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Original Article

α-Asarone ameliorated learning and memory ability in fragile x syndrome model mice via down-regulating p-ERK1/2 expression

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Abstract: Objective: To investigate whether α-asarone can modify learning and memory ability in *Fmr1* knockout mice. Methods: Seventy FVB mice, including sixty knockout mice (KO) and ten wildtype (WT) mice, with an age of 30 days were used. The KO mice were divided into six groups: blank control group, vehicle group, 0.1 mg/kg α-asarone group, 1 mg/kg α-asarone group, 3 mg/kg α-asarone group, 4.5 mg/kg α-asarone group.The WT mice blank control group was the last group. A passive-avoidance assay was utilized to assess learning and memory performance, while western blot was used to detect the expression level of ERK1/2 and p-ERK1/2 in different brain region of FVB mice. The results were analyzed by independent sample t-test and one-way ANOVA. Results: The results of step-through test demonstrated that the KO mice given 3 mg/kg and 4.5 mg/kg had longer latency and less error count compared to vehicle ($P<0.05$). The expression of p-ERK1/2 in hippocampal CA1 and CA3 region in KO mice blank control group was profoundly higher than the WT ($P<0.05$). α-Asarone significantly reduced p-ERK1/2 expression in hippocampus in KO mice at the dose of 1.0 mg/kg, 3.0 mg/kg and 4.5 mg/kg comparing with vehicle ($P<0.05$). However, α-asarone did not change the ERK1/2 expression level in tissue of KO mice. Conclusion: α-Asarone can ameliorate learning and memory ability of *Fmr1* KO mice may be via down-regulating p-ERK1/2 expression. These findings suggest that α-asarone might have a therapeutic effect on FXS and support p-ERK1/2 as a potential therapeutic target.

Keywords: *Fmr1* knockout mice, α-Asarone, ERK1/2, learning and memory

Introduction

Fragile X syndrome, one of the most common X-linked mental retardation monogenic hereditary disease, is most often caused by an abnormal expansion of a trinucleotide repeat (CGG) in the 5' terminal untranslated region of fragile X mental retardation 1 gene (FMR1) leading to little or no expression of FMR-1 protein (FMRP) [1]. FMRP is argued to play a crucial role in refining synapses and dendrites during early brain development [2]. Therefore, deficits in cognition and impairment in memory were frequently observed in FXS patients. The *Fmr1* KO mouse, an effective model for FXS, was initially generated via inserting a Neo cassette into exon 5 of the *Fmr1* coding region of the mouse ortholog in 1994 [3].

It is obvious that mGluR5 is ideally suitable as a drug target for pharmacological treatment of FXS. Many of the therapeutic strategies are based on the mGlu5-mediated signaling pathway, such as 2-methyl-6-phenylethynyl-pyridine (MPEP) and Fenobam, which were able to ameliorate not only the deficits in behaviors but also in memory and learning ability [4, 5]. Although mGluR5 antagonism appears to be promising, the side effects of mGluR5 antagonism need to be paid attention to. Cruz-Martin, et al [6] found

that blocking mGluRs exaggerated spine immaturity in *Fmr1* KO mice. Thus, it is necessary to develop another effective therapy with fewer or no adverse effects for FXS.

α-Asarone (trans-1-propenyl-2,4,5-trimethoxybenzene) is a principal active oil compound isolated from Acorus Gramineus which were reported to be effective in alleviating dementia in Ayurveda, an Indian medicine system [7]. Recently, one research found that α-asarone effectively mitigated cognitive impairment incurred by scopolamine in mice [8]. The other investigators showed that α-asarone could ameliorate spatial memory impairment in β-amyloid (Aβ)-treated rats [9] and meliorate memory deficits in LPS-treated mice [10].

Studies have suggested that lovastatin, a 3-hydroxy-3-methylglutarylcoenzyme A (HMG-CoA) reductase inhibitor, could correct cognitive disorder caused by excessive Ras in neurofibromatosis type 1 model mice [11] and prevent epilepsy induced by reducing the signaling of Ras-ERK1/2 [12]. Lately, an open-label study showed lovastatin dramatically improved the abnormal behaviors in children and adults with fragile X syndrome, especially social skills and learning ability [13]. α-Asarone is also a HMG-CoA reductase inhibitor [14], which has the similar structure to lovastatin. Therefore, in the current study, we attempted to verify whether α-asarone could ameliorate learning and memory deficits in mutant mice and uncover the mechanisms in this process. Importantly, α-asarone may represent the novel drug for FXS therapy in the future.

