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ORIGINAL

EXPLORING THE EFFICACY OF ENRICHED ENVIRONMENTAL TRAINING IN MITIGATING DEPRESSION SYMPTOMS AND PROTECTING HIPPOCAMPAL NEURONS IN CHRONIC UNPREDICTABLE MILD STRESS MODELS: INTEGRATING PHYSICAL FITNESS AND MENTAL HEALTH STRATEGIES

Yiqing Zhang[1], Yangyang Zhang[1], Xiaojun Zhu[1], Qian Ye[1], Zhaodan Gan[1], Guangxu Xu[1,*]

[1] Rehabilitation Medicine Center, the First Affiliated Hospital of Nanjing Medical University, Nanjing 210000, Jiangsu, China.

E-mail: xuguangxu@njmu.edu.cn

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ABSTRACT

Background: Hippocampal neuron damage and impaired regeneration are critical in depression's pathogenesis. An Enriched Environment (EE) has been shown to ameliorate depressive-like behaviors in animals, suggesting its potential as a daily rehabilitation analog for reducing depression incidence in chronic stress conditions through hippocampal neuron protection. This study aims to investigate the effects and underlying mechanisms of EE training on depression induced by chronic unpredictable mild stress (CUMS) in mice. **Method:** Forty mice were randomized into four groups: Control, CUMS, Fluoxetine + CUMS, and EE + CUMS. Depression was induced using single-cage isolation and CUMS for 42 days, with intraperitoneal drug administration during this period. EE training commenced on Day 22, continuing through the modeling phase. Depression behavior was assessed using weight monitoring, the Sucrose Preference Test (SPT), Open Field Test (OFT), and Forced Swimming Test (FST). Hematoxylin-Eosin (HE) staining evaluated hippocampal histopathology. Levels of interleukin 1β (IL-1β), interleukin 6 (IL- 6), phosphorylated phosphatidylinositol kinase (p-PI3K), phosphorylated protein kinase B (p-Akt), and phosphorylated mammalian target of rapamycin protein

(p-mTOR) were analyzed. **Results:** By Day 21, chronic stress elicited depressive behaviors, with more pronounced effects by Day 42 in the CUMS group, validating the model's stability and success. Interventions post-Day 22, Resembling early clinical depression detection, demonstrated that three weeks of fluoxetine and EE training effectively reduced depressive behaviors. Both interventions notably mitigated hippocampal histopathological damage, decreased inflammatory factors, and significantly increased expression of mTOR pathway-related proteins. **Conclusion:** EE training effectively mitigates depressive behavior in chronically stressed mice, primarily through anti-inflammatory actions and modulation of the PI3K/Akt/mTOR signaling pathway. These findings underscore the therapeutic potential of integrating physical fitness and mental health strategies into depression management, emphasizing the importance of environmental and lifestyle factors in neuropsychiatric disorder intervention.

KEYWORDS: EE training; rehabilitation; chronic stress; depression; hippocampus; PI3K/Akt/mTOR pathway; inflammation

1. INTRODUCTION

Depression, a prevalent neuropsychiatric disorder, significantly impacts global health by impairing individuals' emotional well-being, cognitive function, and daily activities. Traditional understanding of its etiology points to a complex interplay of genetic, biochemical, and environmental factors. Among these, the role of stress, particularly chronic unpredictable mild stress (CUMS), has been extensively studied for its capacity to induce depressive- like behaviors in animal models, mimicking the human condition's multifaceted nature (Predictable, Laurencic, & Malone, 2006). The hippocampus, a critical brain region involved in mood regulation and cognitive function, is notably vulnerable to the deleterious effects of chronic stress. Evidence suggests that damage to hippocampal neurons and subsequent impairments in their regeneration are pivotal factors in the onset and progression of depression (Rotenstein et al., 2016). Thus, interventions aimed at protecting these neurons from stress-induced damage or promoting their recovery are of considerable interest in developing effective treatments for depression (Pandarakalam, 2018). In recent years, the concept of an enriched environment (EE) has emerged as a promising approach in this context (Hebb, 1947). An EE is characterized by enhanced sensory, cognitive, and physical stimulation compared to standard laboratory conditions, offering a multifaceted intervention that simulates aspects of daily rehabilitation and physical fitness. Studies have shown that keeping animals in an EE can lead to improvements in cognitive function, neurogenesis, and emotional behaviors, suggesting potential therapeutic benefits for depression. However, the specific mechanisms through which EE training affects depressive behavior and hippocampal neuron integrity, especially under chronic stress conditions, remain to be fully elucidated

(Puthran, Zhang, Tam, & Ho, 2016; Wassie, Azagew, & Bifftu, 2022). Furthermore, the integration of physical fitness and mental health strategies within EE interventions presents a novel approach to depression treatment. Physical activity (Barch et al., 2019) as part of an EE, has been shown to confer numerous benefits on brain health (Averill et al., 2022), including the enhancement of neuroplasticity, reduction of inflammation, and activation of neuroprotective pathways. These effects may synergistically contribute to the amelioration of depressive symptoms and the protection of hippocampal neurons against the adverse effects of chronic stress (Gautam, Tolahunase, Kumar, & Dada, 2019). This study aims to investigate the effects of EE training on depression- like behaviors in mice subjected to CUMS and to explore the underlying mechanisms of hippocampal neuron protection (Gautam et al., 2019). By examining the impact of EE training on inflammatory markers and the PI3K/Akt/mTOR signaling pathway, we seek to provide a comprehensive understanding of how environmental and lifestyle modifications can influence the pathophysiology of depression (Livingston-Thomas et al., 2016). Through this research, we aim to contribute to the development of holistic and integrative treatment strategies that encompass both environmental enrichment and physical fitness as essential components of mental health care (Cho & Kang, 2020; Macartney, Lagisz, & Nakagawa, 2022; Balokhonov, Romanova, & Zemlianov, 2021).

2. Materials and Methods

2.1 Animals

Nanjing Medical University's Experimental Animal Breeding Center provided healthy 6- to 8-week-old male ICR mice, SPF grade, weighing 20.0–25.6 g [Experimental Animal License: SCXK (Su) 2018-0026]. To acclimate the mice to the experimental setting, they were kept under the following conditions for seven days: temperature (22±2) °C, humidity 40%-70%, noise ≤60 dB, 12 h/12 h circadian rhythm, normal diet and water. The Experimental Animal Ethics Committee at Nanjing Medical University authorized the project (IACUC-2009009).

