the HMGB1/TLR4 pathway improves the impaired spatial learning and memory ability of chronic stress-exposed mice in the MWM test

To further evaluate the effect of the HMGB1/TLR4 pathway on cognitive dysfunction in chronic stress-exposed mice, we finally used the MWM test to assess the learning memory ability of each group of mice (Figure 5a). Following 5 d of acquired training, the latency to find the platform tended to decrease from day 1 to day 5 in all groups of mice, and the latency to find the platform in the chronic stress-exposed mice was significantly longer compared with the control mice, and treatment of EP and TAK242 significantly shortened the periods to find the platform in chronic stress-exposed mice (Figure 5b–d). During the test period, the percentage of time spent swimming in the observation area and the number of times crossing the original escape platform were significantly decreased in the chronic stress-exposed mice compared with the control mice, and the treatment of EP and TAK242 markedly increased the percentage of time spent swimming in the observation area and the number of crossings of the original escape platform in the chronic stress mice (Figure 5e, f). There were no significant differences in escape latency (Figure 5g) and average speed (Figure 5h) during the visible platform period between groups of mice, which indicated that there was no difference in the visual acuity and speed between the groups of mice. Together, these data demonstrated that the HMGB1/TLR4 pathway mediates cognitive dysfunction in mice exposed to chronic stress.

2.5 Inhibition of the HMGB1/TLR4 pathway reduces the neuronal damage of hippocampal CA1 region in chronic stress-exposed mice

We then investigated the preliminary mechanisms by which HMGB1/TLR4 pathway mediates cognitive dysfunction in chronic stress-exposed mice. Studies have shown that the damage of

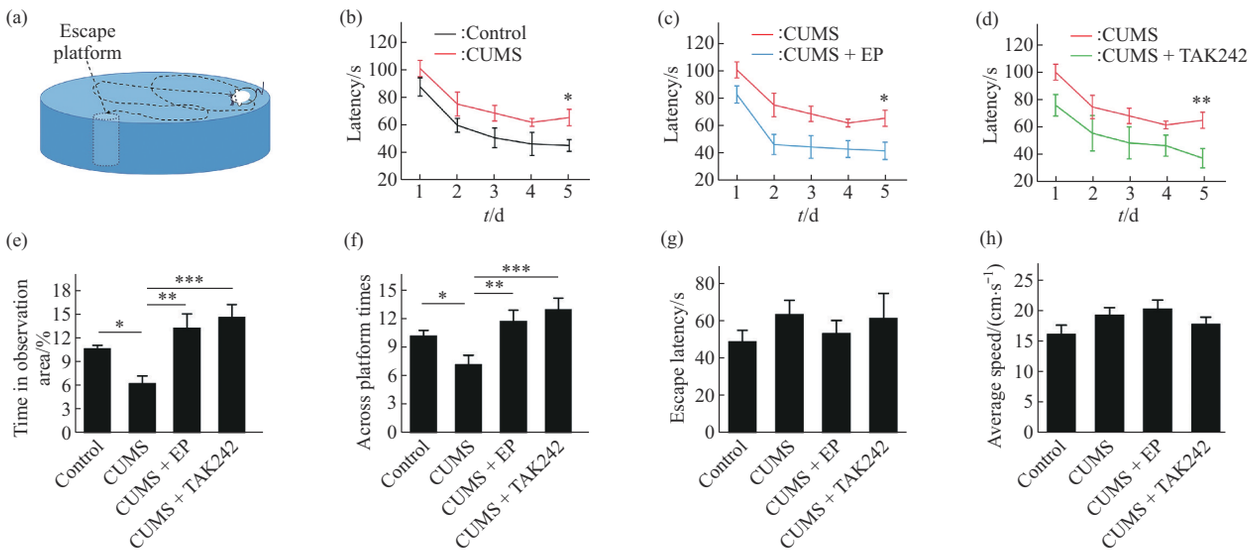

Fig. 5　Effects of EP and TAK242 on the cognitive function of chronic stress−exposed mice in the MWM test