Materials and methods

Animal

The FVB *Fmr1* KO mice and their WT FVB inbred strain mice were obtained from Professor Oostra. Both WT and KO mice were housed separately with a standard environment (23 ± 1°C, 50% ± 5% humidity) and allowed food and water ad libitum with a 12-hour light/dark cycle in the Laboratory Animal Research Center of Guangzhou Medical University.

Experiment procedure

A total of 70 FVB mice (aged 30 d) of both gender, including 60 KO and 10 WT, were used in this research. Before experiments, all mice would be identified to be pure breed according to a detection method used in previous studies [15]. The KO mice were randomly divided into 4 treatment groups (0.1 mg/kg, 1 mg/kg, 3 mg/kg, 4.5 mg/kg), one vehicle group and one blank control group. The WT mice were the other blank control group. There were 10 mice in each group. α-Asarone (List Pharmaceutical Co, Chengdu, China) was dissolved in 0.9% saline and injected intraperitoneally (i.p.) once daily between 9 and 10 am for 9 consecutive days. Vehicle group was injected with an equal volume of the 0.9% saline. Behavioral testing for learning and memory ability were done rightly 30 minutes after injection on day 7 and day 8. And the brain tissues were preparing on day 9. The animal experiments were approved by the Guangzhou Medical University Institutional Animal Care and Use Committee. Also, all efforts were made to minimize the number of experimental animals and ease their suffering.

Step-through passive avoidance test

Learning and memory performance was evaluated in step-through test, which conducted with an experimental box measuring 36×12×12 cm. The experimental box (Chengdu Technology & market Co, Chengdu, China) consisted of six modular shuttle boxes that could start six tests individually at the same time. Each modular shuttle box contained two chambers which were separated from each other by a guillotine door, one chamber was illuminated with bright light and the other was covered with black felt. There was a floor of stainless steel bars in both chambers connecting to an internal shock source.

The first day was the training day, each mouse was allowed to explore the apparatus for 3 minutes with the guillotine door open. After the apparatus acclimatization, the mouse was placed in light compartment back to the guillotine door which was open and a low intensity foot shock (36 v) was delivered in dark compartment for 5 minutes. The whole assay period was 300 seconds. When the mouse stepped into the dark chamber from the light one, it would be punished by a mild foot shock. Before being returned to the cage, the mouse was allowed to stay in dark compartment with another 30 seconds. The memory formed in the 1st day, and learning ability was tested in the following day. For the 1st trial 24 h later, the protocol was repeated but without delivering a

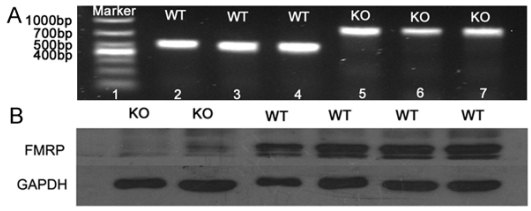

Figure 1. The identification of genotype and Fmrp in WT and KO mice. A. A 468 bp band was found in wildtype mice and a 800 bp band was found in *Fmr1* knock out mice. B. The fragile X mental retardation protein (FMRP) was expressed fully in WT mice and no expression of FMRP was detected in KO mice.

foot shock in dark chamber. When the mouse entered into the dark chamber with its all four paws, the latency was measured. Then, the times that the mouse crossed to the dark side in 300 seconds were the error counts. The behavioral assay was carried out from 10 to 12 am.

Western blotting

On day 9, the mice were decapitated under deep anesthesia (chloral hydrate, i.p., Kemiou Chemical Reagent Co, Tianjin, China) and their brains were removed rapidly at 30 minutes after finishing α-asarone injection.