2.2 Materials and Instruments

Fluoxetine (Sigma, USA); mouse serum interleukin 1β and IL-6 (Neobioscience Biotechnology Co., Ltd.); rabbit anti-mouse phosphorylated PI3K (p-PI3K), PI3K, phosphorylated Akt (p-Akt), Akt, phosphorylated mTOR (p-mTOR) and secondary antibody (Shanghai Biyuntian Biotechnology Co., Ltd.); open field test and forced swimming experiment apparatus (Shanghai Xinruan Technology Information Co., Ltd.); HM525 frozen section machine (Thermo Fisher, USA); CKX53 optical microscope (Olympus, Japan); Tanon automatic chemiluminescence imaging system (Shanghai Tanant Technology

Co., Ltd.), high-speed tissue grinder (Tennant Ltd.), Vmax microplate spectrophotometer (MDC, USA), 96-well flat-bottom microplates, and low-temperature ultracentrifuge (Eppendorf, Germany); 37 °C thermostatic water bath (Grant, UK); and automatic thermostatic enzyme marker (Biotek, USA).

2.3 Animal grouping

The normal control group, the CUMS model group, the fluoxetine + CUMS group, and the EE training +CUMS group, each received 10 mice, for a total of 40 mice. The normal control group underwent 6 weeks of CUMS modeling; starting in the fourth week of modeling, the CUMS + fluoxetine group received an intraperitoneal injection of fluoxetine (10mg/kg), and the CUMS+EE training group received an intraperitoneal injection of saline and EE training for 3 weeks.

2.4 CUMS modeling

According to the literature (Zhou et al., 2020), a depression rat model was prepared using solitary feeding combined with chronic unpredictable mild stimulation (CUMS). Nine stimuli were included ①24 hours of dehydration and starvation; ②24 hours of tilting the rat cage at 45 °; ③reversing day and night; ④moist cage with 200 ml of water and 100 g of sawdust for 24 hours; ⑤ 5 minutes of swimming in water between 4 °C and 8 °C; ⑥ thermal environment at 38 °C-39 °C for 20 min; ⑦ exposure to special odor (air freshener) for 24 h; ⑧ behavioral restriction for 20 min; and ⑨ tail clamping for 1 min. Solitary rearing means single-cage rearing. A CUMS was randomly performed each day to stimulate the animals, and the same stimulus did not occur continuously so that the animals could not predict the stimulus. Solitary rearing combined with the CUMS method was performed for 6 weeks.

2.5 EE training

To simulate clinical EE training (Meijer, Sommer, Spruijt, Van Zutphen, & Baumans, 2007), we independently modified the EE cage reported in the previous literature (Prange et al., 2015): 1. expanding the volume of the EE cage: use a cage with a length × width × height of 60 cm x 35 cm x 47 cm, divided into three platforms, connected by colored jumping boards and climbing ladders; 2. increasing the audio-visual and tactile devices: place a dome hut and a fun magic cube on the third platform, place swings decorated with bells, etc. on the second platform, and install a music player on the bottom platform ; 3. enhancement of independent movement: an exploration maze is placed on the second platform, independent running wheels are installed on the bottom platform by the cage, and a seesaw, a slide with the image of a small elephant-shaped slide, drill tubes and climbing ladders are placed on the bottom platform padding. The toys are cleaned and replaced twice a week and repositioned, the

maze changes every three days, and Mozart K448 "Sonata in D major for two pianos" is played on the music player for 1 hour in a soothing and relaxing volume loop The design of the EE training device based on this cage was granted a national utility model patent (Patent No. ZL 2020 2 3338069.5). Two identical EE cages were made, and the EE training group of 5 mice per cage for 4 hours per day (9:00-11:00,14-16:00), 5 days per week, was simulated for clinical rehabilitation training. (See Figure 1A)

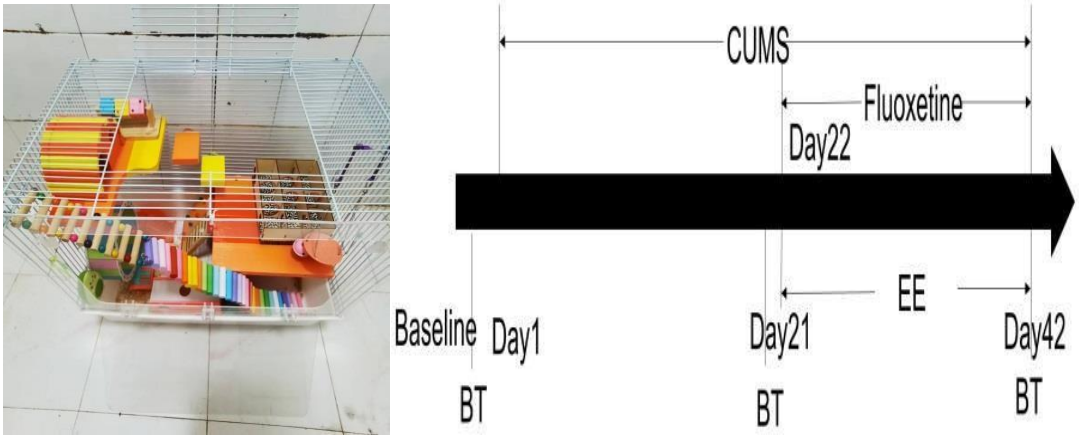


Figure 1: EE training apparatus and experimental procedure. A. Autonomously modified EE cage; B. Experimental procedure. CUMS: chronic unpredictable mild stress process from Day 1 to Day 42; BT: relevant behavioral tests before modeling, on Day 21, and from Day 42; fluoxetine + CUMS group: animals also received fluoxetine at a dose of 10 mg/kg from Day 22 to 42； EE+CUMS group: animals also received EE training intervention lasting from Day 22 to 42.

2.6 Behavioral detection methods (See Figure 1B)

2.6.1 Body mass measurement

Body mass was measured (Schmidt et al., 2010); the experimental animals were weighed on Day 1 before the modeling, at Day 21, and at Day 42.

