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Original Article

ERK1/2 phosphorylation in the hippocampus is associated with abnormal anxiety-related and social behaviors in Fmr1 knockout mice

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Abstract: Fragile X Syndrome (FXS) is one of the leading causes of mental retardation. In order to further investigate the role of phosphorylated ERK1/2 (p-ERK1/2) in abnormal behaviors in FXS, a series of behavioral experiments were used to compare anxiety-related responses and social interactions in Fmr1 knockout (KO) mice and wild type (WT) mice. In the open field test, velocity travel in the central area was increased in the KO mice compared with WT mice ($P<0.01$). Time mice spending in center was significantly decreased in KO mice ($P<0.05$). In the elevated plus maze test, the percentage of number of entrances was significantly decreased in open arm and increase in close arm ($P<0.05$) in KO mice compared with WT mice. In the three-chambered social approach test, KO mice exhibited significantly more approaches to the wire cup containing an acquaintance mouse than WT mice ($P<0.05$). Western blotting was used to detect ERK1/2 and p-ERK1/2 expression in hippocampal CA1 and CA3 region of Fmr1 KO mice and WT mice Expression of pERK1/2 of KO mice markedly increased in the hippocampal CA1 region compared with the WT group ($P<0.05$), while expression of ERK1/2 had no significant difference ($P>0.05$). The results show that ERK1/2 phosphorylation in the hippocampus is associated with abnormal anxiety-related and social behaviors in Fmr1 knockout mice. Altered ERK1/2 phosphorylation may play an important role in abnormal anxiety-related responses and social interactions in FXS patients. These findings suggest that ERK1/2 might be suitable as a new drug target for pharmacological treatment of FXS.

Keywords: Behavior, extracellular signal-regulated kinase (ERK), Fmr1 knockout mouse, hippocampus, fragile X syndrome

Introduction

Fragile X Syndrome (FXS) is one of the leading causes of mental retardation. In FXS, CGG triplet expansion in the fragile X mental retardation gene 1 (Fmr1) prevents the synthesis of the fragile X mental retardation protein (FMRP), causing anatomical and functional alterations, such as abnormal dendrite spines morphology and dysfunctions in synaptic plasticity. FMRP is argued to play a crucial role in refining synapses and dendrites during early brain development [1, 2]. Individuals affected by FXS suffer from mental retardation, learning disabilities, and attention deficit. Patients also show behavioral problems including anxiety, autism, hyperactivity, and aggression [3-5]. Fmr1 knockout mice, which have undetectable levels of Fmr1 mRNA and FMRP, have been demonstrated to be an appropriate model for FXS as exhibiting several of the physical and behavioral characteristics of the human syndrome [6-8].

FMR1 silencing has many consequences, including up-regulation of metabotropic glutamate receptor 5 (mGluR5)-mediated signaling, which contributes to impaired anxiety-related and social behavior of FXS. Reducing group I mGluR signaling should alleviate some of the symptoms of FXS. mGluR5 up-regulates phosphorylation of extracellular signal-regulated kinase (ERK) 1/2 [9, 10]. Evidence has shown that hypersensitivity to mGluR5 and ERK1/2 leads to excessive protein synthesis in the hip-

pocampus of a mouse model of fragile X syndrome [11-14]. In order to further investigate the role of phosphorylated ERK1/2 (p-ERK1/2) in abnormal anxiety-related and social behaviors in the FXS mouse model, a series of behavioral experiments were performed to compare anxiety-related responses and social interactions in Fmr1 KO mice and wild type (WT) mice, and Western blotting to detect the ERK1/2 and p-ERK1/2 expression in hippocampal CA1 and CA3 region of Fmr1 KO mice and WT mice. The results show that abnormalities in synaptic signaling of ERK1/2 in the hippocampus are associated with poor behavior performance in Fmr1 knockout mice. Altered ERK1/2 phosphorylation may play an important role in the abnormal anxiety-related responses and social interactions in FXS patients. These findings suggest that ERK1/2 might be suitable as a new drug target for pharmacological treatment of FXS. This insight suggests the possibility of additional therapeutic targets besides mGluR5 for the treatment of fragile X syndrome.