The hippocampal CA1 and CA3 region were dissected on ice with optical microscope. To gain total tissue lysate, every 100 mg of tissue was mixed with 1000 ul of RIPA lysis buffer, 10 μl phenylmethanesulfonyl fluoride, and 10 μl phosphatase inhibitor (Paragon Biotech Co, Guangzhou, China). Then, the cocktail was homogenized fully on ice and centrifuged at 12000 rpm at 4°C for 5 minutes. After centrifuging, the supernatant was transferred into other centrifuge tubes and stored at -80°C. Protein concentration of the supernatant was determined in triplicate using the bicinchoninic acid protein assay kit (Paragon Biotech Co, Guangzhou, China).

Equal amounts of protein (30 ug) per lane were fractionated electrophoretically by 10% SDS-polyacrylamide gels, and proteins were transferred to PVDF membranes (Millipore, USA). After blocking in 5% slim milk dissolving in TBST for 1.5 hour, the membranes were incubated with rabbit anti-ERK1/2 (1:6000 dilution, Cell Signaling Technology, Beverly, MA, USA) or rabbit anti-P-ERK1/2 (1:2000 dilution, Cell Signaling Technology, Beverly, MA, USA) and mouse anti-GAPDH (1:10000 dilution, Proteintech Group Inc, Chicago, IL, USA) overnight in 4°C. Then, the membranes were developed using horseradish peroxidase-conjugated goat anti-rabbit (1:2000 dilution, Beyotime Institute of Biotechnology, Jiangsu, China) and goat anti-mouse secondary antibodie (1:10000 dilution, Beyotime Institute of Biotechnology, Jiangsu, China) for 2 hour at 25°C, followed by detection with enhanced chemiluminescence by a ECL kit (Bio-Rad, Hercules, CA, USA). The results of western bolt were quantified by Quantity One (Bio-Rad, Hercules, CA, USA). The results of the treatment and control groups were compared on the same gel.

Statistics

Statistical analyses were performed, using the Statistical Package for Social Sciences version 13.0 (SPSS Inc., Chicago, IL USA).

In step-through test, data was presented as mean ± SD. Results between homologous group of KO and WT mice were evaluated using the Independent-Sample T Test while the results of α-asarone treatment groups versus the vehicle group and blank control group of KO mice were analyzed by one-way ANOVA. A probability value of $P<0.05$ was considered statistically significant.

In western blot, one-way ANOVA was performed on the comparisons of optical densities among different groups of KO mice. And the Independent-Sample T Test was used to analyze the data between KO and WT blank control group. A probability value of $P<0.05$ was considered statistically significant.

Results

Confirming each experimental mouse is pure breed

Before each experiment, we used polymerase chain reaction (PCR) and western blot to identify the breed of experimental animals. In PCR, we observed the 468 bp band in WT mice and the 800 bp band in *Fmr1* KO mice (**Figure 1A**). In western blot, WT mice expressed FMRP fully while there was no expression in KO mice (**Figure 1B**). The genotyping results of PCR were completely consistent with those using western

