Neuroendocrinology Letters Volume 36 No. 5 2015
ISSN: 0172-780X; ISSN-L: 0172-780X; Electronic/Online ISSN: 2354-4716
Web of Knowledge / Web of Science: Neuroendocrinol Lett
Pub Med / Medline: Neuro Endocrinol Lett

ORIGINAL ARTICLE

Beneficial effects of enriched environment on behaviors were correlated with decreased estrogen and increased BDNF in the hippocampus of male mice

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Submitted: 2015-06-04 Accepted: 2015-07-11 Published online: 2015-11-29

Key words: **enriched environment; anxiety; learning and memory; estrogen; brain-derived neurotrophic factor**

Neuroendocrinol Lett 2015; **36** (5):490–497 **PMID:** 26707050 NEL360515A11

Abstract

OBJECTIVE: Previous studies reported that environmental enrichment might induce various beneficial effects in the central nervous system. However, the effect of environmental factors on endogenous estrogen level was not investigated. The present study was designed to examine the effect of enriched environment on endogenous estrogen in hippocampus and behavioral outcomes.
METHODS: Behavioural measurements, including open field, elevated plus maze and Morris water maze, were used to evaluate anxiety and learning and memory of the male C57BL/6J mice that were housed in enriched environment for five months. In addition, the estrogen and brain-derived neurotrophic factor (BDNF) expression in the hippocampus were measured.
RESULTS: We found that environmental enrichment decreased anxiety-like behaviors and facilitated spatial learning and memory in male C57BL/6J mice. In addition, the mice raised in enriched environment showed decreased endogenous estrogen levels both in the hippocampus and plasma compared to controls. Furthermore, our results indicated that environmental enrichment up-regulated BDNF mRNA expression level in the hippocampus.
CONCLUSION: In conclusion, environmental enrichment decreased anxiety-like behaviors and facilitated spatial learning and memory in male C57BL/6J mice. Lastly, environmental enrichment up-regulated BDNF mRNA expression level in the hippocampus and decreased plasma estrogen level. The possible mechanism remained to be determined.

INTRODUCTION

It was reported that environmental enrichment could induce various beneficial effects on behavioral performances, such as decreasing depressive-like behaviors and improving learning and memory (Maesako *et al.* 2012). At the cellular and molecular levels, it has been observed that rodents exposed to enriched environment displayed increases in cortical weight and thickness (Rosenzweig *et al.* 1964), size of the cell soma and nucleus, length of dendritic spines (Kozorovitskiy *et al.* 2005) and synaptic size and number (West & Greenough 1972). Moreover, Environmental enrichment has been shown to improve neurological function and cognitive performance in several disease animal models, such as Alzheimer's disease (AD) (Jankowsky *et al.* 2005; Fang *et al.* 2011; Fang *et al.* 2010), Parkinson's disease (PD) (Bezard *et al.* 2003), Huntington's disease (HD) (Spires *et al.* 2004), and stroke (Johansson 1996). Evidence indicated that a large number of gene expression could potentially participate in the benefits of EE on the brain (Rampon *et al.* 2000). Brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family, was one of molecular markers that were up-regulated in the brain induced by EE (Cancedda *et al.* 2004). It was highly expressed in hippocampus (Binder & Scharfman 2004) and was a key modulator of neuronal survival, synaptic efficacy, neuronal connectivity and use-dependent plasticity (Barde 1994; Lu & Chow 1999). Numerous studies demonstrated that BDNF participated in the beneficial effects induced by environmental enrichment (Rossi *et al.* 2006; Sun *et al.* 2010).

Estrogen, particularly 17β-estradiol (E2), had a wide range of effects in the brain and its therapeutic potential for mood and cognition impairment has been recognized for some time (Gibbs 2010; Liu *et al.* 2011). It also could modulate the level of BDNF in the brain (Sohrabji *et al.* 1995; Liu *et al.* 2012). Estrogen replacement in ovariectomized female rodents has been shown to affect BDNF mRNA expression in the hippocampus (Blurton-Jones & Tuszynski 2006). In addition, it was reported that the cognitive and neural response to estrogen was influenced by environmental factors (Gresack *et al.* 2007). Previous studies indicated that environmental enrichment might induce various beneficial effects in the central nervous system (van Praag *et al.* 2000). On the other hand, estrogen has been associated to neuroprotective features in the brain (Baudry *et al.* 2013). So, the link between enriched environment and his effect on estrogenic levels would be interesting. However, the effect of environmental factors on endogenous estrogen level was not investigated up to date.

The purpose of the present study was to investigate whether the level of endogenous estrogen in the hippocampus was affected by environmental enrichment in mice, as well as to analyze the correlations between the estrogen level and behavioral outcomes.