In conjunction with the administration of chronic stress treatment, mice were injected intraperitoneally with PBS (lysate control, 0.1 ml/d), EP (50 mg/kg, once a day), or TAK242 (0.3 mg/kg, twice a week) for four consecutive weeks. (a) The schematic diagram of Morris water maze. (b−d) The periods to find the hidden platform during 5 d in the acquisition trial of MWM test were recorded. (e, f) The percentage of time in the observation area (the middle ring of the target quadrant) (e) and the number of times that the mice crossed the platform (f) in the probe phase of MWM test were recorded. (g, h) The escape latency to locate the platform (g) and the average speed of mice (h) in the visible platform trail were recorded. Values are mean± SEM (n=6−9). $^{*}P$<0.05, $^{**}P$<0.01, $^{***}P$<0.001.

neurons in the CA1 area of the hippocampus is closely related to cognitive function[36-39]. Therefore, we observed the number of neuronal cells in the CA1 area of mice in each group by HE staining. The number of neuronal cells in the CA1 area of chronic stress-exposed mice was significantly reduced compared with the control mice, and intraperitoneal injection of EP or TAK242 abolished this change (Figure 6a, b). These data indicated that the HMGB1/TLR4 pathway mediates neuronal cell injury in the CA1 area of chronic stress-exposed mice.

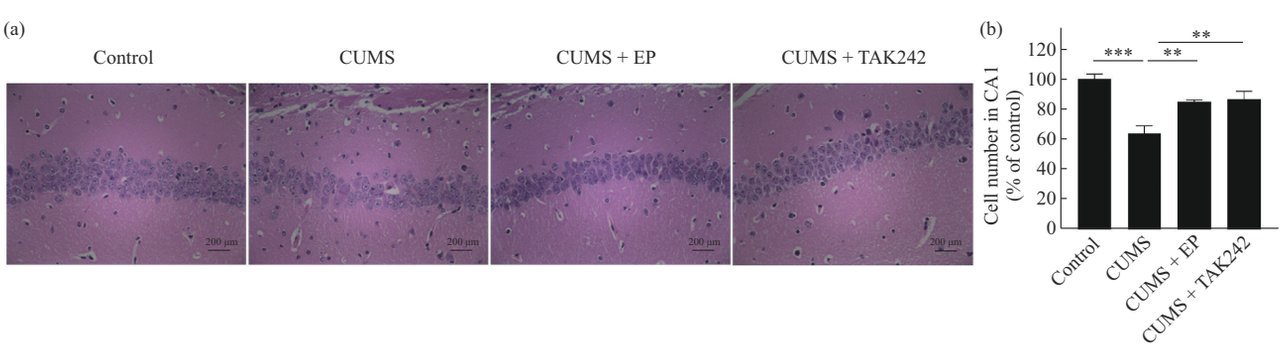

Fig. 6　Effects of EP and TAK242 on the neurons of hippocampal CA1 region in chronic stress−exposed mice

In conjunction with the administration of chronic stress treatment, mice were injected intraperitoneally with PBS (lysate control, 0.1 ml/d), EP (50 mg/kg, once a day), or TAK242 (0.3 mg/kg, twice a week) for four consecutive weeks. (a) Representative image of hematoxylin-eosin (HE)-stained sections from a mouse hippocampus in the CA1 region. (b) Comparison of the number of neuronal cells in the CA1 region of mice in each group. Values are mean±SEM (n=3). $^{**}P$<0.01, $^{***}P$<0.001.

2.6 Inhibition of the HMGB1/TLR4 pathway reduces the protein expressions of apoptosis-related proteins in the hippocampus of chronic stress-exposed mice

Apoptosis is considered as a common mode of programmed cell death affecting neuronal injury[40-41], we then further examined the protein expression levels of hippocampal apoptosis-related proteins BAX and BCL2 in all groups of mice. The expression of the pro-apoptosis protein BAX protein was upregulated and the expression of the apoptosis-inhibiting protein BCL2 was down-regulated in the hippocampus of chronic stress-exposed mice compared with the control mice, whereas treatment of the EP or TAK242 blocked these alterations (Figure 7a, b). Together, these results further indicated that the hippocampal HMGB1/TLR4 pathway mediates apoptosis in chronic stress-exposed mice.