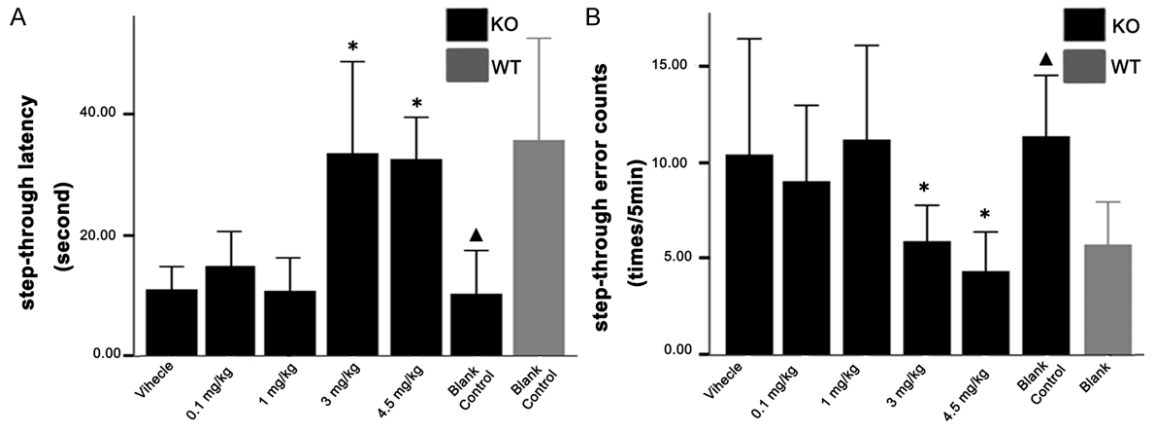

Figure 2. Effect of α-asarone on latency and error count of KO mice and WT mice in the step-through test. A. The latency of the blank control group of KO mice were significantly shorter than the WT mice (▲P<0.05); The latency of α-asarone treatment groups with 3 mg/kg and 4.5 mg/kg were clearly prolonged compared with the vehicle group (*P<0.05). B. The error count of the blank control group of KO mice were significantly increased than the WT mice (▲P<0.05); α-asarone treatment groups with 3 mg/kg and 4.5 mg/kg significantly reduced the error count compared to vehicle group (*P<0.05).

blot, which confirmed the experimental animals pure breed absolutely.

The Fmr1 KO mice have learning and memory deficit

The step-through test was carried out over two days. Day 1 was the phase of memory acquisition, and then learning ability was tested on day 2. The data that displayed above was from day 2 while data of the 1st day not shown. In the step-through test, the latency for the blank control group of KO mice were significantly shorter than the WT mice (P<0.05; **Figure 2A**). Likewise, the error count were significantly increased in the KO mice compared with the homologous WT group (P<0.05; **Figure 2B**).

α-Asarone prolongs the latency and reduces the error count in KO mice

Neither the latency nor error count between the blank control group and vehicle group of KO mice was statistically significant (P>0.05; **Figure 2A**, **2B**). And compared with the vehicle group, the latency of α-asarone treatment groups, especially at dose of 0.1 mg/kg, 3 mg/kg, 4.5 mg/kg, were clearly prolonged, but only at dose of 3 mg/kg and 4.5 mg/kg showed statistical significance (P<0.05; **Figure 2A**). Interestingly, α-asarone treatment groups with 3 mg/kg and 4.5 mg/kg significantly reduced the error count (P<0.05; **Figure 2B**) compared to vehicle group while the treatment groups with 0.1 mg/kg and 1 mg/kg had no significant difference (P>0.05; **Figure 2B**).

The p-ERK1/2 is highly expressed in Fmr1 KO mice

After the behavioral tests, we detected the ERK1/2 and p-ERK1/2 expression in hippocampal CA1 and CA3 region of FVB KO mice and WT mice. Compared to WT mice, the ERK1/2 level in both CA1 and CA3 region of KO blank control group was slightly decreased but neither of them had the significant difference (P>0.05; **Figure 3A**). However, the level of p-ERK1/2 expression in both CA1 and CA3 region was markedly increased and both of them had the statistical significance (P<0.05; **Figure 3B**).

α-Asarone down-regulates the expression of p-ERK1/2 in Fmr1 KO mice

After 9-days consecutive treatment with different doses of α-asarone, no statistical significance were found in both ERK1/2 and p-ERK1/2 expression in hippocampal CA1 and CA3 region between vehicle and blank control group of KO mice (P>0.05; **Figure 4A**, **4B**). Interestingly, compared to the vehicle, the expression of p-ERK1/2 in CA1 and CA3 region was decreased profoundly and had statistical significance in treatment groups at the doses

