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ERK/MAPK-mediated alleviation in cognitive dysfunction in chronically stressed mice treated with ethyl acetate extracts of *Cynomorium Songaricum*

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This study explored the neuroprotective mechanism of ethyl acetate extracts of *Cynomorium Songaricum* Rup (ECS) in improving the cognitive dysfunction in chronically stressed mice. We tested the spatial learning and memory ability through water maze, the expression level of phosphorylated extracellular signal regulated kinase 1/2(P-Erk1/2) and phosphorylated cAMP response element protein (P-CREB) of mitogen-activated protein kinase (MAPK) signal pathway in the hippocampus. Besides, the expression of synaptophysin (Syn) and postsynaptic density protein 95 (PSD-95) was assessed. We also detected the long-term potentiation (LTP) by neurotic electrophysiology, observing the morphological change of hippocampal neurons by HE staining. The results showed that ECS could improve the learning and memory disorders caused by chronic stress in mice. This may be related to the raise in the expression of Syn and PSD-95 by activating the expression of P-Erk1/2 and P-CREB in MAPK signaling pathway.

Keywords: Ethyl acetate, *Cynomorium songaricum* (ECS), Cognitive function, Chronic stress, LTP, MAPK, Synaptic plasticity

INTRODUCTION

It has been reported that chronic stress has an influence on cognition, particularly on spatial memory and learning ability [1, 2]. Some studies revealed that this reaction might be correlated to hippocampal morphology and function [3]. Interestingly, this morphologic and functional change differs sexually: When ovarian hormones are removed by ovariectomy (OVX), chronic stress produces robust dendritic retraction in CA3 neurons, as the dendritic pruning extends beyond the traditional apical region found in males, and can be detected in the basal arbors of some of the CA3 neuronal subtypes [4]. Estrogen probably plays an important role in these distinct responses. One of the ways through which estrogen works in the central nerve system involves G-coupled protein estrogen receptor, GPER, which mediates the rapid signaling pathways of estrogen, including ERK/MAPK pathway. ERK/MAPK pathway activation leads to synaptic plasticity change that accordingly induces cognition variation [5, 6].

Cynomorium Songaricum Rup. is a well-know Chinese herb medicine which is used for treating aging and weakness syndrome. In our previous study we found that ethyl acetate extract of *Cynomorium Songaricum* (ECS) can protect the SK-N-SH cells from cytotoxicity induced by amyloid β (Abeta or Aβ) 25-35. Moreover, it shows antioxidant effect *in vitro*. By *in vivo* test it was found that the extract can improve spatial cognition in aging rats through estrogen-like effect [7,8]. Whether ECS can improve the cognition induced by chronic stress and its probable mechanism needs to be explored.

In this study, the effect of ECS on cognition was assessed *in vivo*. Besides, we also tested the key protein expression that is correlated to synapse plasticity and the probable pathway, which will give us clues to explain how ECS works in cognitive disorder induced by chronic stress.

EXPERIMENTAL

Drugs and reagents

The sources of various drugs and reagents are given here: P-Erk1/2 antibody (ABCAM), P-creb (CST), Synaptophysin antibody (ABCAM), PSD-95 (ABCAM), β-actin (COMBIN), RIPA lysate (BEYOTIME), PMSF (BEYOTIME), protease inhibitors (ROCHE), BCA protein quantification kits (THERMO), prestained protein markers (THERMO). NaCl (SIGMA, Lot No. SLBJ9883V); KCl (SIGMA, Lot No. SLBH5524V); $MgSO_4$ (SIGMA, Lot No. 091M02151V); D-glucose (SIGMA, Lot No. SLBD 9496V); $CaCl_2$ (SIGMA, Lot No. SLBK 9976V); $NaHCO_3$ (SIGMA, Lot No. SLBF3956V); NaH_2PO_4 (SIGMA, Lot No. 20140409); HEPES (SIGMA, Lot No. I1114).

Extraction method

Accurately weighed 20 kg of *Cynomorium songaricum* decoction pieces bought from Anguo Chinese herbal medicine market, Hebei province, China were used for extraction. Each time, 160 kg of

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70% ethanol solution was added to the decoction pieces, then heated and refluxed three times, for 1.5 h each time. The solution was recycled and concentrated under reduced pressure till no alcohol taste. Then petroleum ether and ethyl acetate were used for extraction. The solution was recycled and the ethyl acetate polar part was extracted.