MATERIALS AND METHODS

Animals and housing

Sixteen male C57BL/6J mice aged 3 weeks were purchased from Shanghai Slaccas animal laboratory. The mice were weaned at the age of 3 weeks and shipped at aged 4 weeks. The mice were maintained in a 12-h light-dark cycle (lights on at 7:00 am) at a temperature of 22±1 °C. Food and water were available ad libitum. After another week of standard housing, the mice were randomly assigned to either standard or enriched housing conditions. Standard housing conditions consisted of a 25 cm×15 cm×14 cm acrylic box with sawdust containing groups of 4 mice. Enriched housing conditions consisted of 47 cm×35 cm×37 cm acrylic box with sawdust containing 8 mice. The EE apparatus contained one running wheel and a variety of objects, including wood and plastic objects, tunnels, hiding places and nesting materials. The objects were changed every other day. All experiments were carried out in accordance with the Animal Care and Use Committee of the University of Science and Technology.

Behavioral tasks

All behavioral tasks were performed during the light phase of cycle in a new room similar to the housing room. Before each test, the animals were given a 20-min adaptation period to become familiar with the test room. Every task was run on separate days. In each task, the mice were tested in a random order. The mice in both groups were handled one week before the first task and the handling time for each mouse is comparable. All of the tasks were monitored by a video camera.

Open field (OF) test

To detect spontaneous locomotor activity, exploration and anxiety-like behaviors, an open field procedure was performed according to previous descriptions, with minor modification (Chen *et al.* 2004; Stewart *et al.* 2014; Meng *et al.* 2011). An open, wooden box (50 cm×50 cm×25 cm) with smooth surface was used in the present study. For each trial, an animal was placed into one of the four corners, facing the corner, and was permitted to explore the environment for 5 minutes.

Elevated plus maze (EPM) test

The EPM procedure was taken from Walf et al (Walf & Frye 2007; Meng *et al.* 2015). Based on the design, the maze (made of Plexiglas) consisted of two opposite closed arms (30 cm×6 cm) enclosed with walls (15 cm in height) and two opposite open arms (also 30 cm×6 cm, without walls) that forms a plus shape. The whole apparatus had a central arena (6 cm×6 cm) and was elevated to 80 cm above the floor. Each mouse was placed in the central arena of the maze facing an open arm and allowed to explore the maze for 5 minutes.

Morris water maze (MWM)

The MWM was used for testing spatial learning and memory (Chen *et al.* 2004). The pool (1.2 m in diameter) was filled with opaque water and surrounded by complex maze cues. The escape platform (10 cm in diameter) was placed in the center of a designated quadrant with its top positioned 1 cm below the water surface. In the hidden-platform test, mice were trained daily by four trials for 7 days. A probe test was given on the eighth day. The starting point of each trial was designed according to the method of Vorhees and Williams (Vorhees & Williams 2006). The mice were allowed to swim freely until they climbed onto the platform, and they were given a rest for 30 s on the platform. If the mice were unable to find the platform within 60 s, they were guided to the platform and allowed to stay for 30 s. During the probe test session, the hidden platform was removed from the pool, and the mice were allowed to swim in the maze for 90 s. The latency to the platform in the hidden-platform test, the duration in and the frequency to the target quadrant in the probe trial were analyzed.

Tissue preparation and estrogen measurement

Tissue preparation was performed according to previous descriptions, with minor modification (Shi *et al.* 2014). Two days after the last behavioral test, the mice were anesthetized with 5% chloral hydrate, 5 ml/kg weight, and decapitated. Both blood and hippocampus samples were prepared for 17β-estradiol measurement. Blood was collected in tubes containing heparin sodium as an anticoagulant and centrifuged at 4 °C (6000 rpm for 10 min). After separation, the plasma was stored at –70 °C until assayed with an enzyme-linked immunosorbent assay (ELISA) kit for mouse 17β-estradiol (Biosource, Carlsbad, CA ,USA) (Meng *et al.* 2012). Bilateral hippocampi were separated. One hippocampus was homogenized for E2 measurement; the other was stored in –80 °C until it was used for extracting mRNA. To prepare E2 samples of hippocampus tissue, the right hippocampus was homogenized (15 passes in a Teflon-glass homogenizer; 600 RPM) in PBS and centrifuged at 4 °C (12000 rpm for 10 min). The supernatant was removed and stored at –80 °C for 17β-estradiol measurement. To determine the gross hippocampal protein, the precipitation was lysed in a cocktail containing RIPA buffer (Roche, Indianapolis , IN, USA) for 30 min and centrifuged at 4 °C (12000 rpm for 10 min). Then, the supernatant was removed and stored at –80 °C for gross protein measurement by the Lowry method. The E2 concentration in the hippocampus was normalized by the gross protein.