2.6.2 SPT

Forty-eight hours before the experiment, acclimatization to 5% sucrose water was performed, and after twenty-four hours of fasting and dehydration, the sugar water/pure water consumption test was performed at 8:00-9:00 a.m. One bottle of water with 1% sucrose and one bottle of water with no added sugar were given at the same time, the locations of the bottles of water were exchanged at 30 minutes, and the remaining amount was measured after 1 h of drinking (Krishna, Dodd, & Filipov, 2016; Moore, Beidler, & Hong, 2018).

The total fluid consumption, sugar water consumption and sugar water preference percentage were calculated for the animals. The ratio of sugar water

consumed to the total amount of fluid consumed multiplied by 100 indicates a preference for sugar water. Sugar water preference experiments were performed on Day 1 before modeling and on Day 21, and Day 42.

2.6.3 OFT

The mice were kept in the middle square positioned at the base of the open container (size: 50cm×50cm×40cm) for 5 minutes, and the inside of the open box was split into 36 identical squares. Then, the activities of the mice were recorded over 5 min (Mello et al., 2013). The number of horizontal crossings was the number of mice crossing the bottom block with three paws;

The number of uprights corresponded to the number of times the two forelimbs left the ground upright. After completion of the experiment, the mice were removed, and the equipment was cleaned by spraying a 10% ethanol solution in a wooden box to eliminate the effect of their movement marks on later tests. Absence field experiments were tested on the 43rd day of modeling.

2.6.4 FST

Individual mice were placed in a glass tank with a 10 cm diameter and 30 cm depth at the temperature of the water being 25±2 °C for 6 minutes, and the entire amount of time spent unmoving of swimming was recorded for the next 4 minutes. On day 43 of the modeling process, the experiment involving being forced to swim was carried out (McHugh, Sugarman, Meyer, Fitzmaurice, & Greenfield, 2020).

2.7 Histopathological examination of the hippocampus

After the behavioral index test, blood stored in a low-temperature refrigerator was taken from the intraocular canthus vein of mice to prepare serum. After the mice killed, the brain was severed and placed upon ice, and hippocampal tissues were separated; one part was kept in a refrigerator at -80 °C, and the other part was fixed, gradient dehydrated, embedded and sectioned, and HE staining was performed by conventional methods.

2.8 Inflammation Index Detection

The supernatant was filtered after 15 minutes of centrifugation at 4 °C. Serum and hippocampus tissues were added according to the directions of the IL-1 and IL-6 kits to the appropriate reagents in the IL-1β and IL-6 kits respectively, 450 nm was chosen as the measurement point for optical density values, and enzyme standardization equipment was used for the measurement. Using the standard curve, IL-1β, IL-6, and other inflammatory response markers were computed.

2.9 Hippocampal Protein Expression Assay

In order to detect alterations in PI3K/Akt/mTORC1 signaling pathway-related proteins in hippocampus tissues, immunoblotting was utilized. Protein was extracted, electrophoretic ally separated, transferred to a membrane, exposed primary antibodies overnight at 4 °C with 2 hours of with 3% skim milk incubation, rinsed with TBST, one hour of incubation was carried out at room temperature with secondary antibodies, washed with TBST, and developed by ECL exposure. Image analysis using Image was done. The protein concentration was measured based on the gray ratio between the target protein and the GAPDH internal standard.

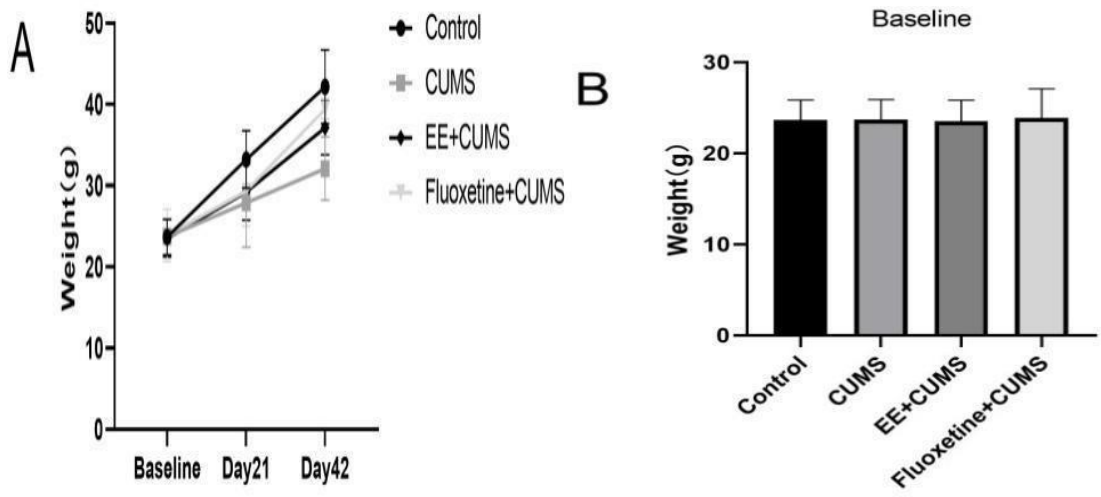
2.10 Data Processing

Graph Pad Prism 8.0 was utilized to conduct empirical analyses on all of the data after it was first summarized using the mean and standard deviation. The sample means of each group were put side by side using a one-way ANOVA, and a significance threshold of 0.05 was utilized to find out whether or not there was a meaningfully significant difference between the groups.

3. Results

3.1 Effect of EE training on body weight.

Before the onset of modeling, there was not a discernible change in body weight among the groups (P>0.05). On the 21st day of CUMS, the body mass of the remaining three groups dropped relative to the control group (P<0.05), but there were no major dissimilarities between the groups (P>0.05). On the 42nd day of modeling, the body mass of mice in the CUMS group decreased by a large margin than the typical control group (P<0.01). When contrasted with the CUMS group, mice in the CUMS + fluoxetine and CUMS+EE training groups gained substantially more weight (P <0.01) (Figure 2).