Animals

For this study, 30 ICR female mice in SPF level having an age of 56-62 days were offered by Beijing Vital River Laboratory Animal Technology, Beijing, China (Permit No: SCXK 2012－0001). This study was approved by the University Ethical Committee on Research Practice at Beijing University of Chinese Medicines and performed in accordance with approved standards of laboratory animal care and use in experiments.

Animal grouping and drug administration

The experimental animals were randomly divided into control group, model group and ECS group. Each group consisted of 7 rats. After a week of adaptive feeding, 0.06 mL/10 g of 7% chloral hydrate was used for anesthesia and bilateral ovaries were removed. After 7 days, drug administration was started. Seven kinds of methods were used to stimulate model group and ECS group, they were tail clamp, day and night reversed, restraint, cold stimulation, heat stimulation, water deprivation for 24 h, fasting for 24 h. ECS group was fed with 0.47 mg/ml of drug, and the dosage was 0.1 ml/10 g, 10 times of the adult oral dose. The drug was dissolved in distilled water under ultrasound.

Index detection

Morris water maze test. This spatial learning and memory test involved pre-experiment for 1 day, place navigation test for 5 days, and space exploration experiment for 1 day. The platform was located in the southwest quadrant. In the place navigation test, the southeast, northeast, and northwest quadrants were the entry points into the water. The time of searching platform, also called escape latent period, was recorded. For example, if mice did not find the platform within 60 s, they would be led to the platform by the experimenter, and the escape latent period was recorded as 60 s. Space exploration experiment withdrew platform on the 6^{th} day, while the water entry point was on the opposite side of the platform, and the number of crossings across the original platform was recorded.

Western Blot. The hippocampal tissue protein of mice was extracted for protein quantification, 12% separation gel and 5% concentrated gel was prepared. Wet turning method was used, with 80 V 0.5 h, and 100 V 1 h electrophoresis. Transmembrane conditions comprised 75 V constant voltage for 1.5 h, and 0.45 μm PVDF membrane. The closure of the primary antibody was incubated for one night. On next day, the membrane was washed and the second antibody was incubated. Finally, the gel imaging system was exposed.

Neurotic electrophysiology. The brain slice was prepared by using the hippocampus of two mice in each group for neurotic electrophysiology LTP test.

Hematoxylin and eosin (HE) staining. Rats were randomly selected from each group for morphometric analysis, anesthetized with 7% chloral hydrate, perfused with PBS (phosphate buffer saline, 0.1 M, 4°C) and again perfused with 4 % paraformaldehyde through the ascending aorta, until stiffening of tail and limbs. Afterwards, the brains were divided into two parts: One part was stored with 4% paraformaldehyde for 7 days and other part was with 2.5% glutaraldehyde. Former part was sliced into coronal sections of 4 μm thickness for hematoxylin-eosin (H & E) staining, while the latter one was used for electron microscope study.

Statistical method

The results of the behavior test were statistically analyzed by SPSS version 20.0 software. The experimental data were expressed as $\bar{x} \pm s$, and single-factor analysis of variance was performed. LSD was used for multiple comparisons. If $P < 0.05$, the difference was statistically significant.

RESULTS

Morris water maze test

The results from the orientation navigation experiment showed that the escape latency of each group was gradually shortened, and there was non-significant ($P > 0.05$) difference in escape latency during 1-4 days for each group. On the 5^{th} day of the experiment, compared with the female control group, the incubation period of the female model group was significantly ($P < 0.05$) longer. Compared with the female model group, the escape latency of female ECS mice group was significantly ($P < 0.05$) shorter. Space exploration experiment results showed that compared with the female control group, the times of crossing platform in the female model group was decreased, and the difference was statistically significant ($P < 0.01$). Compared with the female model group, the times of crossing platform in the female ECS mice group increased, and the difference was statistically significant ($P < 0.01$) (Table 1).

