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RESEARCH ARTICLE

Phenotype analysis and rescue on female FVB.129-*Fmr1* knockout mice

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Abstract **Fragile X syndrome (FXS) is the most common monogenic cause of intellectual disability and a cause for autism. FXS females report milder phenotypes and a lower rate of cognitive problems compared to males. This is most likely because most females are heterozygous, while males are hemizygous for the disease. Thus, most preclinical studies have been completed in males. As there is major interest in testing experimental drugs for FXS, it is imperative to determine whether females in animal models used for research, present behavioral alterations that might translate to humans in order to confirm that experimental drugs have an effect on both genders. In our study we describe behavioral phenotypes in homozygous FXS female mice developed on the FVB.129 background. We focused on detection of hippocampal-mediated cognitive abilities and other behaviors described for FXS. Our research shows that, while female FVB.129-*Fmr1* knockout mice present normal learning, they have impaired memory, as well as susceptibility to audiogenic seizures. In agreement with previous reports in rodents and humans, significant levels of the small GTPase Rac1 were found in FXS female mice. Because Rac1 is involved in neuronal development, plasticity and behavior, we additionally aimed to pharmacologically inhibit Rac1 and determine whether observed phenotypes are rescued. Treatment of female FVB.129-*Fmr1* knockout with a Rac1 inhibitor abolished behavioral deficits, bringing phenotypes to control levels. Our results suggest that female FVB.129-*Fmr1* knockout mice display behavioral impairments that resemble FXS in humans. Moreover, those behavioral shortfalls might be associated with alteration of plasticity involving excessive Rac1 function, since pharmacological reduction of Rac1 normalizes previously altered phenotypes to control levels.**

Keywords autism, small GTPases, behavior, Fragile X syndrome, animal models

Introduction

Autism is considered a neurodevelopmental disorder with genetic and physiologic components, which seems to affect more males than females. For the female population it manifests mostly when there is a biological threshold that is crossed. Numerous investigations have been published regarding Fragile X syndrome (FXS), one autistic illness described by cognitive deficiencies and neuronal abnormalities. The majority of that research shows behavioral dysfunction in males carrying a mutation in the *Fmr1* gene that encrypts for FMRP, protein that is missing in FXS (Zhao et al., 2005; Baker et al., 2010; Pietropaolo et al., 2011). Not many studies have been performed in females with FXS trying to avoid hormonal modulation and other confounders. Indeed, it has been stated that estrogens might positively regulate learning and memory. These female hormones appear to be capable of affecting memory formation by regulating plasticity in the hippocampus (Bi et al., 2000; Bi et al., 2001; Zhao and Brinton, 2007; Fernandez et al., 2008; Lewis et al., 2008; Fan et al., 2010; Zhao et al., 2010, 2012; Fortress et al., 2013). Estrogens, acting over G-protein-coupled estrogen receptors (GPERs) are involved in local protein synthesis and other epigenetic processes that affect

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overall hippocampal cells. The fact that estrogen has an effect on hippocampal cells, as well as epigenetic processes and local protein synthesis, appears to be crucial for cognitive abilities, at least in rodents. G-protein-coupled estrogen receptors (or GPERs), as well as estrogen synthesized in the hippocampus, appear to have a role in hippocampus-mediated memory formation. *In vitro* studies have shown that activation of estrogens promotes dendritic spine remodeling (Hasegawa et al., in press; Kramár et al., 2009; Srivastava et al., 2008), and regulation of spinogenesis favoring memory formation. Moreover, the discovery that estrogen can be synthesized and released within the hippocampus (Hojo et al., 2004; Kretz et al., 2004; Prange-Kiel et al., 2006) raises the exciting possibility that learning-induced endogenous estrogen synthesis by hippocampal neurons may stimulate the rapid molecular alterations that are necessary for memory formation. This might explain the frequent differences observed between male and female despite both suffering an identical condition, such as autism.