RNA extraction, reverse transcription and real-time PCR

Total RNA was extracted from frozen hippocampus by TRIzol reagent (Invitrogen, Carlsbad, California, USA) according to previous descriptions (Liu *et al.* 2013). The purified RNA was quantified by One drop spectrophotometer. 1ug total mRNA was reverse transcribed using RT kit for real time PCR (Takara, Otsu, Shiga ,Japan), according to the instructions of the manufacturer. Real time PCR was performed using SYBR Green Mix on ABI StepOne Applied Biosystems. Thermo cycle conditions were as follows: 1 cycle at 95 °C 5mins, and 40 cycles 15 s at 95 °C and 1 min 60 °C. The forward and reverse primer for BDNF and aromatase were as follows: BDNF, forward 5'-GCTTATTCTGGTGAGTACTAAGTCTTAATGAGT-3' and reverse 5'-GACAAAAGTCTTTATTTATTAACACCTTATTG-3'. Aromatase, forward 5-TTAATGAAAGCATGC GGTACC-3 and reverse 5-GGAAGTACTCGAGCCTGTGC-3. Relative BDNF and aromatase mRNA level was determined using the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

The data were presented as means ± S.E.M. The latency to find the platform in Morris water maze was analyzed with repeated-measures ANOVA with one between-subject (Environment) and one within-subject (days) factors. Other significant differences between the means were calculated by the independent-sample T-test. The correlation analysis was performed by the Pearson's correlation test. The following levels of statistical significance were noted: $*p<0.05$, $** p<0.01$.

RESULTS

Open field test

After five months' separated housing in standard and enriched environment, the two groups of mice showed different levels of anxiety. Compared with control animals, EE mice showed longer duration (Figure 1B $p<0.05$), shorter latency (Figure 1C, $p<0.05$) and higher frequency (Figure 1D, $p<0.05$) to the inner area in the open field (OF) task. There was no statistical differences in locomotor activity (Figure 1E, $p>0.05$) and exploratory behaviors (Figure 1F, $p>0.05$) in the OF test.

Elevated plus maze task

In elevated plus maze test, a lower level of anxiety-like behaviors was found in the EE mice compared with that in standard housing animals. The EE mice showed a longer duration in both open arms (Figure 2B, $p<0.05$) and center area (Figure 2D, $p<0.05$) of the maze, which were the main measures in this test. They also traveled longer distance in center area in EPM test (Figure 2E, $p<0.05$).

Morris water maze

Morris water maze test was used to test the spatial learning and memory of mice (Figure 3). Overall, mice raised in EE conditions performed better in the spatial learning and memory in the MWM test. Repeated-measures ANOVA revealed that both the EE mice and control mice improved in the spatial learning across the day-to-day acquisition trials ($F(6,102)=22.14$, $p<0.001$).