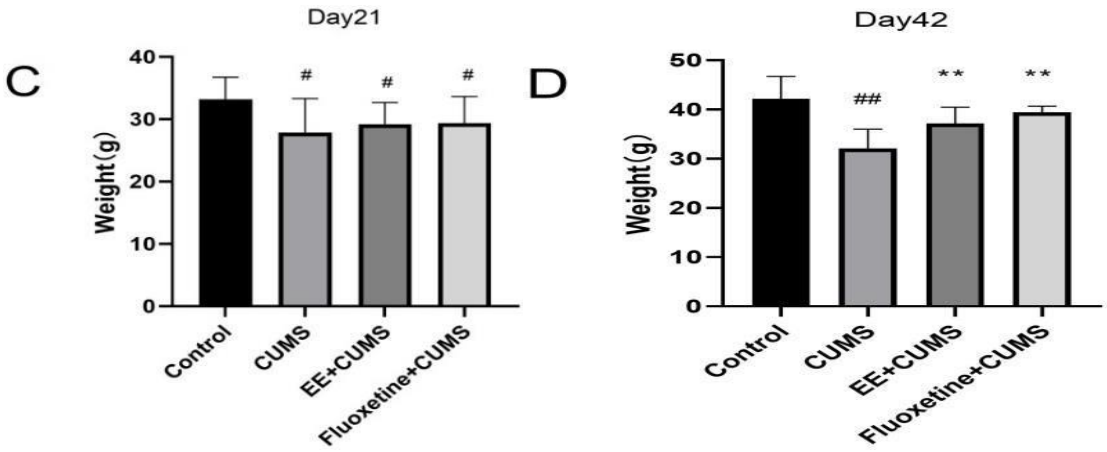
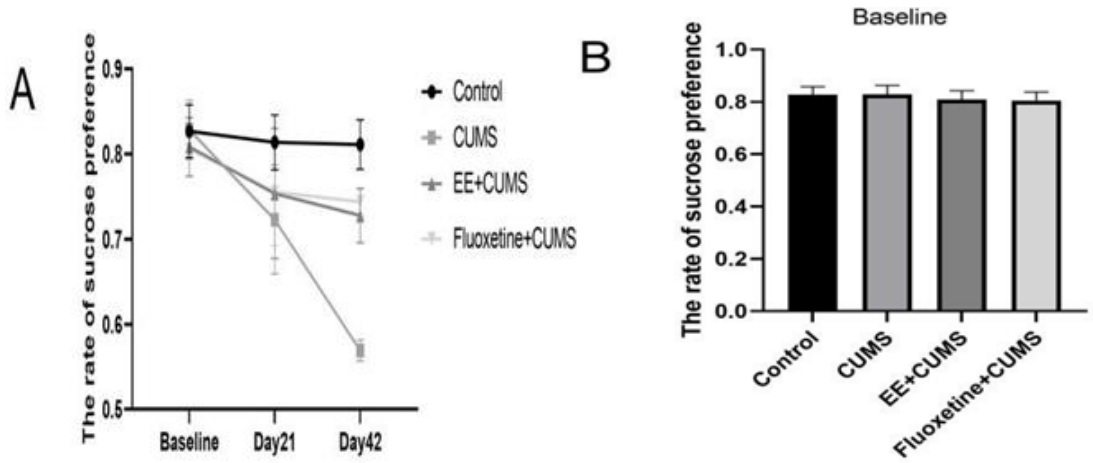


Figure 2: Effect of EE training on body weight: body mass assay values are communicated as the mean ± SD (n=10/group). A shows the change in body weight of each group from before modeling to Day 42 of modeling. B, C, D show the significant differences in body weight of the control group, CUMS group, fluoxetine+ CUMS group and EE+CUMS group before modeling, Day 21 of modeling, and Day 42 of modeling respectively. Significant differences are indicated by "*P" "*P<0.05", "**P<0.01" in contrast to the CUMS group; Significant deviations which can be seen when compared to the group serving as the control are expressed as "#P"," #P<0.05", "##P <0.01".

3.2 Effect of EE training on the SPT

Prior to modeling, the findings of this investigation indicated that there were no significant distinctions in sugar water preference across the groups (P>0.05). On the 21st day of CUMS, the sugar water preference of the remaining three groups declined relative to the group serving as a control (P<0.05), but there were no major dissimilarities across groups (P>0.05). On the 42nd day of modeling, the sugar water preference of mice within the CUMS group was considerably lower than that of the control group (P<0.01), while the sugar water preference of mice in the CUMS + fluoxetine and EE training groups was considerably higher than those of the CUMS group (P<0.01) (Figure 3).