Regarding FXS, there are few reports indicating that females have also attenuated capabilities similar to the ones described for their male counterparts (Lesniak-Karpiak et al., 2003; Baker et al., 2010). In the laboratory, most investigations describing preclinical studies have been performed using a FXS male rodent model developed on the C57BL/6 background (Ding et al., 2014) for example. Currently, a new mouse model developed on the FVB.129 background is available, in which there is also no stable FMRP protein produced, as described for other models. Behaviorally, this FVB.129-*Fmr1* knockout male mice present hyperactivity, learning and memory deficit, impaired cued conditioning behavior, altered startle response and pre-pulse inhibition, as well as high susceptibility to audiogenic seizures (Zhao et al., 2005; Pietropaolo et al., 2011). While there is evidence regarding the phenotypic alteration in FXS male model, few studies have explicitly explored similar behaviors in female KO mice, especially in the model developed in the FVB.129 strain. Thus, herein FVB.129-*Fmr1*-knockout and FVB.129 control female were tested for several memory paradigms as well as for susceptibility to suffer audiogenic seizures, a strong phenotype described for males. Female FVB.129-*Fmr1* knockout (KO) mice were found to have deficient memory and altered auditory skills as reported previously for their male counterpart. Furthermore, as recently reported in FXS humans (Fatemi et al., 2013) and male KO mice (Bongmba et al., 2011), levels of the small GTPase Rac1 were found to be elevated in the female KO mice. In an attempt to normalize levels of Rac1 to decode whether this protein has a role in behavioral deficits, we observed that treatment with Rac1 inhibitors improved cognitive capabilities and protected against audiogenic seizures outcomes. Our results indicate that, in order to test potential therapeutic effects, both genders of this FVB.129-*Fmr1* knockout mice could be used to help detect benefits on rescuing behavioral deficits.

Materials and methods

Animals

Female and male breeders for these experiments were attained from The Jackson Laboratories (Bar Harbor, ME). Mice were crossed and maintained at the University of Houston facility. For the experiments described here, only female mice were used, as wild type controls FVB.129P2-Pde6b + Tyrc-ch/AntJ ([WT];Jackson Laboratories Stock# 004828) and FXS mice FVB.129P2-Pde6b + Tyrc-ch Fmr1tm1Cgr/J ([KO]; Jackson Laboratories Stock# 004624). Mice were kept in groups and maintained under a 10:14 light/dark cycle under precise humidity (50%–55%) and temperature (21°C). Food and water were provided *ad libitum*. When appropriate, drugs used for experiments were dissolved in saline and injected intraperitoneally in a total volume of 50–100μLat the concentration indicated. All our experiments were executed in agreement with the Public Health Service policies and the Animal Welfare Act, with animal use protocols approved by the University of Houston Institutional Animal Care and Use Committee (IACUC).

Behavioral analysis

Different cohorts of female WT and KO mice on FVB.129 background were tested (4–6 months old). One group was tested on elevated plus maze, prepulse inhibiton and hot plate (n = 7–8/group). A second cohort was used to test for susceptibility to audiogenic seizures (n = 10–13/group). The third group was tested for prepulse inhibition and fear conditioning (n = 7/group) after being treated with either saline solution or a Rac1 inhibitor (NSC 23766 at 5 mg/kg bw for 10 days). A fourth cohort was treated with either saline solution or a Rac1 inhibitor (NSC 23766 at 5 mg/kg bw for 13 days) and tested for susceptibility to audiogenic seizures (n = 7/group). All tests were conducted between noon and 5pm.

Elevated plus maze (EPM)

The EPM measures anxiety levels in an animal. The EPM comprised two open arms and two closed arms (30 cm in length each) joining together through a central zone, all elevated 40 cm above the floor. Mice were positioned in the center and given five minutes to travel the entire maze. A computer recorded the number and duration of entries to open and closed arms by the animal (Noldus Ethovision XT). If animals remained significantly more time in the closed arms, that was measured as a sign of anxiety.