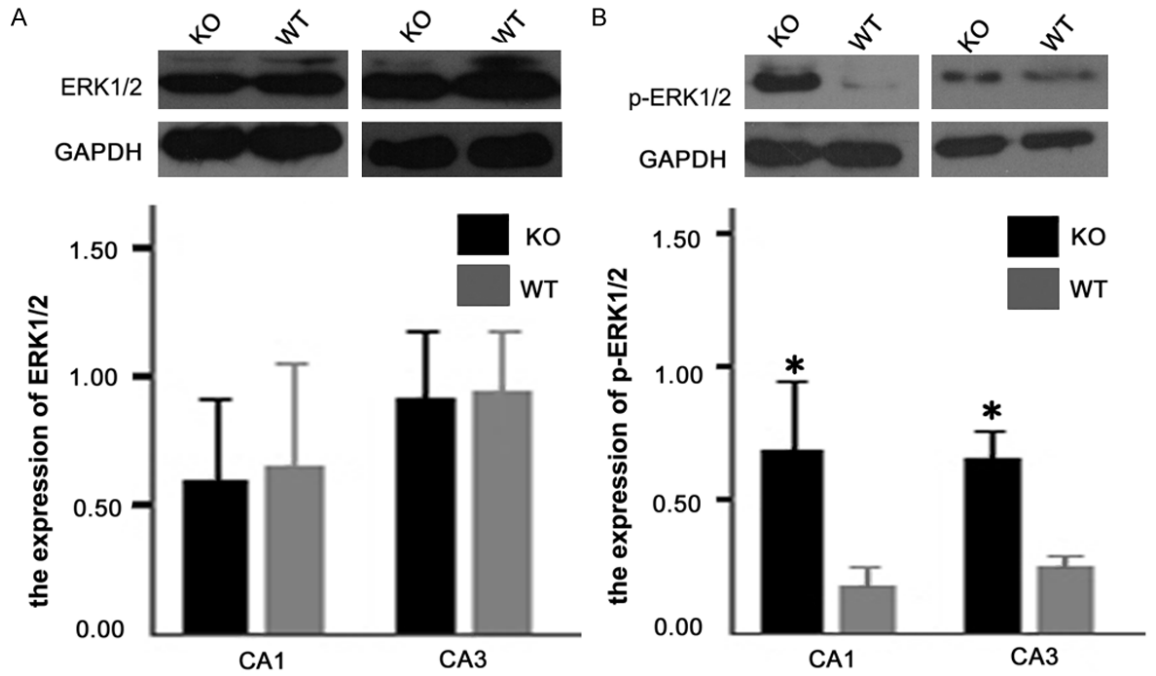

Figure 3. The expression of ERK1/2 and p-ERK1/2 in hippocampal CA1 and CA3 region of KO and WT mice. A. Compared to WT mice, the ERK1/2 level in both CA1 and CA3 region of KO blank control group was slightly decreased but neither of them had the significant difference ($P>0.05$). B. The level of p-ERK1/2 expression in both CA1 and CA3 region was markedly increased and both of them had the statistical significance.

of 1 mg/kg, 3 mg/kg and 4.5 mg/kg ($P<0.05$; **Figure 4A**, **4B**), but there was no significant difference in 0.1 mg/kg treatment group ($P>0.05$; **Figure 4A**, **4B**). However, we did not find any significant changes of ERK1/2 expression in either CA1 or CA3 region in different doses of α-asarone treatment groups when compared to the vehicle ($P>0.05$; **Figure 4A**, **4B**).

Discussion

Fragile X syndrome (FXS) is the most common inherited cause of intellectual disability that affects all major ethnic groups and races [1]. Thus, it is crucial to find the safe and effective treatment for FXS due to the rapidly growing patient population and the consequent huge burden on affected individuals, their families and care givers, and society as a whole. *Fmr1* KO mice share many similar symptoms with FXS patients, which make them be the perfect animal model for studying the features of FXS and drug therapeutics of this disease. Evidence from our early study showed that *Fmr1* KO mice exhibited defects in passive avoidance tests [16]. Irwin et al [17] and Galvez et al [18] found that *Fmr1* KO mice existed dendritic spine immaturity and learning and memory deficit. Results in our present research displayed that the latency for the blank control group of KO mice was significantly shorter and the error count was more than WT mice, showing that KO mice had learning and memory impairment which was in accordant with previous studies [19].

Excessive mGluR activation has been identified to be the main cause for the cognitive disorder in FXS [20]. Interestingly, mGluR agonist, dihydroxyphenylglycine, can activate ERK. And ERK activation plays a critical role in mGluR-dependent long-term depression. Therefore, aberrant mGluR activation may be responsible for the abnormal activation of ERK pathway in FXS.