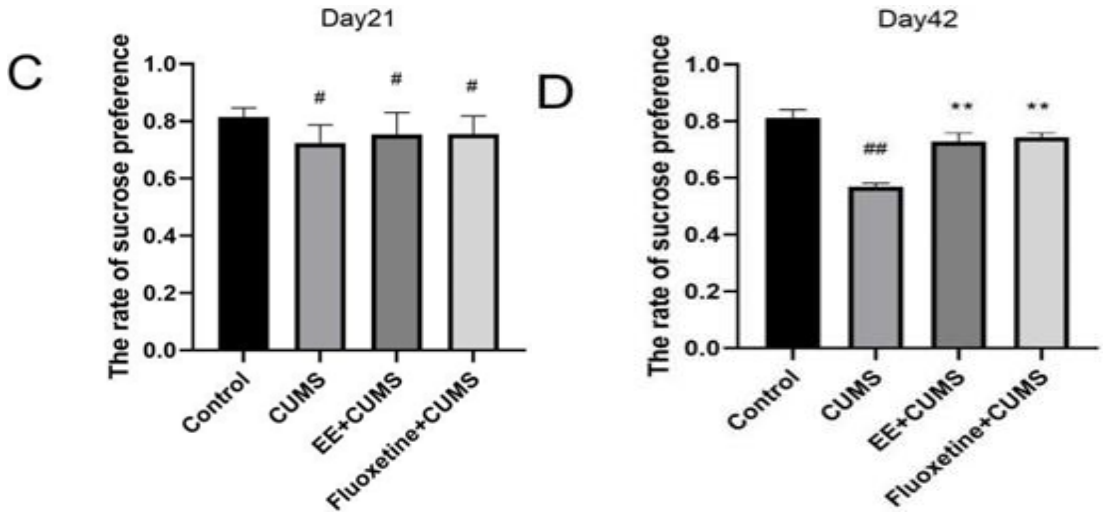
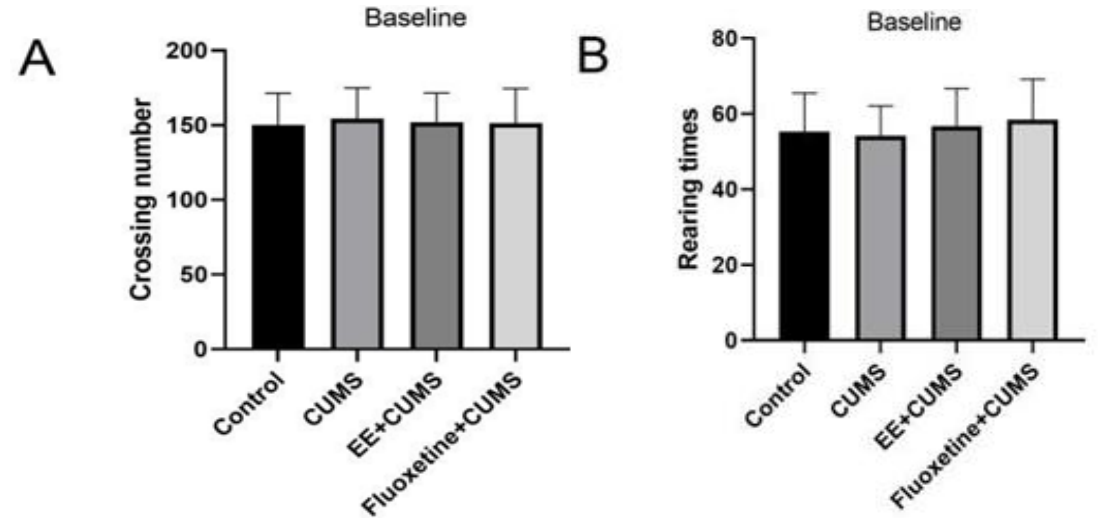


Figure 3: Effect of EE training on SPT: the sugar water preference is communicated as the mean ± SD (n=10/group). A shows the change of each group from before modeling to Day 42 of modeling. B, C, D show the significant differences in SPT of the control group, CUMS group, fluoxetine+ CUMS group and EE+CUMS group before modeling, Day 21 of modeling, and Day 42 of modeling respectively. Significant differences are indicated by "*P" "*P<0.05", "**P<0.01" in contrast to the CUMS group; Significant deviations which can be seen when compared to the group serving as the control are expressed as "#P","#P <0.05", "##P <0.01".

3.3 Effects of EE training on the OFT and FST

On the 43rd day of modeling, the number of horizontal traversing frames and the number of vertical activities of mice in the CUMS group were drastically reduced than those of the control group (P<0.01), whereas the time spent swimming without moving was much longer than what was recorded for the control group (P< 0.01). The mice in the fluoxetine hydrochloride and EE training groups had substantial increases in the number of horizontal traversal frames, the number of vertical actions(P<0.01), while the time of swimming immobility was reduced (P<0.01) (Figure 4).



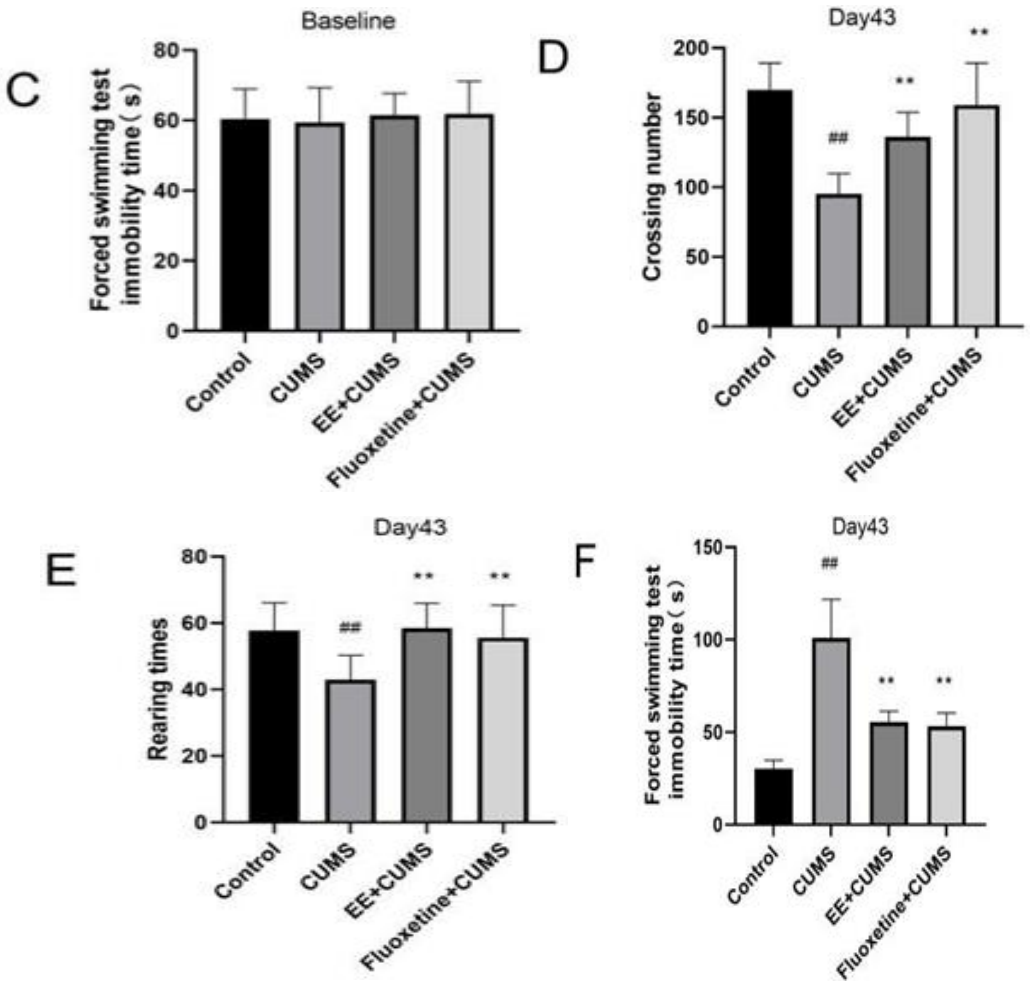


Figure 4: Effect of EE training on the OFT and FST: the detection values of the OFT and FST are represented in the form of the mean ± SD (n=10/group). A, B, C, D, E, F show the results of the number of horizontal crossing squares, the number of vertical movements, and the duration of swimming immobility for the control group, CUMS group, fluoxetine + CUMS group, and EE+CUMS group before modeling, on Day 43 of CUMS. Significant differences are indicated by "*P" "*P<0.05", "**P<0.01" to contrast with the CUMS group; Significant deviations which can be seen when compared to the group serving as the control are expressed as "$^{\#}$P"," $^{\#}$P <0.05", "$^{\#\#}$P <0.01".