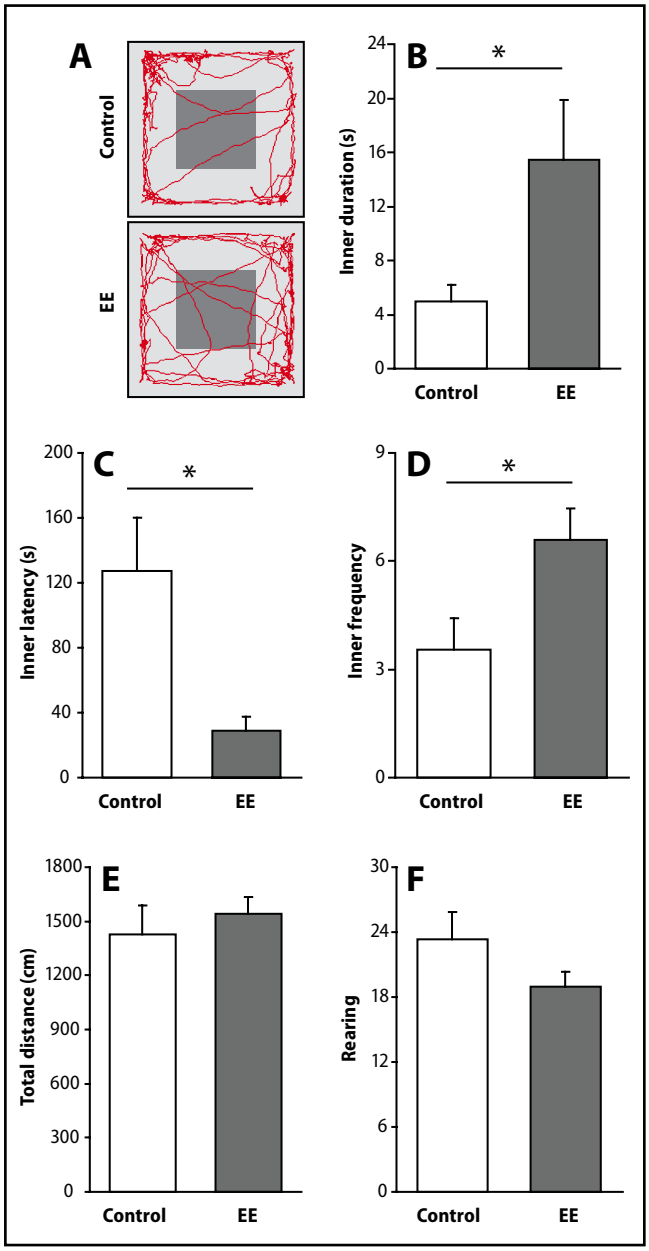

Fig.1. Open field task. (A) Representative activity tracers for enriched environment (EE) treated and control (CON) mice during 5 min task. The duration in the inner area (B), the latency (C) and frequency (D) to enter the inner area, total distance traveled (E) and rearing (F) were analyzed for the task. The data represent means ±S.E.M. Independent-sample T-test, *p<0.05.

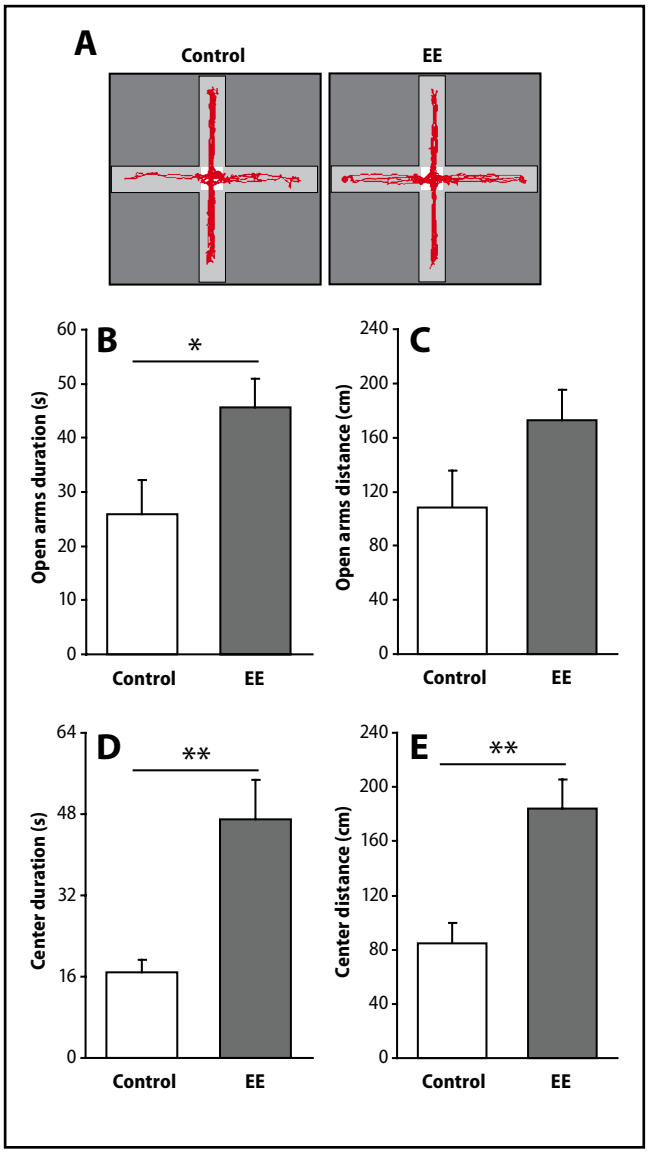

Fig. 2. Elevated plus maze task. (A) Representative activity tracers for enriched environment (EE) treated and control (CON) mice during 5 min task. The duration (B) and distance (C) that the mice travelled in the open arms (B and C) and the frequency (D and E) of travelling in the open arms were analyzed. The data represent means ±S.E.M. Independent-sample T-test, *p<0.05, **p<0.01.

Compared with control animals, EE mice showed a better performance in the spatial learning and memory in the acquisition trials (F (1,17)=639.45, p<0.001). Though no significant difference was found in the target quadrant duration, the frequency to the target quadrant in EE mice was higher than that of control mice in the probe trial (Figure 3C, p<0.05).