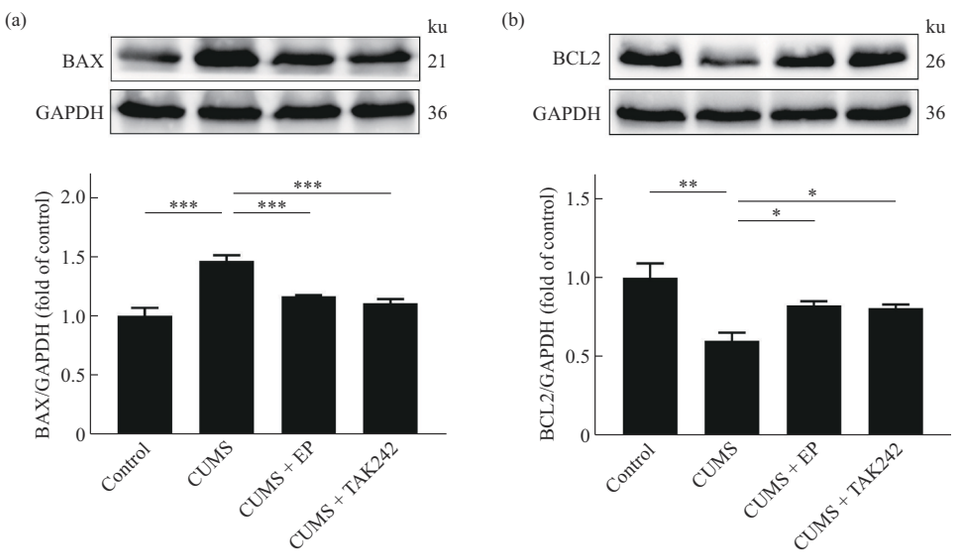

Fig. 7 Effects of EP and TAK242 on the expressions of apoptosis-related proteins in chronic stress-exposed mice

In conjunction with the administration of chronic stress treatment to mice, mice were injected intraperitoneally with PBS (lysate control, 0.1 ml/d), EP (50 mg/kg, once a day), or TAK242 (0.3 mg/kg, twice a week) for four consecutive weeks. The protein expressions of BAX (a) and BCL2 (b) in the hippocampus were detected by Western blot. Values are mean±SEM (n=5). $^{*}P$<0.05, $^{**}P$<0.01, $^{***}P$<0.001.

3 Discussion

The present study aimed to determine the role of hippocampal HMGB1/TLR4 pathway in chronic stress-induced cognitive dysfunction. Our results found that the HMGB1/TLR4 pathway was enhanced in the hippocampus of chronic stress-exposed mice. Inhibition of hippocampal HMGB1/TLR4 pathway induced by intraperitoneal injection of EP and TAK242 ameliorates cognitive dysfunction and reduces hippocampal apoptosis in chronic stress-exposed mice. These findings suggested that the hippocampal HMGB1/TLR4 pathway mediates cognitive impairment and apoptosis induced by chronic stress.

Prolonged stress can disrupt the body's internal balance and cause various physical and psychological disorders[42]. Stress can also trigger cognitive dysfunction and the onset and development of neurodegenerative diseases such as Alzheimer's disease particularly in vulnerable individuals[43-44]. Cognitive dysfunction due to stress is a growing concern, so it is important to elucidate the mechanisms of stress-induced cognitive impairment. Numerous studies have shown that HMGB1 can impact cognitive function by regulating TLR4, and an increase in systemic HMGB1 levels has been linked to cognitive dysfunction[45], indicating that the HMGB1/TLR4 pathway may be involved in cognitive dysfunction during stress. First, our research found

that the expressions of HMGB1 and TLR4 were upregulated in the hippocampus of chronic stress-exposed mice, which is consistent with previous studies[45-46]. Gu *et al*.[34] found that EP and TAK242 ameliorate inflammation in chronic stress mice by downregulating the expressions of HMGB1/TLR4 pathway-related proteins. Furthermore, we found that inhibition of the hippocampal HMGB1/TLR4 pathway ameliorates cognitive dysfunction in chronic stress-exposed mice. These data suggest that the hippocampal HMGB1/TLR4 pathway mediates chronic stress-induced cognitive impairment. A large number of studies have demonstrated the regulatory role of the HMGB1/TLR4 pathway in stress-induced depression[46-49]. Furthermore, a study finds that targeting HMGB1/TLR4 pathways mediates cognitive dysfunction in Alzheimer's disease[20]. Similarly, the HMGB1/TLR4 pathway has been found to mediate cognitive impairment caused by sleep deprivation[23] and diabetes[22]. Combining the above findings with our results strongly suggests that the HMGB1/TLR4 pathway mediates chronic stress-induced cognitive dysfunction.