3.4 Effects of EE training on the hippocampal tissue structure of chronic stress mice

Figure 5 depicts the outcomes. In the normal group, the number of neuronal cells in the CA3 region of the hippocampus was large, neatly distributed, with full cytoplasm, fewer intracytoplasmic spaces, and consistent coloration. In the CUMS group, the tissue was edematous, and the number of neuronal cells was reduced, with solidified nuclei and vacuoles. The number of neurons was restored, and their arrangement gradually tended to be neat and regular, and the damage to the cell body was significantly improved in both the fluoxetine + CUMS and EE+CUMS groups (Figure 5).

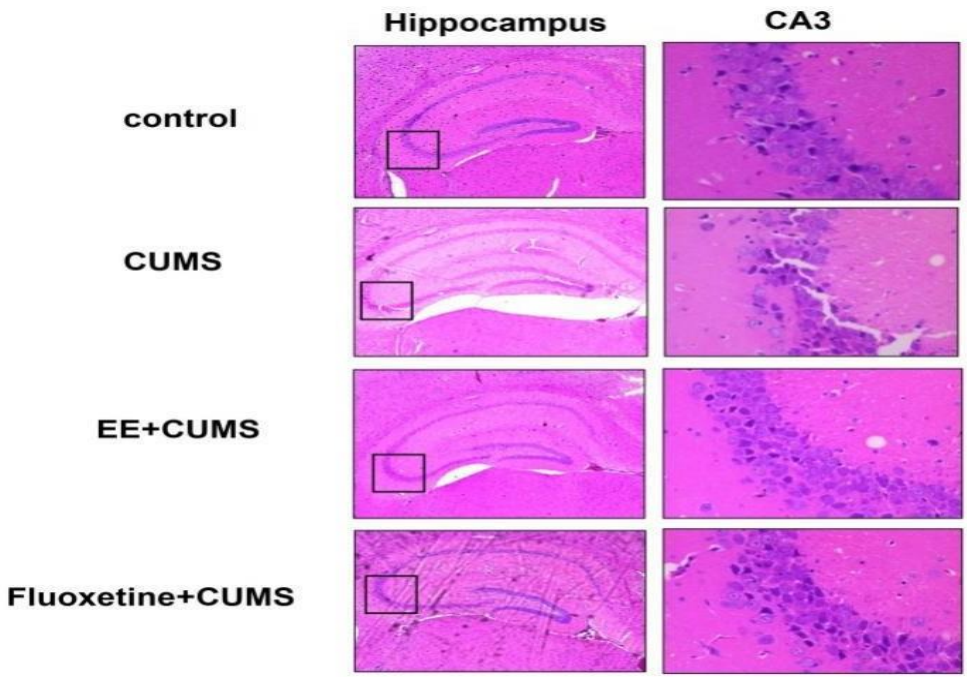
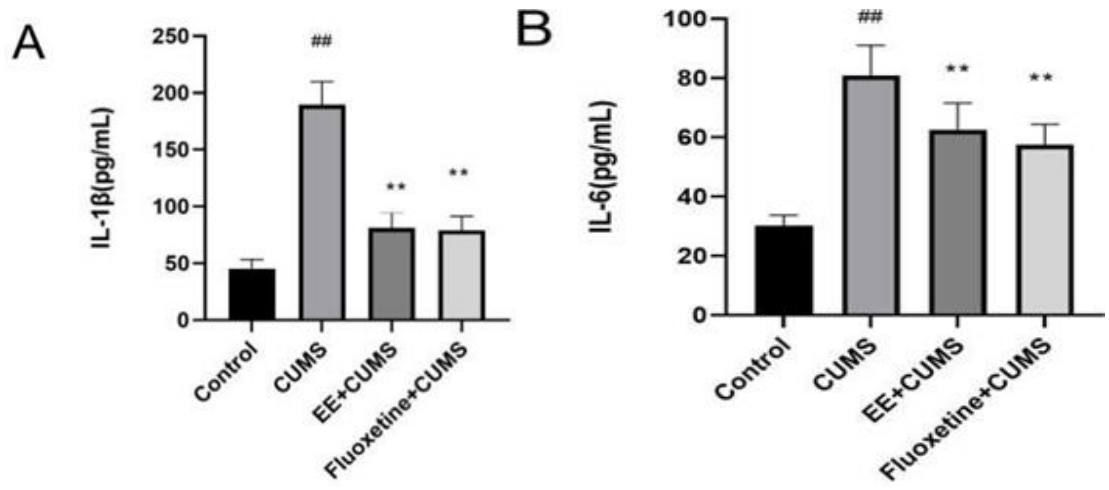


Figure 5: HE staining results of the CA3 area where the hippocampus is located in each group of mice: show the pathological changes within the CA3 region in the control group's hippocampus, CUMS group, EE +CUMS group and fluoxetine + CUMS group.

3.5 Effects of EE training on serum and hippocampal inflammatory factors

For each mouse group, we evaluated the proportions of inflammatory factors in blood and hippocampal tissue. Statistically significant variations ($P<0.01$) were observed between the amounts of IL-1β and IL-6 in the hippocampal tissues of mice in the model group and those of animals in the control group. When contrasted to the model group, the levels of IL-1β and IL-6 in the CUMS + fluoxetine and CUMS+EE groups were a considerable amount decreased ($P<0.01$), indicating that EE training effectively reduced the levels of inflammatory markers in the serum and hippocampal tissues of chronically stressed mice (Figure 6).