Materials and methods

Subjects

FVB Fmr1 KO mice and the WT FVB inbred strain mice were obtained from Professor Oostra. Select *FMR1* KO homozygote (-/-) and WT homozygote (+/+) FVB inbred line mice. 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. The operation and breeding procedures of animals in this study conformed to animal breeding management standards of Guangdong Province and Guangzhou Medical College Laboratory, and followed humanitarian principles. Animal experiment was performed at the Institute of Neuroscience, Second Affiliated Hospital of Guangzhou Medical College, China. Experimental processes were maintained quiet indoors. Experimental procedures were performed in accordance with the Guidance Suggestions for the Care and Use of Laboratory Animals, issued by the Ministry of Science and Technology of the People's Republic of China.

Genotype identification

In order to ensure that the strains were pure bred, the correct targeting and integration was confirmed by genotyping using polymerase chain reaction (PCR) and electrophoresis. Primer 5'-AGTCATGCTATGGATATCAG-3' and 5'-TGGGCTCTATGGCTTCTGA-3' were designed to detect KO mice. Primer 5'-GTGGTTA-GCTAAAGTGAGGATGAT-3' and 5'-CAGGTTTGTT-GGGATTAACAGATC-3' were designed to detect WT mice.

Visual placing responses

Before the detailed behavioral assays, visual acuity was evaluated using visual placing responses, because poor visual acuity influences the results of most behavioral tests. When suspended by the tail and lowered toward the table, all mice raised their heads and reached out their forelimbs for the surface of the table, indicating no difference in visual acuity between groups.

Open field test

The open field test was described previously [15, 16]. Each test mouse was placed in the center of an open field arena (70 × 70 cm each) illuminated at 600 lux and allowed to explore for 5 minutes. Each parameter in 5 min was evaluated with a computer video-tracking system (Smart v2.5.21; Panlab, Spain).

Elevated plus maze test

The elevated plus maze test was described previously [17-19]. The elevated plus-maze was elevated to a height of 50 cm above the floor and consisted of two open arms and two enclosed arms (30 × 5 cm each) that extended from a central area (5 × 5 cm). The enclosed arms were surrounded by 15-cm-high walls. Each test mouse was placed in the central area facing one of the open arms and allowed to explore for 5 min. Each parameter was analyzed using a computer video-tracking system (Smart v2.5.21).

Three chambered social approach test

To investigate sociability and social novelty preference, the three-chambered social approach test was described previously [20-22]. Sociability is defined as the test mice tend to social contact with a familiar mouse versus an inanimate object. Furthermore, social novelty preference is defined as the test mice tend to social contact with a novel stranger versus a

familiar mouse. The three-chamber apparatus was a transparent plastic box with two transparent acrylic partitions with a rectangular opening (6 × 6 cm). The acrylic partitions divided the box into three chambers (left, center, and right; 20 × 40 × 23 cm each), which were illuminated at 120 lux. Each side chamber contained a cuboid wire cage (7.2 × 7.7 × 17 cm each) in the corner to hold a stimulus mouse. The wire cage consisted of wire bars, allowing minimal contact between mice, and preventing fighting. A white bottle was placed on the top of the wire cage to prevent the test mouse from climbing to the top of the cage. All stimulus mice were same background, age, gender and weight mice, which were acclimated to the wire cages for 10 min before beginning the test. The test consisted of three phases: acclimation, sociability, and social novelty preference. During the acclimation phase, a test mouse was placed in the center chamber and allowed to freely investigate and habituate to all three chambers and wire cages for 10 min. A familiar littermate was then placed in one of the wire cages. A wire cage containing a small ball was placed in the other side of three-chamber as a non-social and inanimate object. During the second phase, the test mouse was placed in the center chamber and allowed to freely investigate all three chambers for 10 minutes. The second phase was referred to sociability test. Subsequently, a novel unfamiliar mouse introduced as a novel stranger mouse was replaced for the small ball within another wire cage in the other side of the three-chamber apparatus during the sociability test. During the third phase, the test mouse was placed in the center chamber and allowed to freely investigate all three chambers for 10 minutes. The third phase was referred to as the social novelty preference test. Movement of the test mouse was recorded with a video camera and movement tracks were analyzed with Smart v2.5.21. Each parameter, including number of events, duration, and mean time per event in test was measured.