Startle response/prepulse inhibiton (PPI)

This test is based in the reflex all animals have to flinch in response to a sudden loud sound. This reflex decreases if a low sound (pre-pulse) is given before the loud sound. A pre-

pulse inhibition of startle response test (San Diego Instruments, San Diego, CA) was used to assess the animal's general reflexes (startle) and acoustic skills. The test session lasted for about 16 minutes during which the mice were given seven trial types repeated five times to make a total of six trials. Mice were positioned in a plexiglass tube enclosed in a sound-attenuated startle box and allowed 5 min to acclimate before the test session (Spencer et al., 2011). Trial types included (1) no stimulus test presented to measure baseline movement; (2) a startle alone test of 120 dB for 40 ms, to measure maximum startle response; (3) five test composed of different prepulse sounds for 20 ms (74, 78, 82, 86, or 90dB) given 100 ms before the startle stimulus of 120 dB. Repetition of trial types was done in a pseudo-random manner with an inter-trial interval of about 10–20 s. The amplitude of the mouse's movement upon hearing the sound was measured by an electrostatic sensor located directly below the plexiglass cylinder (Crawley, 2000). The startle response was recorded every 1 ms for a total of 65 ms following the start of the startle stimulus. The maximum startle amplitude was averaged 6 trials/mouse. This parameter was used to calculate the percent PPI as previously described (Paylor and Crawley, 1997).

Hot plate

A hot plate analgesic meter (Columbus Instruments, Columbus, Ohio) assessed the animals' analgesia response as an indicator of a sensory-perceptual function. This response is vital to learning in fear conditioning paradigm. Mice were individually placed onto a hotplate (25.4 cm × 25.4 cm) preheated to 55°C. Latency to respond by jumping, hind paw licking or hind paw flicking was measured, and at that time the animal was immediately removed from the hotplate. The tested mouse was also removed from the hot plate if after 30 s there was no response.

Fear conditioning

The Video Freeze Fear Conditioning (Med Associates Inc., St Albans, Vermont) was used for conditioning fear learning. The behavioral testing involved context-dependent learning (association of a particular setting with a 0.75 mA foot shock for 2 s) and cued-dependent learning (association of a particular tone with a 0.75 mA foot shock for 2 s). Mice were placed in a conditioning box (13 × 10.5 × 13 cm) prepared with a 28V light, a sound speaker, and a floor that had 19 metal rods all equally spaced. The box was placed inside sound-proof cubicles (56× 50 × 41 cm) with background noise-generating fans to reduce peripheral noises. In this 7-min test, a 30-s tone (80 dB, 2 kHz) headed a 2-s scrambled foot shock. The shock was presented at 2, 4 and 6min after session commencement. The occurrence of freezing was measured every 10 s throughout training. The occurrence of jumping after the foot shock was also recorded. This task can assess contextual conditioning (dependent on the hippocampus) and conditioning to the tone (mostly hippocampal-independent and amygdala-dependent task). To test for context, the animals were brought back to the training box 24 h after training for another 7-min test session. As part of this test, shocks were not administered. The occurrence of freezing as an indication of learning and recall was measured every 10 s. To test for cue/tone conditioning, the test box was altered by covering the floor and walls, as well as adding a different odor to produce a perceived novel test environment. The animals were introduced into this modified novel box for a 7-min session 24 h after training. The same tone provided during training was continuously administered after 3 min of being in the novel box, and lasted for 3 min. Freezing was measured during testing session. After testing was completed the animals were returned to their home-room in their home cages. The percentage freezing data was obtained as an index of learning and memory.