ERK (Extracellular-signal regulated protein kinase), including ERK1 and ERK2, is one key member of the mitogen-activated protein kinase (MAPK) family of serine/threonine kinase in mammalian, functioning in regulating a series of biochemical processes, such as cell proliferation, cell differentiation, and apoptosis [21]. It is activated after phosphorylation of tyrosine and threonine residues by the extracellular

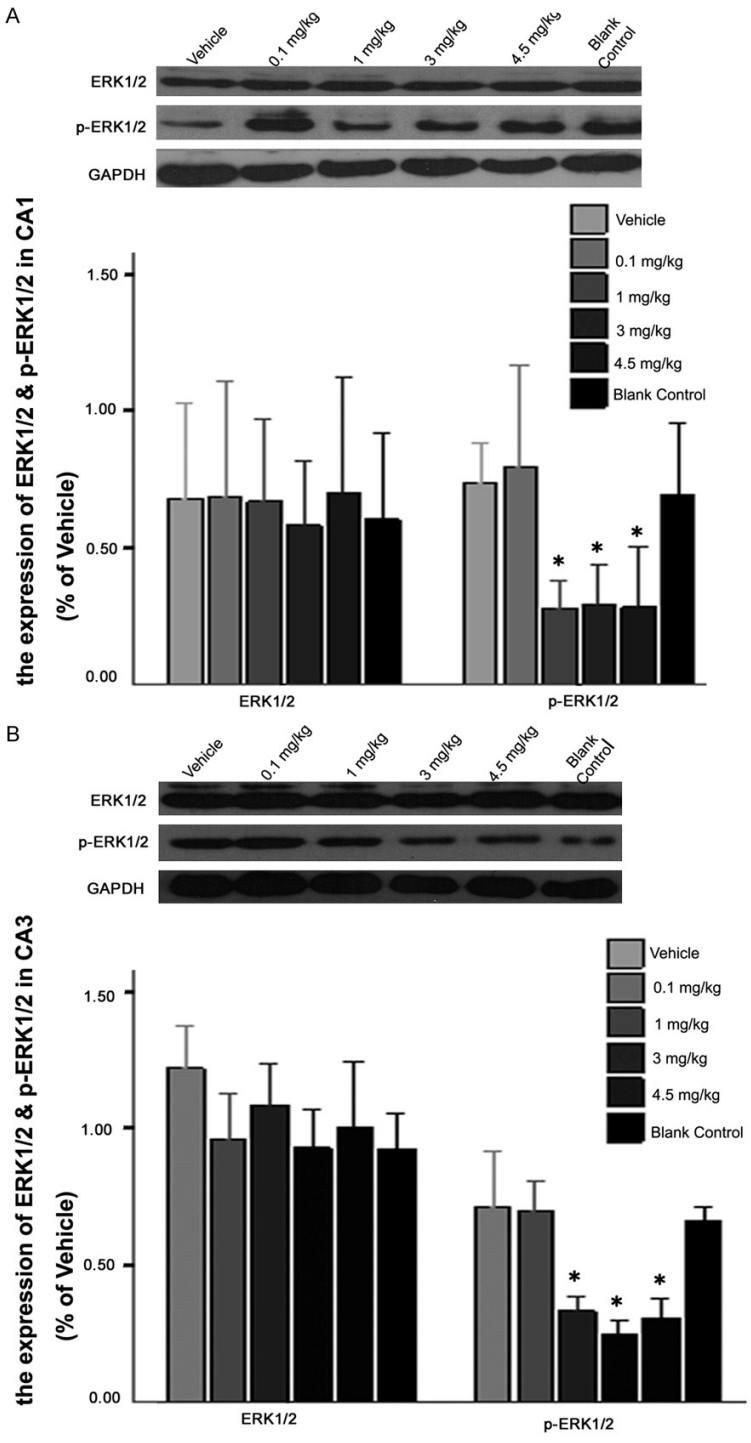

Figure 4. The effect of different doses of α-asarone on ERK1/2 and p-ERK1/2 levels in hippocampal of Fmr1 knockout mice. A, B. Compared to the vehicle, the expression of p-ERK1/2 in CA1 and CA3 region were decreased profoundly and had statistical significance in treatment groups at the doses of 1 mg/kg, 3 mg/kg and 4.5 mg/kg ($P<0.05$); There was no significant changes of ERK1/2 expression in either CA1 or CA3 region in different doses of α-asarone treatment groups when compared to the vehicle ($P>0.05$).

ligands such as neurotransmitters, growth factors and hormones, then its active form subsequently phosphorylate cytoplasmic targets or nuclear's [22]. ERK is abundantly expressed in central nervous system (CNS) and distributed in brain areas that related to learning and memory behavior in human, such as neocortex, hippocampus and striatum. Thomas et al [23] reported that ERK regulated synaptic activity and structural and functional plasticity, and was implicated in both long-term potentiation (LTP) and long-term depression (LTD), which is respectively considered to underlie memory and maintain neural plasticity. Mazzucchelli, et al [24] revealed that the latency for the ERK1 mutant mice was significantly prolonged in long-term retention test in avoidance tasks compared to WT mice. These findings above suggest that ERK1/2 may contribute to cognitive deficit and dendritic spine immaturity in FXS.