Western blotting

The mice were decapitated under deep anesthesia (chloral hydrate, i.p., Kemiou Chemical Reagent Co, Tianjin, China) and their brains were removed rapidly. 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 μL 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 12,000 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 (1:10,000 dilution, Cell Signaling Technology, Beverly, MA, USA) or rabbit anti-P-ERK1/2 (1:10,000 dilution, Cell Signaling Technology, Beverly, MA, USA) and mouse anti-GAPDH (1:10,000 dilution, Protein-tech Group Inc, Chicago, IL, USA) overnight in 4°C. Then, the membranes were developed using horseradish peroxidase-conjugated goat anti-rabbit (1:10,000 dilution, Beyotime Institute of Biotechnology, Jiangsu, China) and goat anti-mouse secondary antibody (1:10,000 dilution, Beyotime Institute of Biotechnology, Jiangsu, China) for 2 hours at 25°C, followed by detection with enhanced chemiluminescence by a ECL kit (Bio-Rad, Hercules, CA, USA). The results of Western blot were quantified by Quantity One (Bio-Rad, Hercules, CA, USA). The results of 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). Data are presented as mean ± SD. The Independent-Sample T Test was used to analyze the data between KO and WT control group. A probability value of $P<0.05$ was considered statistically significant.

Results

Laboratory animal model testing and visual placing responses

The KO and WT mice PCR results are shown in **Figure 1**. KO mice showed amplification of about 800-bp DNA fragments. WT mice showed

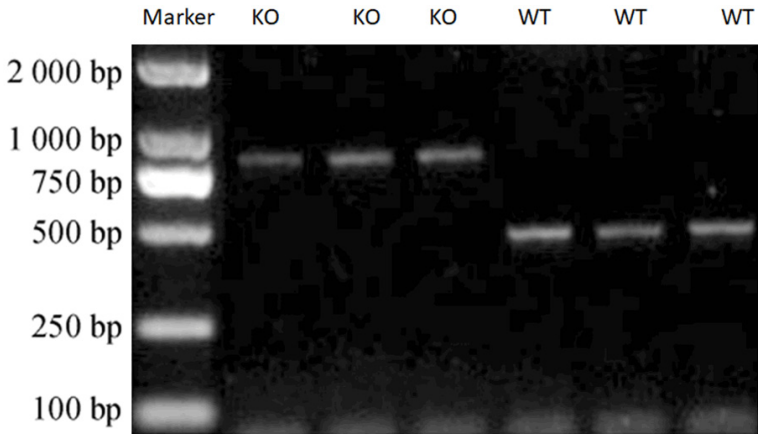

Figure 1. PCR amplification of Fmr1 fragment of KO/WT mice.

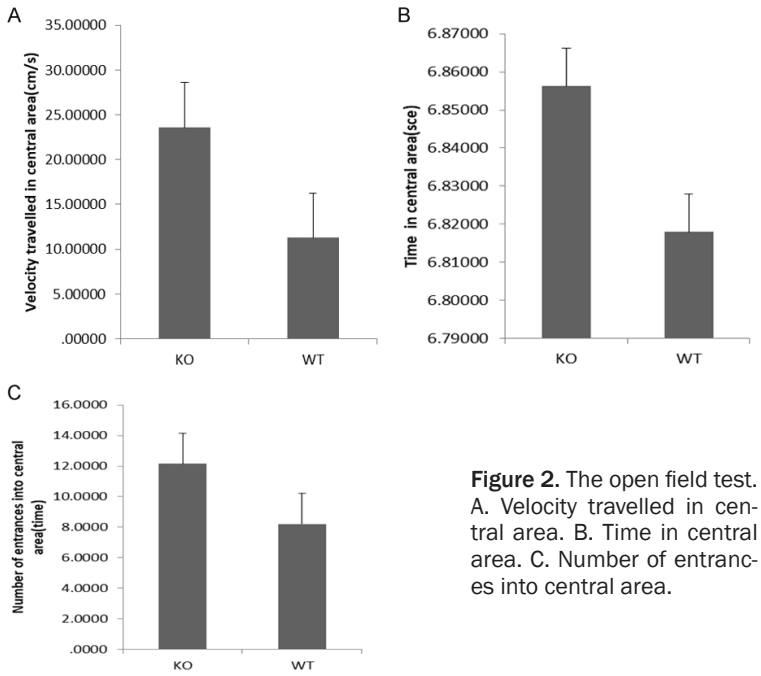

Figure 2. The open field test. A. Velocity travelled in central area. B. Time in central area. C. Number of entrances into central area.

amplification of about 468 bp DNA fragments. KO mouse showed irritability hyperactivity, and aggression, and prone to epilepsy. Male mice exhibited macroorchidism. All mice raised their heads and reached out their forelimbs for the surface of the table, indicating no difference in visual acuity between groups.