Induction of audiogenic seizures (AGS)

This test is based on the possibility of inducing convulsive seizures by applying intense auditory stimuli. Animal models of FXS have demonstrated a robust susceptibility to audiogenic seizures starting with wild running and followed by clonic seizures that progress to tonic seizures ending in the death of the animal. Female mice were placed individually into a cylinder located inside a sound-proof chamber with a window that allows surveillance of the animal. The mouse was then exposed to a sound level of 120 dB from an alarm for 5 min. Mice were observed and scored during the entire test to determine when they were suffering either no response/just wild running (considered a negative outcome), or clonic/tonic seizure followed by death caused by respiratory arrest (considered as positive response).

Brain extraction and protein isolation

For biochemical determinations, mice were subjected to decapitation in order to obtain the brains, which were rapidly dissected and flooded in ice-cold cutting saline solution (5 mM glucose, 110 mM sucrose, 60 mM NaCl, 28 mM $NaHCO_3$, 3 mM KCl, 1.25 mM NaH_2PO_4, 7 mM $MgCl_2$, and 0.5 mM $CaCl_2$, 0.6 mM ascorbate) saturated with 95% O_2/5% CO_2. Different areas of the brain were isolated (cortex, hippocampus, brain stem and cerebellum) and maintained always on ice or stored at − 80°C if needed, until analyzed. Samples were homogenized in homogenizing buffer (HBC; 10 mM Hepes, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 50 mM NaF, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 10 mg/mL leupeptin, 2 mg/mL aprotinin, 1 mM microcystin-LR, and 200 nM calyculin A). Samples were subjected to 1500 *g* for 10 min at 4°C to exclude debris. Protein concentration in the samples was determined by the Bradford assay. Brain lysates were then stored at − 80°C for subsequent biochemical analyses.

Electrophoresis and Western-blot analysis

Protein lysates were mixed with Laemelli buffer containing β-

mercaptoethanol and boiled for 5 min at 95°C followed by standard Western blotting protocols. An equivalent volume of protein (10 μg) from each lysate was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE, 12%) using a Mini Protean II system (BioRad, Hercules, CA) followed by sample electrophoretic transfer to a polyvinylidene difluoride (PVDF) membrane. Transferred samples in membranes were blocked by incubating them in Tween 20-Tris buffered saline (T-TBS, 50 mM Tris-HCl [pH 7.5-8.0], 150 mM NaCl, and 0.1% Tween 20) containing 5% non-fat milk. Incubation was held for 1 h at room temperature. Membranes were then incubated with Rac1 antibody (1:10000 from Millipore, Billerica, MA) overnight at 4°C. Samples were rinsed in T-TBS three times for 15 min. Blots were then incubated with a secondary antibody (horseradish peroxidase-conjugated goat antimouse IgG [1:5000] from Promega, Madison, WI) followed by three times T-TBS washing and then visualized using enhanced chemiluminescence (ECL, Amersham Biosciences, Piscataway, NJ) exposed to X-Omat Blue film (Kodak, Rochester, NY) for 15 s to 1 min.

Statistical analysis

Results are presented as means±standard error of the mean (S.E.M). One-way ANOVA was used to compare the mean of behavioral parameters between the WT and KO, followed by Tukey's post hoc test (Prism, GraphPad Software, Inc., CA). A $p \leqslant 0.05$ was considered statistically significant.

Results

Female FVB.129-*Fmr1* KO mice show a mild defect in pre-pulse inhibition (PPI)

As patients suffering FXS present auditory hypersensitivity, we tested our female mice on the startle response and pre-pulse inhibition test to determine whether they have any auditory impairment. We observed that female FVB.129-*Fmr1* KO mice present an altered reaction to auditory stimuli. These female FVB.129-*Fmr1* KO mice present a deficiency in acoustic response compared to the female FVB.129 control mice. Female KO mice exhibited a decreased startle response (Fig. 1A). Analysis of the startle response to a loud noise alone (120 dB) showed that female FVB.129-*Fmr1* KO mice had a decreased response to this auditory stimuli compared to the female FVB.129 WT control group. Additionally, significant differences were found in PPI between WT and KO mice. Female FVB.129-*Fmr1* mutant mice showed statistically significant enhanced inhibition to a tone when a pre-pulse was applied, which appeared at all pre-pulses higher than 82 dB (Fig. 1B). Percentage pre-pulse inhibition increased with increased pre-pulse sound for WT and KO, indicating that mice, in all groups, were not only able to hear (proper hearing ability) but were able to distinguish between the different pre-pulses (auditory threshold). However, comparing the different pre-pulses between the genotypes, we observed that there was a significant difference between WT and KO (Fig. 1B). These results indicate that while these female KO mice are able to hear, the loss of FMRP affected most likely the auditory threshold of these mice.