Recently, it has been found that chronic stress can induce apoptosis, which is closely related to cognitive impairment. There is no doubt that we next explore the relationship between pathways and apoptosis in chronic stress. Therefore, we examined the neuronal cell damage and the expression of apoptosis-related proteins in the CA1 region of the hippocampus of chronic stress mice. Our research shows that the HMGB1/TLR4 pathway mediates chronic stress-induced apoptosis. Recent studies have shown that inhibiting the HMGB1/TLR4/NF-κB signaling pathway is a key mechanism for inhibiting neuronal apoptosis and inflammatory cytokine release[50]. After extracellular HMGB1 binds to the receptor TLR4, the receptor activates NF-κB transcription factors, which are required for the expression of some important inflammatory mediators, such as IL-6, IL-1β, and TNF-α, and overexpression of HMGB1 has pro-apoptotic effects[51]. Current research has reported that dozens of genes regulate the process of apoptosis. Among them, BCL2 is a protein that can inhibit the expression of specific genes and BAX can promote apoptosis. *BCL2* and *BAX* are two pairs of genes with opposing functional roles, and the balance of their expression determines whether the relevant cells survive or die[52]. As shown, the expression of BAX protein was upregulated and the expression of BCL2 protein was down-regulated in the hippocampus of chronic stress mice. This was reversed with the use of EP, a specific inhibitor of HMGB1, and TAK242, a selective inhibitor of TLR4. Furthermore, inhibition of the HMGB1/TLR4 pathway mediates the protective effect of Panaxynol on myocardial ischemia-reperfusion-induced apoptosis[53]. Alpha-lipoic acid reduces myocardial ischemia-reperfusion injury and apoptosis by downregulating HMGB1 expression[54]. Similar studies have shown HMGB1/TLR4 pathway that mediates diabetes-induced apoptosis[17]. All of these reinforce our point that the HMGB1/TLR4 pathway mediates apoptosis in cognitively impaired chronic stress mice.

However, there are certain limitations to our experiments. We did not use any HMGB1/TLR4 pathway agonists in our experiments to further validate our conclusion. There has been no verification of the conclusion in cellular models and transgenic mice. Future studies are required to determine whether the HMGB1/TLR4 pathway promotes hippocampal apoptosis to mediate chronic stress-induced cognitive impairment. Furthermore, the upstream mechanism of the HMGB1/TLR4 pathway is unclear. Interestingly, lactate levels have recently been found to be elevated in chronic stress rats[55] and lactylation influenced by lactate could regulate protein function and stability[56]. Therefore, we will next investigate whether chronic stress enhances the HMGB1/TLR4 pathway by promoting lactylation.

4 Conclusion

In conclusion, we demonstrated that the HMGB1/TLR4 pathway mediates cognitive impairment in chronic stress mice, partly through inhibiting hippocampal apoptosis. Our findings not only contribute to the understanding of the role of the HMGB1/TLR4 pathway in central function, but also provide new targets and ideas for ameliorating stress-induced cognitive dysfunction.

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海马HMGB1/TLR4通路介导慢性应激小鼠的认知功能障碍*

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摘要 目的 慢性应激可引起认知功能障碍，但其机制尚不清楚。研究已经证实，HMGB1/TLR4通路与认知障碍密切相关。本研究旨在探讨HMGB1/TLR4通路是否参与慢性应激诱导的认知功能障碍。**方法** 采用慢性不可预知应激（CUMS）小鼠模型，连续4周每天随机给予不同类型的应激。采用新物体识别实验、Y迷宫实验和Morris水迷宫实验检测认知功能。蛋白质印迹法（Western blot）检测HMGB1、TLR4、BCL2和BAX蛋白的表达。苏木精-伊红（HE）染色观察海马CA1区神经元损伤。**结果** 暴露于CUMS的小鼠海马HMGB1和TLR4蛋白表达明显升高。此外，根据新物体识别实验、Y迷宫和Morris水迷宫实验，丙酮酸乙酯（EP，HMGB1的特异性抑制剂）和TAK242（TLR4的选择性抑制剂）抑制HMGB1/TLR4通路可减轻慢性应激小鼠的认知障碍。此外，注射EP和TAK242还能缓解慢性应激小鼠海马细胞凋亡的增加。**结论** 海马HMGB1/TLR4通路参与了慢性应激诱导的细胞凋亡和认知功能障碍。

关键词 慢性应激，认知功能障碍，HMGB1/TLR4通路，细胞凋亡，海马

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