Altered thigmotaxis and anxiety-related in the Fmr1 knockout mouse

To evaluate spontaneous motor activity, exploratory behaviors, and emotional responses in a novel environment, the open field test was performed. During the 5-minute test session, in central area, the velocity travelled were increased in KO mice compared with WT mice (**Figure 2A**, T=3.587, p=0.002). Time mice spending in center was significantly decreased in KO mice (**Figure 2B**, T=-2.239, p=0.036). In addition, although the number of entrances into central area of mice was not significantly different between groups, there were trend towards increased number in central area in KO mice compared with WT mice (**Figure 2C**, T=2.011, P=0.057). In a novel, brightly lit arena, mice prefer the periphery to the central areas and tend to run or walk along the wall, a behavior called thigmotaxis. The results suggest that thigmotaxis mice exhibiting was significantly increased in KO group compared with WT group mice.

To investigate anxiety-related behaviors, the elevated plus maze test was performed. In the elevated plus maze test, the percentage of number of entrances was significantly decreased in open arm and increased in the close arm (**Figure 3A**, T=-2.719, p=0.015; T= 2.719, p=0.015;) in KO mice compared with WT mice. In addition, the distance travelled totally in open arm and close arm were significantly decreased in KO group mice compared with WT mice (**Figure 3B**, T=-2.180, p=0.044). These results suggest that KO mice displayed enhanced motor activity in close arm with anxiety-related behaviors.

Elevated levels of social anxiety in the presence of unfamiliar partners

To examine social behaviors, including the sociability and social novelty preference, we performed the three-chambered social approach test. In the sociability test, a familiar (acquaintance) littermate was placed within a wire cage, which permitted visual, olfactory, audito-

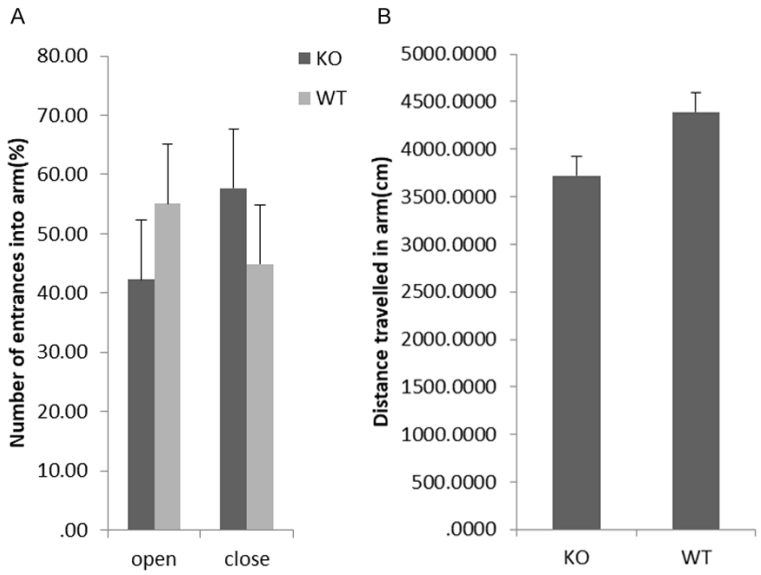

Figure 3. The elevated plus maze test. A. The percentage of number of entrances into arm. B. The distance travelled in arm.

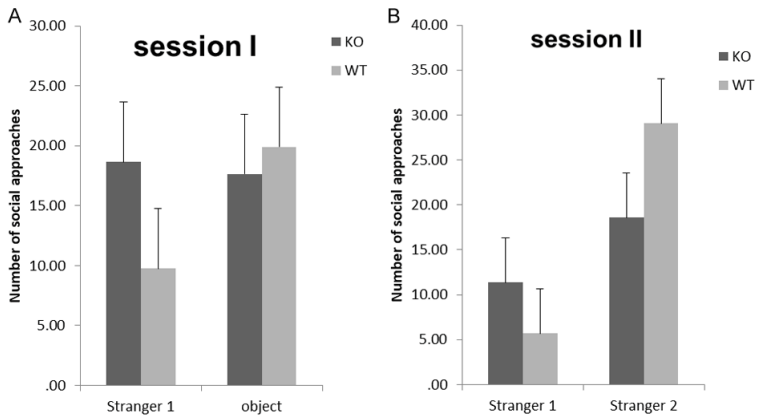

Figure 4. The three-chambered social approach test. A. Number of social approaches in the sociability test. B. Number of social approaches in the social novelty preference test.