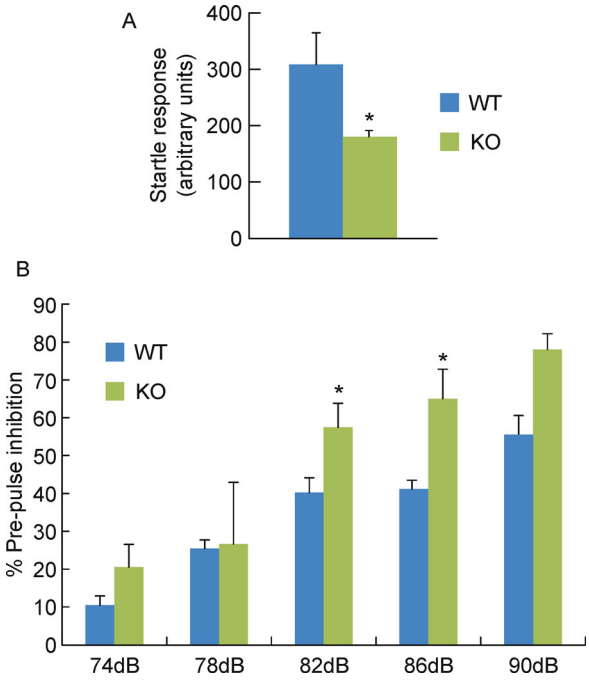

Figure 1 Aberrant startle response phenotypes of FVB.129-*Fmr1*-knockout female mice compared to wild type female FVB.129 controls. (A) Graph represents the impaired startle response of FVB.129-*Fmr1* KO female mice, measured as a reflex where subjects flinch upon the hearing of a sudden, loud sound. (B) Pre-pulse inhibition is altered in female *Fmr1* KO mice (* $p < 0.05$ WT vs. KO; n = 7–8/group).

Female FVB.129-*Fmr1* KO mice do not display anxiolytic behavior in the elevated plus maze

To determine the anxiety-like traits in female FVB.129-*Fmr1* KO mice, we chose to subject these animals to the elevated plus maze paradigm. This test is based on the normal predisposition for mice to move around a novel environment while at the same time avoiding the hostile nature of open environments (Pellow et al., 1985). Mice normally prefer the security of the closed arms but they adventure to explore the open arms due to their curiosity (Crawley, 2000). Both groups, WT and KO animals, present the same exploratory behaviors in terms of distance traveled (Fig. 2A) and speed (Fig. 2B). However, KO showed an inclination toward spending more time in the open arms compared to WT, indicating that KO females might be less anxious. However, statistical analysis showed these differences to be not significance (Fig. 2C). Taken together, these results confirm