BDNF mRNA expression and correlation analysis

It showed that the level of BDNF in the hippocampus was higher in the EE mice compared to the controls (Figure 4, p<0.01). In addition, the mRNA expression level of BDNF in the hippocampus was significantly correlated with the frequency (Figure 5A, r=0.50, p<0.05) and the latency (Figure 5B, r=0.50, p<0.05) to the inner area in OF test and the mean latency to the platform (Figure 5C, r=0.60, p<0.05) in the MWM test. There was no correlation between the BDNF mRNA with other behavioral measures as shown in Table 1.

Endogenous estrogen level in hippocampus and plasma and aromatase mRNA expression

After finishing the behavioral tasks, the estrogen level in the hippocampus was measured. Both in the hippocampus and plasma, the 17β-estradiol level was lower in the EE mice than that in the control mice (Figure 6,

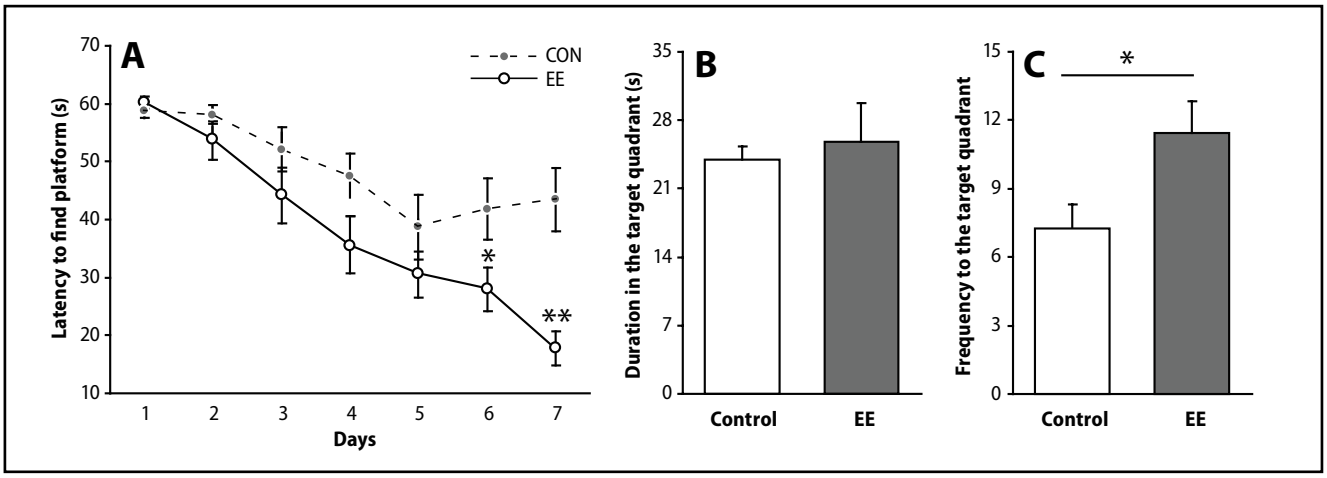

Fig. 3. Learning and memory in the hidden version of the Morris water maze. The latency to the platform (A), the duration in (B) and the frequency to (C) the target quadrant during the probe trial were analyzed. The data represent means ± S.E.M, *p<0.05.

Tab.1. Correlation analysis between the measures of behavioral tests with the E2 and BDNF mRNA level in hippocampus*.

Tasks	Measures	Pearson's correlation test p-value	
		E2	BDNF
Open field	Inner duration	0.719	0.067
	Inner latency	0.325	**0.039**
	Inner frequency	0.078	**0.049**
	Rearing	0.060	0.562
	Total distance	0.518	0.434
Elevated plus maze	Open arms duration	0.217	0.082
	Open arms distance	0.279	0.132
	Center duration	**0.009**	0.171
	Center distance	0.055	0.219
Morris water maze	Latency to the platform	**0.004**	**0.015**
	Duration in the target quadrant	0.207	0.211
	Frequency to the target quadrant	0.062	0.669

* The correlation analysis was performed by the Pearson's correlation test.

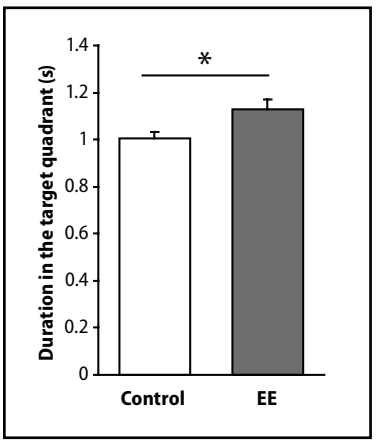

Fig. 4. Relative level of BDNF mRNA level in hippocampus measured by real time PCR method. The data represent means ±S.E.M. Independent-sample T-test; *p<0.01.