Table 1. Comparison of learning and memory behavior of all groups (n = 7, $\bar{x} \pm s$)

Group	Latency to platform (s)					In Quadrants Southwest Frequency
	Day 1	Day 2	Day 3	Day 4	Day 5	
Control	26.0 ± 12.7	20.7 ± 7.9	18.3 ± 7.2	11.0 ± 4.3	10.0 ± 4.1	8.3 ± 0.82
Model	36.7 ± 14.7	26.0 ± 12.6	25.6 ± 11.4	18.0 ± 12.9	22.0 ± 7.8*	4.8 ± 2.0**
ECS	33.9 ± 15.3	24.4 ± 8.4	20.3 ± 9.7	15.3 ± 7.9	13.0 ± 6.8$^{\#}$	7.2 ± 0.4$^{\#\#}$

Note: Compared with control, $^{*}P < 0.05$; $^{**}P < 0.01$; Compared with model, $^{\#}P < 0.05$; $^{\#\#}P < 0.01$.

Western Blot

The results showed that the Syn protein expression of female model group was significantly ($P < 0.01$) lower than that of the female control group, and the Syn protein expression of the female ECS mice group was significantly ($P < 0.05$) higher than that of female model group. Besides, the PSD-95 protein expression of female model group was significantly ($P < 0.05$) lower than that of female control group, and the PSD-95 protein expression of the female ECS mice group was significantly ($P < 0.05$) higher than that of female model group (Figure 1 and Table 2). P-Erk1/2 protein expression in female model group was significantly ($P < 0.01$) lower than that in female control group, and the content of P-Erk1/2 protein expression in the female ECS mice group was significantly ($P < 0.01$) higher than that of female model group. In addition, P-creb protein expression in female model group was significantly ($P < 0.05$) lower than that in female control group, and the P-creb protein expression of the female ECS mice group was significantly ($P < 0.05$) higher than that of female model group (Figure 2 and Table 2).

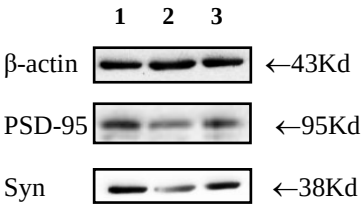


Fig.1. Expression of PSD-95 and Syn in hippocampus (1-control; 2-model; 3-ECS)

Neurophysiological results

The results showed that the slope percentage of a field excitatory postsynaptic potential (fEPSP) in the model group was significantly ($P < 0.01$) lower than that in the control group, suggesting that the learning and memory function of the model group mice may be defective.

Table 2. Comparison of proteins expression of all groups (n = 4, $\bar{x} \pm s$)

Group	Syn	PSD-95	P-Erk1/2	P-creb
Control	0.9± 0.2	0.9 ± 0.2	1.3 ± 0.2	1.1 ± 0.3
Model	0.4± 0.2**	0.5 ± 0.1*	0.4 ± 0.1**	0.7 ± 0.1*
ECS	0.8 ±0 .1$^{\#}$	0.9 ±0 .3$^{\#}$	1.3 ± 0.3$^{\#\#}$	1.1 ± 0.3$^{\#}$

Note: Compared with control, $^{*}P < 0.05$; $^{**}P < 0.01$; Compared with model, $^{\#}P < 0.05$; $^{\#\#}P < 0.01$.

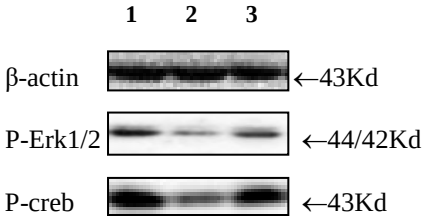
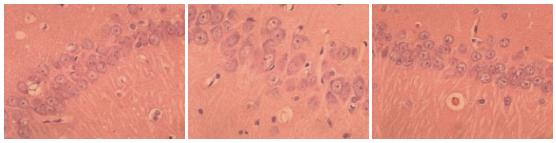


Fig.2. Expression of P-Erk1/2 and P-creb in hippocampus (1-control; 2-model; 3-ECS)

The slope percentage of fEPSP in the female ECS mice group was significantly ($P < 0.01$) higher than that in the model group, arguing that ECS can improve the cognitive dysfunction in chronically stressed mice after castration (Table 3, Figure 3).