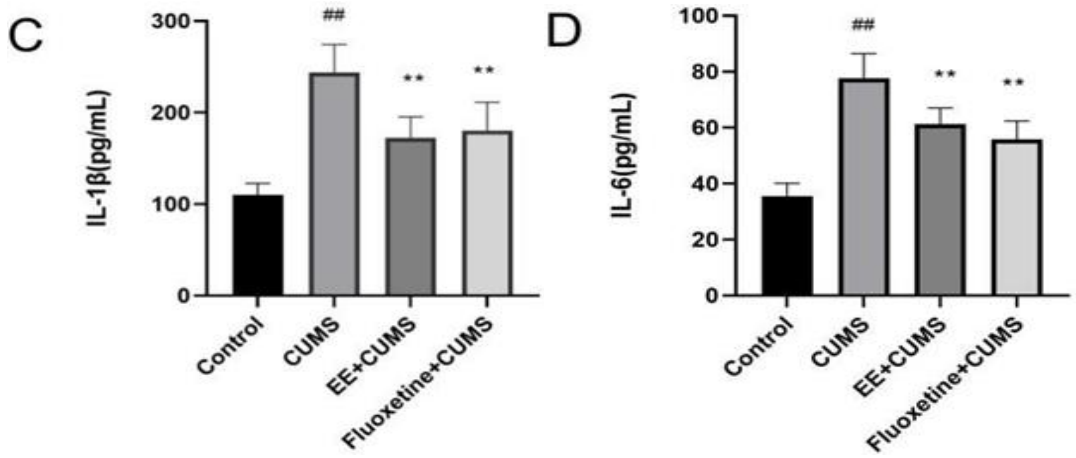
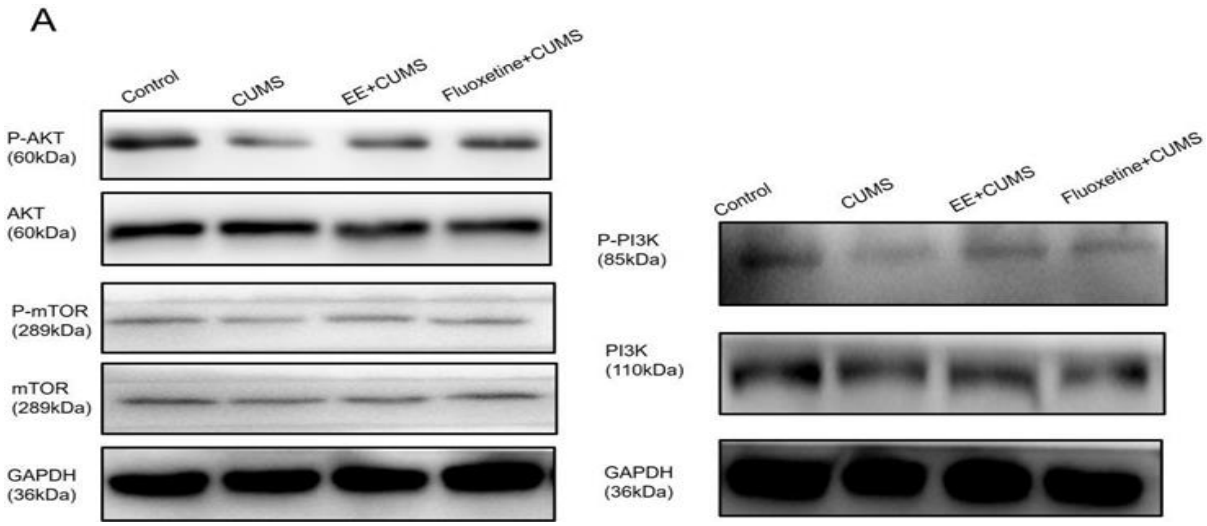


Figure 6: Effects of EE training on serum and hippocampal inflammatory factors: the detection values of inflammatory factors are represented in the form of mean ± SD (n=10/group). A, B, C and D show the results of serum and hippocampal IL-1β and IL-6 in the control group, CUMS group, fluoxetine + CUMS group and EE+CUMS group, respectively. Significant differences are indicated by "*P" "*P<0.05", "**P<0.01" in contrast to the CUMS group; Significant deviations which can be seen when compared to the group serving as the control are expressed as "#P","#P <0.05", "##P <0.01".

3.6 Effects of EE training on the hippocampal mTOR pathway

The levels of phosphorylated forms of PI3K, Akt, and mTOR, respectively, in the proteins in the hippocampus of CUMS animals were substantially lower when compared to the healthy controls (P<0.01), indicating that the starting up of the activation of the PI3K/Ak/mTOR pathway in model animals was partially suppressed. Proteins called p-PI3K, p-Akt, and p-mTOR in the hippocampus of the CUMS + fluoxetine and CUMS+EE training groups were elevated as compared to the group that served as the control (P< 0.01). Expression levels of phosphorylated forms of PI3K, Akt, and mTOR in the signaling pathways was elevated in the hippocampi of the fluoxetine and EE training groups (Figure 7).