ry, and some tactile contact without fighting, in one side of the three-chamber apparatus. A wire cage containing a small ball was placed in the other side of three-chamber as a non-social and inanimate object. There was no significant difference between two groups for the duration of social interaction initiated by the subjects, although KO mice exhibited significantly more approaches to the wire cup containing an acquaintance mouse than WT mice (**Figure 4A**, T=2.331, p=0.026). In the social novelty preference test, the first familiar mouse used in the sociability test remained within the same cage. A novel unfamiliar mouse introduced as a novel stranger mouse was replaced for the small ball within another wire cage in the other side of the three-chamber apparatus. KO mice exhibited significantly more approaches to the wire cup containing an acquaintance mouse than WT mice (**Figure 4B**, T=2.295, p=0.029), although the result was not statistically significant for the duration of social interaction initiated by the subjects, too.

Up-regulating p-ERK1/2 expression in the Fmr1 knockout mouse

To detect the ERK1/2 and p-ERK1/2 expression in hippocampal CA1 and CA3 region of FVB KO mice and WT mice, Western blotting was used. Interestingly, the ERK1/2 level in both CA1 and CA3 region of the KO group was slightly decreased but not statistically significant compared to WT mice (**Figure 5A**; P>0.05). However, the level of p-ERK1/2 expression in both CA1 and CA3 region was markedly increased though only of CA1 had the statistical significance (**Figure 5B**; T=2.230, P=0.03).

Discussion

FXS is the most common inherited cause of intellectual disability that affects all major ethnic groups and races. 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 [6-8].

Results in our present research displayed that KO mice had elevated levels of social anxiety in the presence of unfamiliar partners which was in accordant with previous studies. However,