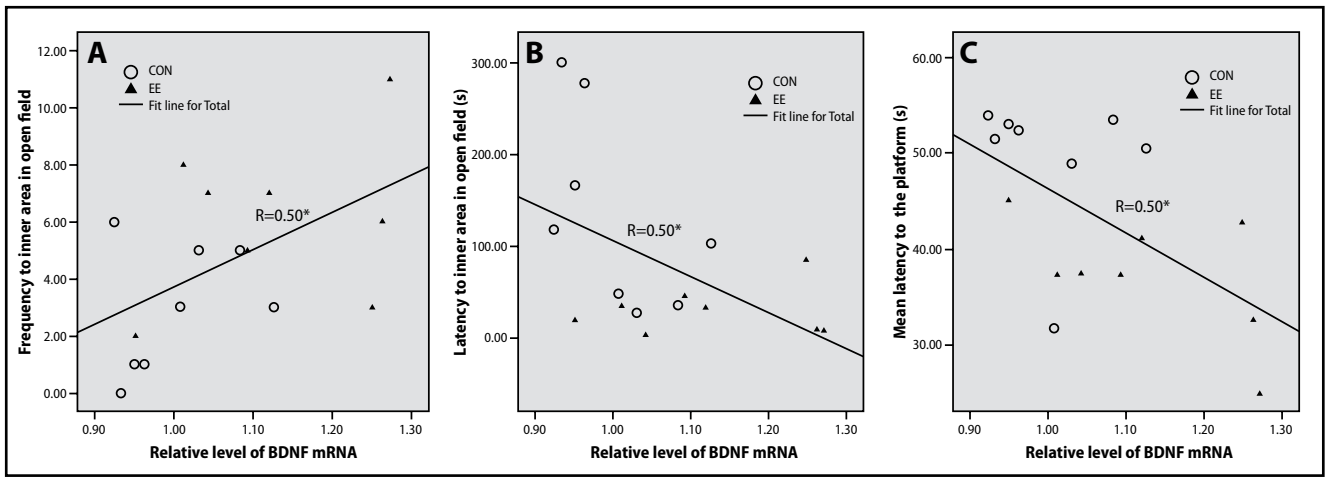

Fig. 5. Correlations between BDNF mRNA level in hippocampus and measures in behavioral tests. (A), Correlations between BDNF mRNA level and the frequency to inner area in the open field. (B), Correlations between BDNF mRNA level and the latency to inner area in the open field. (C), Correlations between BDNF mRNA level and the latency to the plat form in Morris water maze. The correlation analysis was performed by the Pearson's correlation test, *p<0.05.

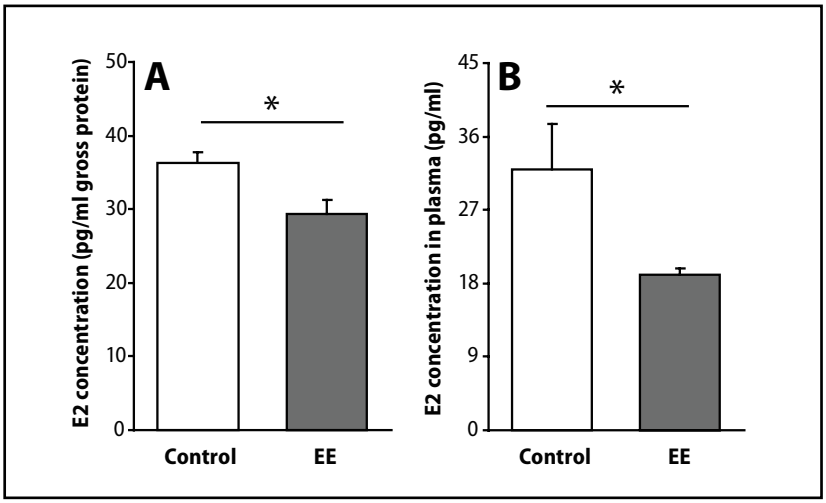

Fig. 6. Estrogen level in the hippocampus (A) and plasma (B) of EE mice and standard housing mice. The data represent means ±S.E.M. Independent-sample T-test; *p<0.01.