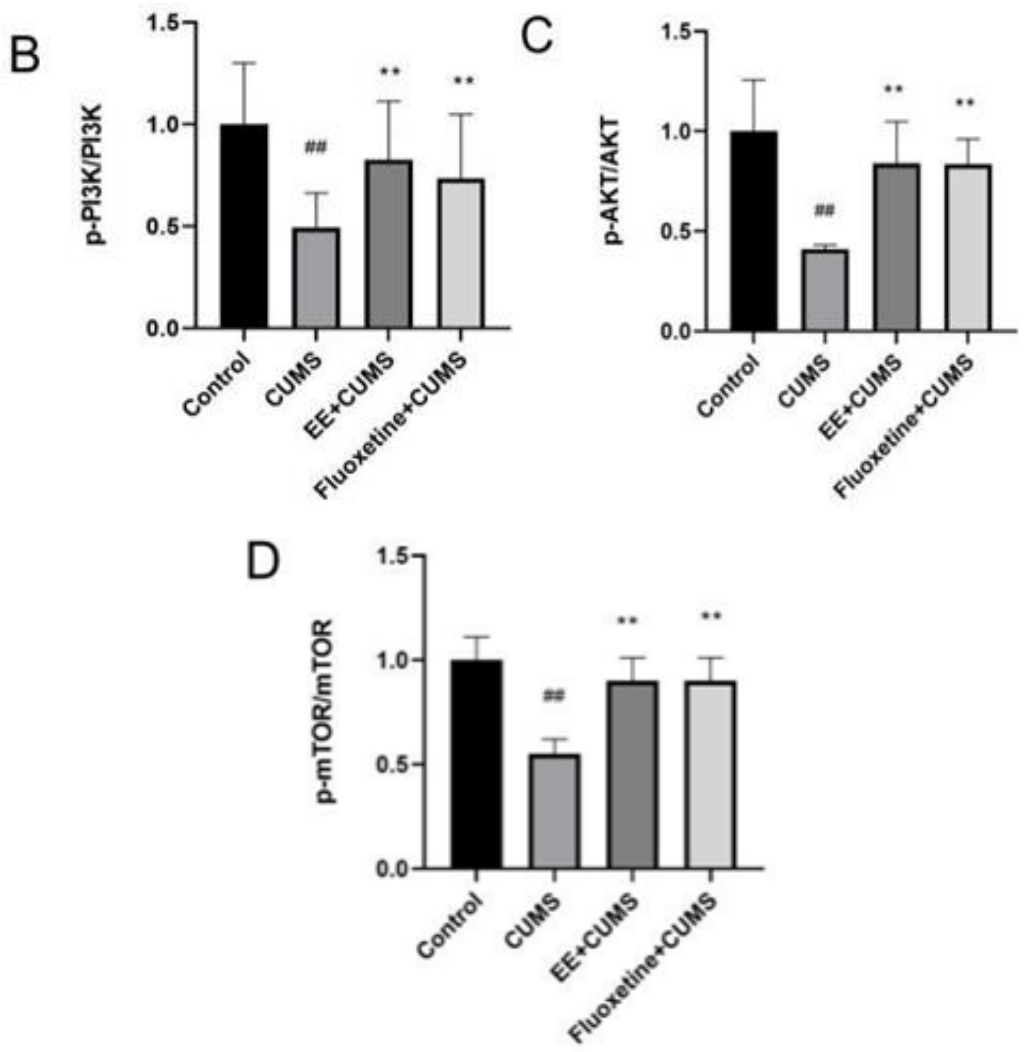


Figure 7: Effects of EE training with hippocampal-associated proteins: the detection values of hippocampal-associated proteins are represented in the form of the mean ± SD (n=10/group). Significant differences are indicated by"*P" "*P<0.05", "**P<0.01" to contrast with the CUMS group; Significant deviations which can be seen when compared to the group serving as the control are expressed as "#P","#P <0.05", "##P<0.01".

4. Discussion

Depression is a prevalent mental disease with clinical manifestations of depressed mood, delayed behavior, decreased learning and memory, loss of pleasure, and decreased body mass, and its pathogenesis is not fully understood (Anacker et al., 2013; Guehne, Stein, & Riedel-Heller, 2015). Long-term, chronically stressful stimuli are associated with the development of depression, and long-term, chronic stress can result in the atrophy, degeneration, or death of hippocampus neurons in the brain. According to the findings of the current investigation, body mass represented the general survival quality of mice, as well as T the OFT, SPT, and FST are the classic experiments used to evaluate depressive behavior. The open box experiment

and forced swimming experiment can reflect mice's behavioral despair, whereas the sugar water preference experiment can reflect a lack of pleasure. Chronic stress on Day 21 of modeling caused the emergence of depressive behavior in mice, simulating the detection of early depressive states in the clinic and the time of timely treatment intervention. Depressive symptoms were more obvious in the CUMS group on Day 42 of modeling.

Depression modeling was stable and successful, while 3 weeks of fluoxetine and environmental enrichment training were effective in alleviating depressive symptoms and reducing the occurrence of depression. Studies indicate that hippocampal tissues are closely related to the emotion regulation of the body; hippocampal tissues in depression model animals often show more obvious pathological damage, and antidepressants can reduce depressive symptoms by improving pathological damage to hippocampal tissue. The results of the present study of a chronic stress depression model showed that EE training significantly improved the structure of pyramidal cells and neuronal damage within the hippocampal CA3 region of mice, promoted an increase in the quantity of hippocampal neuronal cells and improved neuronal plasticity. It has been found that increased inflammatory cytokines in the peripheral and central areas of depressed patients lead to enhanced immune pathological damage and that disorders of central neurotransmitter metabolism are correlated with disorders of central cytokines. The present study showed that enrichment training significantly reduced IL-1β and IL-6 levels in hippocampal tissues.

In almost all eukaryotic cells, the PI3K/Akt/mTOR signaling route is present. mTOR activity is crucial for neuronal growth, differentiation, and development; inhibiting mTOR pathway activity can diminish neuronal activity and produce neuronal damage. Chronic unexpected stress has been demonstrated to lessen the manifestation of mTOR and Akt-phosphorylated proteins in rat hippocampus tissues, and abnormalities in the mTOR signaling pathway predispose rats to depression. Enrichment training significantly activated PI3K/Akt/mTOR, as seen by the upregulation of the ratio of p-PI3K, p-Akt, and p-mTOR proteins to total proteins.

5. Conclusion

Our findings illuminate the therapeutic potential of enriched environment (EE) training as a non-pharmacological intervention for depression induced by chronic stress. The efficacy of EE training in reducing depressive behaviors in mice subjected to chronic unpredictable mild stress (CUMS) is notably linked to its anti-inflammatory effects and the modulation of the PI3K/Akt/mTOR signaling pathway. These results underscore the significance of incorporating physical fitness and mental health strategies into the treatment and management of depression.

By highlighting the role of environmental and lifestyle modifications, our study advocates for a broader approach to depression therapy, one that complements traditional pharmacological treatments with interventions aimed at enhancing the living environment and promoting physical activity. Such integrative strategies could offer a more holistic and effective means of combating depression, potentially reducing the reliance on medication and its associated side effects, while fostering resilience against stress-related psychiatric conditions.

Funding

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