Experimental studies have reported that the basal level of p-ERK in hippocampal synaptoneurosomes of *Fmr1* KO mice increased [25, 26]. Wang, et al [22] likewise found that the p-ERK and MEK1/2 highly expressed in brain tissue both in FXS patients and *Fmr1* KO mice. In our work, as expected, compared with WT blank control group, the expression of p-ERK1/2 of KO mice profoundly increased in both hippocampal CA1 and CA3 region while the expression of ERK1/2 had no significant difference.

Accumulating evidence suggested that α-asarone have cognitive enhancing effects that can be used to remedy learning and memory impairment [27]. Kumar et al [8] did intra-

peritoneal injection of α-asarone in scopolamine amnesic mice for 15 days, and these mice were tested with step-through passive avoidance test and the Y-maze test. Results showed that α-asarone ameliorated memory and cognitive function as indicated by prolonging the transfer latency time and spontaneous alternation time in behavioral testing above. Shin et al [10] did administer α-asarone orally in C57BL/6 mice before the LPS injection once a day for 3 days, and learning and memory deficit was evaluated by the Morris water maze test. They showed that α-asarone not only significantly prolonged the swimming time spent in the target and peri-target zones but also increased the number of target heading and memory score in the Morris water maze, which indicated α-asarone may be beneficial to cognitive impairment. Based on previous studies, in present research we used the step-through passive avoidance test to evaluate learning and memory performance of FVB KO mice. After 4 different doses of α-asarone (0.1 mg/kg, 1 mg/kg, 3 mg/kg, 4 mg/kg) i.p. injection for 9 continuous days, the *Fmr1* KO mice at dose of 3 mg/kg and 4.5 mg/kg exhibited an increased latency and a decreased error count comparing to vehicle group. The results from the step-through test implied α-asarone's ameliorating effect on learning and memory deficit in *Fmr1* KO mice.

For better understanding the related mechanisms of α-asarone's ameliorating effect on cognitive impairment in *Fmr1* KO mice, we used western blotting to detect the expression level of ERK1/2 and p-ERK1/2 in hippocampal tissue of mice. In the present study, α-asarone down-regulated the expression level of p-ERK1/2 in s both hippocampal CA1 and CA3 region in *Fmr1* KO mice, especially at dose of 1 mg/kg, 3 mg/kg and 4.5 mg/kg. From the results of our current study, α-asarone improved the learning and memory dysfunction in *Fmr1* knockout mice which may be related to the reduction of p-ERK1/2. However, 1 mg/kg of α-asarone did not rescue the passive avoidance deficit in KO mice. According to one of our early research, 9~24 mg/kg of α-asarone made the FVB KO mice sedation, while concentration of 3~6 mg/kg was beneficial to improve the abnormal behavior of KO mice [28]. Combining with the results of behavioral test and western blot, we inferred that the dose of 0.1 mg/kg and 1 mg/kg might be too low to change the behavior in KO mice or the number of the experimental mice in low dose group were not enough to cause a statistically change.

Conclusion

In summary, this study supports the hypothesis that α-asarone effectively ameliorates learning and memory ability of *Fmr1* KO mice. And the effect of α-asarone was found to be mediated via by down-regulation of p-ERK1/2 in hippocampus in KO mice, which indicated that the involvement of p-ERK1/2 contributed to the learning and memory deficit in FXS model mice. More importantly, our findings would provides a new thought, new targets and new theory basis for the treatment of fragile X syndrome. Further studies would be required to establish effective and safe dosage regimen of α-asarone for FXS model mice treatment and for FXS patients in the future.

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Disclosure of conflict of interest

None.

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