HE staining

The results showed that the pyramidal cells in the hippocampal CA1 region of the model group were disordered, sparse, the cell volume was reduced, the nucleus was fixed and the cytoplasm was deep stained. The pyramidal cell morphology of the hippocampal CA1 region of the ECS group was normal, arranged balanced and neatly, with clear structures (Figure 4).



1 2 3

Fig.4. Neurological form of pyramidal cells in hippocampus from control group (1), model group (2), ECS group (3) HE staining, X40.

DISCUSSION

Cynomorium songaricum is the main herb for nourishing kidney and enriching essence. Recent studies have shown that *Cynomorium songaricum* can promote cell regeneration and differentiation, and help synaptic growth and memory function improvement, with significant antiaging effect [9, 10]. ECS is a mixture of various antioxidant ingredients [11], and was used as the research medicine in this experiment. Estrogen plays an important role in learning and memory, neuroprotection, emotional cognition and other aspects [12]. Thus, this study involved the removal of bilateral ovary of mice to simulate the rapid

withdrawal effect of estrogen, then combined with chronic stress to create cognitive dysfunction model, and to study estrogen-mediated signaling pathways, such as MAPK pathway.

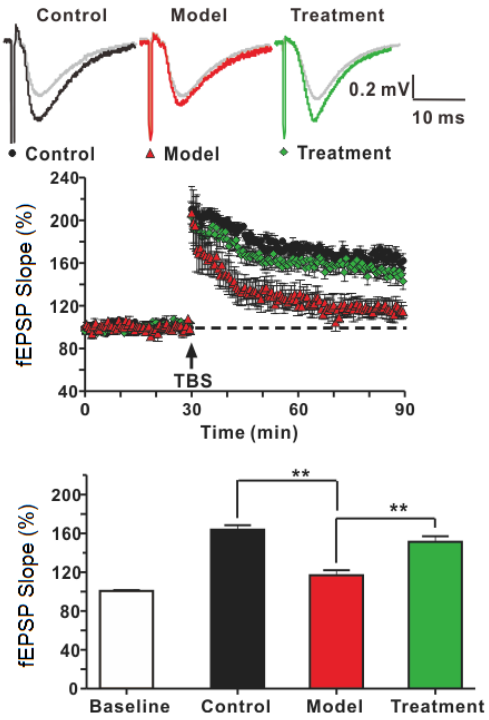


Fig.3. LTP in all groups

Table 3. Comparison of LTP of all groups

Group	Control (n = 6)	Model (n = 5)	ECS (n = 6)
Before	100.1 ± 1.2	99.7 ± 1.8	102.1± 1.4
After TBS	163.9 ± 4.5	116.8 ± 5.1**	151.3 ± 5.8$^{\#\#}$

Note: Compared with control, $^{*}P < 0.05$; $^{**}P < 0.01$; Compared with model, $^{\#}P < 0.05$; $^{\#\#}P < 0.01$.

The synaptic plasticity of the central nervous system is closely related to the learning and memory, so the number and density of dendrites in the nerve cells are very sensitive to changes in estrogen concentration, and hippocampal neurons can produce new dendrites and synapses when estrogen level is increased [13]. This experiment was intended to detect the functional plasticity and structural plasticity of synapse, and explore the neuroprotective effect of ECS in hippocampus histomorphology.

MAPK pathway is involved in cell growth, proliferation, apoptosis and other physiological processes, and affects synaptic plasticity. After its signal cascade, Erk1/2 is phosphorylated, forming P-Erk1/2. It induces the phosphorylation of nuclear transcription factor, creb, and triggers the biological change to play a biological effect [14].

Synaptic plasticity refers to the dynamic changes in the information transfer efficiency of synapses with the changes in neuronal activity, including synaptic structural plasticity and synaptic functional plasticity. Functional plasticity directly affects life activities, and structural plasticity is the morphological basis of functional plasticity. The structural plasticity is mainly manifested as the growth and the increase of density. The main manifestation of synaptic functional plasticity is neurophysiological LTP [8]. A large number of experimental studies [15, 16] have proved that changes in synaptic plasticity can affect learning and memory.