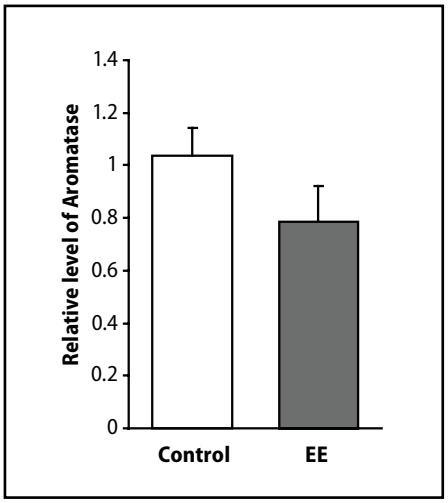

Fig. 7. Relative level of aromatase mRNA in hippocampus measured by real time PCR method. The data represent means ±S.E.M.

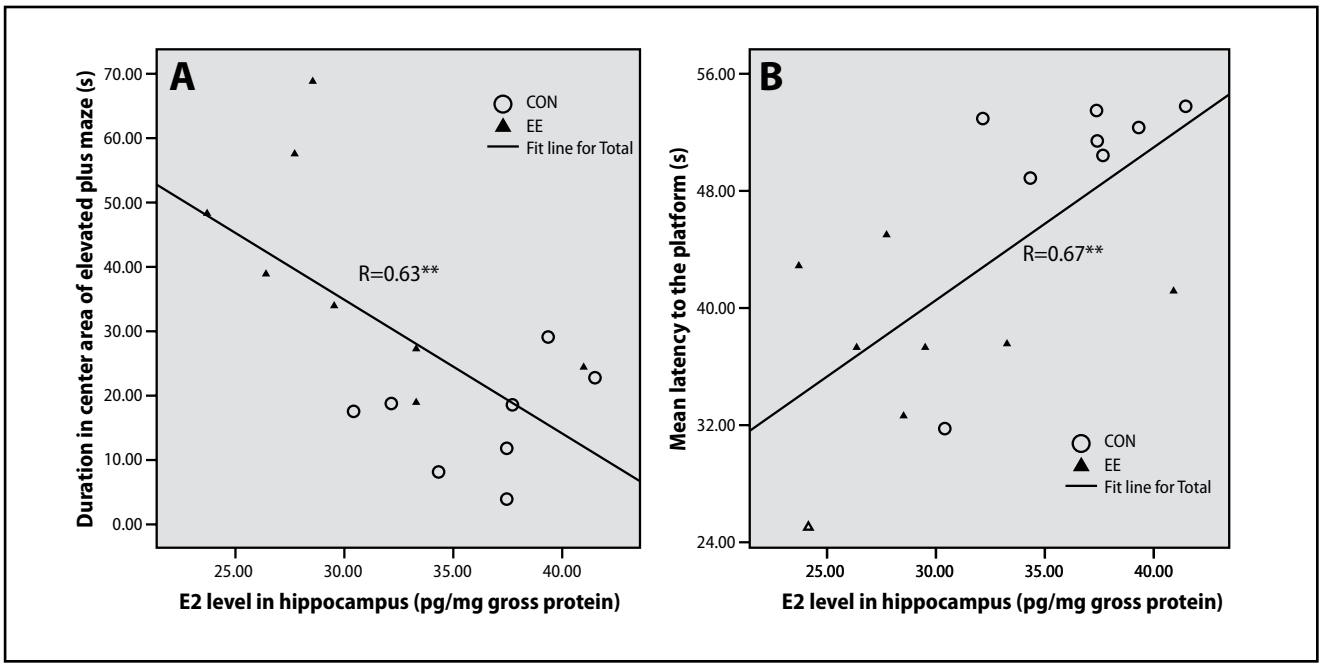

Fig. 8. Correlations between E2 level in hippocampus and measures in behavioral tests. (A), Correlations between E2 level and duration in center of the elevated plus maze. (B), Correlations between E2 level and latency to the platform in the Morris water maze. The correlation analysis was performed by the Pearson's correlation test, **p<0.01.

p<0.05). There was a decrease tendency in hippocampal aromatase expression in EE mice, though it failed to reach a statistical significant at p=0.05 level (Figure 7). Furthermore, the hippocampal estrogen level was negative correlated with the duration in the center area of EPM (Figure 8A, r=0.63, p<0.01) and positive correlated with the average latency to the platform in MWM (Figure 8B, r=0.67, p<0.01). We did not find correlations between the hippocampal estrogen level and other behavioral measures (Table 1). There was a trend in the correlation between the E2 and BDNF mRNA expression level (Figure 9, r=0.49, p=0.056).

DISCUSSION

The present study demonstrated that the mice exposed to an enriched environment showed decreased anxiety-like behaviors and facilitated spatial learning and memory. Furthermore, we found that there was an increased level of BDNF mRNA and a decreased level of E2 in the hippocampus of EE mice compared to controls. In addition, Pearson correlation analysis revealed that the levels of hippocampal BDNF and E2 were correlated with behavioral measures for anxiety and learning.