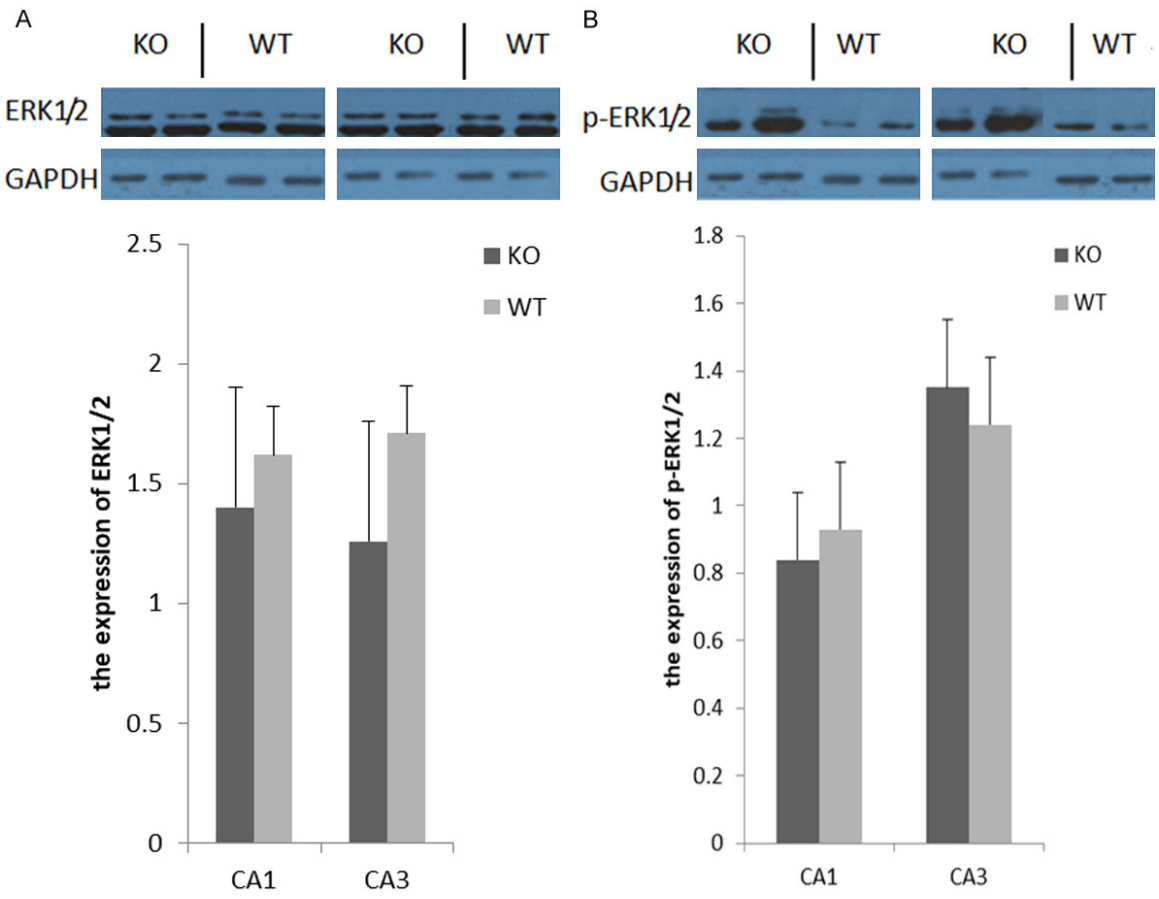

Figure 5. Expression of ERK1/2 and p-ERK1/2 in hippocampal CA1 and CA3 region of KO and WT mice. A. Level of p-ERK1/2 expression. B. Level of p-ERK1/2 expression.

Fmr1 KO mice respond with increased social approach with a very familiar partner rather than the duration of social interaction initiated by the subjects. These data are in line with the view of Corinne M. Spencer et al. that the most consistent and robust difference is the number of active approaches rather than the time spent in interactions initiated by the subject mouse. Once the approach has occurred, the duration of interaction depends on the cooperation of both the mouse that initiated the interaction and the recipient mouse. Thus, regardless of the interest of the initiating mouse, if the partner is not cooperative, the duration of the interaction will be shorter [20-22].

In the present study, the Fmr1 knockout mouse showed hyperactivity with thigmotaxis and enhanced motor activity in close arm with anxiety-related behaviors. These results are different to those reported that Fmr1 KO mice showed decreased anxiety-like responses in open-field and light/dark tests [23]. However, these different behavioral phenotypes we observed in the mouse models are consistent with behaviors observed in individuals with FXS. There are several possible explanations for these results. FXS patients may exhibit cognitive impairment, hyperactivity, attention deficits, social difficulties and anxiety, and autistic-like behaviors. The degree to which patients display these behaviors varies considerably and is influenced by family history, suggesting that genetic modifiers play a role in the expression of behaviors in FXS. The genetic background of the laboratory mouse contributed to the differences in the experimental results. Several studies have examined behavior in a mouse model of FXS in which the Fmr1 gene has been ablated. For many of these phenotypes, sometimes the effect size was greater in particular backgrounds, perhaps due to different baseline responses in the WT mice, variations in the genetic background, age, gender, and reproduce

times, and experimental conditions involving laboratory mice [24].

It has been shown that excessive mGluR activation is the main cause for the cognitive disorder in FXS and mGluR agonistcan can activate ERK. ERK activation plays a critical role in mGluR-dependent long-term depression [13, 25, 26]. Therefore, aberrant mGluR activation may be responsible for abnormal activation of the ERK pathway in FXS. ERK, 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 [9, 10, 27]. It is activated after phosphorylation of tyrosine and threonine residues by the extracellular quently phosphorylate? cytoplasmic targets or nuclear. 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 [28, 29]. Experimental studies have reported that the basal level of p-ERK in hippocampal synaptoneurosomes of Fmr1 KO mice increased. Wang, et al. likewise found that the p-ERK and MEK1/2 were highly expressed in brain tissue both in FXS patients and Fmr1 KO mice [11-13, 30-34]. In this research, expression of pERK1/2 of KO mice markedly increased in hippocampal CA1 region compared with the WT group, while the expression of ERK1/2 had no significant difference. ERK was present in a form indicating elevated activity in the FX mouse hippocampus.

In summary, this study shows that Fmr1 knockout mice have elevated levels of social anxiety, hyperactivity with thigmotaxis, and enhanced anxiety-related behaviors. They share many similar symptoms with FXS patients. Additionally, up-regulating p-ERK1/2 expression in the Fmr1 knockout mouse was observed, which suggested that a core defect in FXS is leaky translation in response to ERK1/2 activity. ERK1/2 phosphorylation in the hippocampus is associated with abnormal anxiety-related and social behaviors in Fmr1 knockout mice. These findings raise the possibility that ERK has a substantial treatment potential for the treatment of fragile X syndrome. This insight suggests the possibility of additional therapeutic targets besides mGluR5 for the treatment of fragile X syndrome.

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Disclosure of conflict of interest

None.

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