Erk1/2 is one of the main members of the MAPK family, and is involved in the body's stress response [17]. P-Erk1/2 is the product of phosphorylated Erk1/2. ERK, after phosphorylation, can transfer a variety of extracellular signals step by step to the nucleus, and activate a variety of nuclear transcription factors. It is also involved in cell growth, development, division, differentiation and other processes [18]. When p-Erk1/2 enters the nucleus, and acts on the transcription factor, it can phosphorylate nuclear transcription factor creb, other protein kinases and other substrates. It regulates the transcription of related genes, and participates in many physiological processes such as cell growth, development, division and cell function synchronization [19]. Studies have shown that P-Erk1/2 has neuroprotective effects [20], and ERK signal transduction pathways may be involved in the process of learning and memory [21].

CAMP response element binding (CREB) protein is a target protein for phosphorylation in the MAPK signaling pathway [4]. This transcription factor exists in the nucleus and can be activated by P-Erk1/2 (i.e., P-CREB). It is widely expressed in the nerve cells. The regulation and transcription functions are closely related to the level of phosphorylation itself, and exist in non-phosphorylated form in the nucleus, with no transcriptional activity. When the mitogen-activated protein kinase (MAPK) is activated, the CREB site can be identified to activate the phosphorylation level reaction and activate the transcriptional activity [4]. CREB plays an important role in the axon growth [22], which is also important in synaptic plasticity, learning and long-term memory [23-25].

Syn is a kind of sugar-containing transmembrane structural protein on synaptic vesicle membrane. It participates in synaptic vesicle exocytosis and the fusion process of synaptic vesicles and the anterior membrane of synapses, and is closely related to the release of neurotransmitters [26]. At the same time, Syn also participates in synaptic formation, and is closely related to the plasticity of synapses [27,28]. In embryonic development, the expression of Syn is consistent with the formation of synapses, and is a sign of synaptogenesis in which the number and distribution density can indirectly reflect the density of synapses. Its expression can be used as a specific marker for presynaptic terminals to reflect the density and distribution of synapses [29].

PSD-95 is a scaffolding protein with a molecular

weight of 90 to 95 kDa found in the synaptic dense region of glutamate synapses. As a postsynaptic membrane marker [30, 31], it is the main protein involved in NMDA receptor-mediated long-term potentiation (LTP) formation and learning and memory maintenance [32]. LTP is the main form of synaptic plasticity, and the biological basis of learning and memory [33]. The studies have shown that PSD-95 plays an important role in synaptic development, stabilization, and plasticity [32]. The removal of PSD-95 gene can cause changes in mouse synaptic LTP and learning and memory dysfunctions [34].

The behavior results of the experimental water maze test showed that the number of crossings across platform in the ECS group was significantly ($P < 0.01$) increased. In the animal behavior index, ECS showed alleviated spatial memory impairment in chronically stressed mice after emasculation. The results of the Western blot test showed that the expression of p-Erk1/2 ($P < 0.01$) and p-creb ($P < 0.05$) was increased in the ECS group. In the signal pathway cascade reaction ECS showed activation of the MAPK pathway and exhibited estrogen protection. The expression of Syn ($P < 0.05$) and PSD-95 ($P < 0.05$) was also increased. In the synaptic plasticity-related proteins, the extract of ECS showed an increase in the expression of cognitive related synaptophysin. Nerve electrophysiological LTP results showed that the slope percentage of fEPSP in ECS group was significantly ($P < 0.01$) higher. In addition, ECS showed a function of improving spatial memory impairment in chronically stressed mice after emasculation. The results of HE staining showed that the pyramidal cell morphology of hippocampal CA1 region in ECS group was normally arranged, balanced, neatly and structurally clear, which reduced the neuronal damage in hippocampus and had neuroprotective effect.

CONCLUSION

The extract of ECS, by activating MARK pathway, may increase the expression of p-Erk1/2 and its downstream signal factor p-creb, and the expression of synaptic plasticity-related proteins Syn and PSD-95, and improving the synaptic structural and functional plasticity, protect the hippocampal pyramidal cell morphology, play a neuroprotective effect, and finally improve the cognitive dysfunction in chronically stressed mice after emasculation.

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Conflict of interest*: The authors declared that they have no conflicts of interest to this work.*

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