Existing studies revealed that environmental enrichment had a the beneficial effect on anxiety-like behavior (Fox *et al.* 2006). In addition, environmental enrichment has been shown to protect against the negative effects of maternal separation on stress reactivity (Francis *et al.* 2002; Zhang *et al.* 2012). In consistent with these studies, we reported that long-term environmental enrichment decreased anxiety-like behavior using the open field task and the elevated plus maze. In addition, the finding that EE mice showed a better learning ability compared to controls in the Morris water maze test was consistent with other reports (Pang & Hannan 2013).

Previous studies have demonstrated that BDNF participated in the beneficial effects induced by environmental enrichment (Rossi *et al.* 2006). Our finding that there was a higher level of BDNF mRNA in the hippocampus of EE mice compared to controls was consistent with other report (Ickes *et al.* 2000). Furthermore, Pearson's correlation test showed that the mRNA expression level of BDNF in the hippocampus was significantly correlated with the frequency and the latency to the inner area in OF test and the mean latency to the platform in the MWM test. These results further revealed the relationship between the molecular mechanism in the brain and the beneficial effects on behaviors induced by environmental enrichment. It was the limitation of our present study that we only detected the mRNA levels of hippocampal BDNF. The changes of the protein levels of BDNF and other interested targets caused by enriched environment would be further investigated in the future.

Extensive studies have been performed to investigate the role of E2 in modulating plasticity and function of hippocampus (Bi *et al.* 2000). To further illustrate the source of hippocampal E2 in our mice model, we measured the aromatase (key enzyme converting testosterone to E2) expression level in the hippocampus and circulated E2 level in the plasma. Interestingly, our results demonstrated that E2 level was lower in the hippocampus of EE mice compared to controls. In addition, the E2 level in the hippocampus was negatively correlated with the duration in center area in EPM test and positively correlated with the mean latency to the platform in MWM. However, we found that there was no statistical significance in the expression level of hippocampal aromatase in EE mice compare to controls. Surprisingly, several animal studies demonstrated that the inhibition of endogenous E2 level by aromatase inhibitors did not impair but rather improve learning and memory in several tasks (Alejandre-Gomez *et al.* 2007; Aydin *et al.* 2008). The possible explanation was that the effect of E2 after exposure to enriched environment was associated with its receptors, such as estrogen receptor alpha (ERα), estrogen receptor beta (ERβ), and G protein –coupled estrogen receptor (GPER). Estrogen receptors were widely distributed in several memory-associated brain regions such as the hippocampus and cerebral cortex, but they were not uniformly distributed throughout the brain (Mitterling *et al.* 2010).

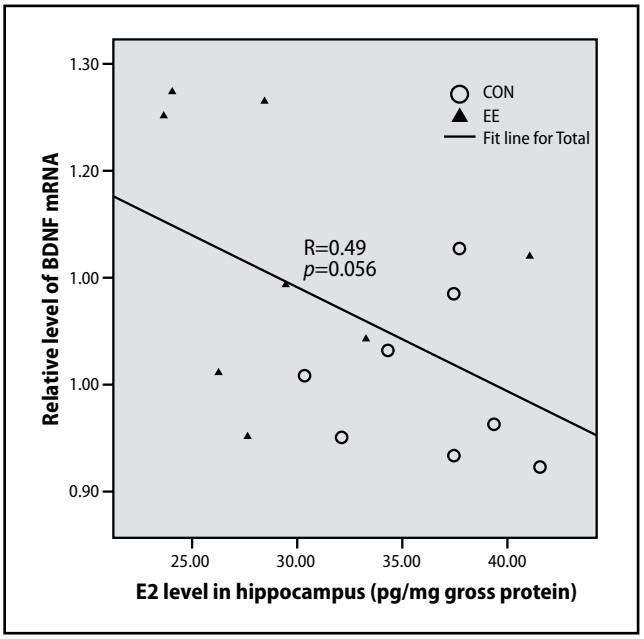

Fig. 9. Correlations between BDNF mRNA level and E2 level in hippocampus. The correlation analysis was performed by the Pearson's correlation test.

In conclusion, the current study showed that environmental enrichment decreased anxiety-like behaviors and facilitated spatial learning and memory in male C57BL/6J mice. Furthermore, our results indicated that environmental enrichment up-regulated BDNF mRNA expression level in the hippocampus and decreased plasma estrogen level. The specific mechanism remained to be determined.

ACKNOWLEDGMENT

This study was financially supported by National Basic Research Program of China (2012CB947602), National Natural Science Foundation of China (31200802) and Natural Science Foundation of Anhui province (1308085MH125).

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