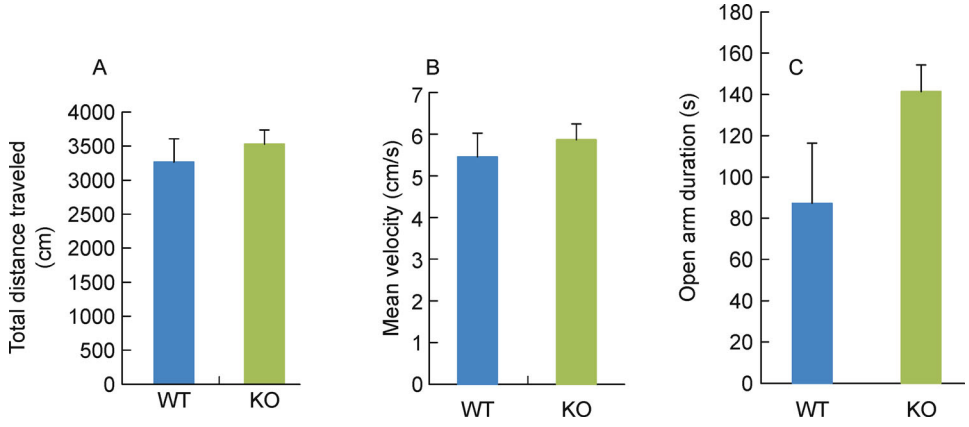

Figure 2 Evaluation of anxiety traits in the elevated plus maze. FVB.129-*Fmr1*-knockout female mice presented no anxiety phenotypes as compared to wild type female FVB.129 controls using the elevated plus maze and measuring (A) total distance traveled and (B) velocity on this travel. (C) Female FVB.129-*Fmr1* KO mice presented a trend toward spending more time within the open arms compared to controls (n = 7–8/group).

that the loss of FMRP in female FVB.129-*Fmr1* KO mice did not lead to the development of significant anxiety related behaviors as measured in the elevated plus maze.

Higher levels of the small GTPase Rac1 in the brain of female FVB.129-*Fmr1* KO mice

In general, FXS is characterized by an increased protein translation. Research has indicated that FXS patients present a range of phenotypic characteristics that relate to cell growth, and actin cytoskeleton organization. It is well known that small GTPases of the Rho family, such as Rac1, are key regulators in these processes. Consistent with the idea that FXS is a disease of exaggerated protein translation, previous work in our laboratory has shown that levels of Rac1-GTP (active form of Rac1) are elevated in males *Fmr1* knockout mouse in the C57Bl6/J strain (Bongmba et al., 2011). Additionally, other laboratories have reported that humans with FXS have also significant levels of the small GTPase Rac1 (Fatemi et al., 2013). Thus, we also examined whether levels of Rac1-GTP are altered in this female FXS model developed in the FVB.129 background. In agreement with previous results in *Fmr1* knockout males, we found that, at basal conditions, levels of Rac1-GTP were higher in several brain regions of *Fmr1* knockout female mice (Fig. 3), mainly the hippocampus, cerebellum, brain stem and cortex, as compared to FVB.129 control females.

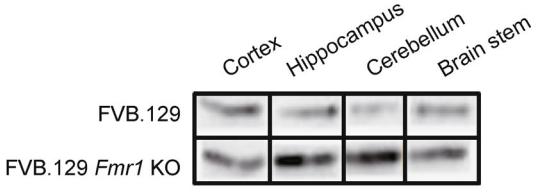

Figure 3 Rac1 expression levels in FVB.129-*Fmr1* knockout mice. Representative western blot depicting elevated Rac1 expression in brain tissue of female FVB.129-*Fmr1* knockout mice compared to FVB.129 female control group. Tubulin, run as control, demonstrated equal protein loading (not shown) (Rac1 ~22 kDa; n = 5/group).

Female FVB.129-*Fmr1* KO mice show increased predisposition to audiogenic seizures

One of the most robust phenotypes that have been described in male animal models is the high susceptibility to induction of audiogenic seizures (AGS), with FXS patients suffering also from seizures. *Fmr1* KO mice are very susceptible to seizure activity, and this type of activity depends on the developmental state of the brain (Michelson and Lothman, 1989). Male and female juvenile animals have a very dissimilar timing with respect to development of the brain. Thus, to determine whether female FVB.129-*Fmr1* KO mice suffer from audiogenic seizures, we tested these groups of females for their susceptibility to AGS as described before. While animal models for FXS raised in other backgrounds showed these AGS early in their development, we found that adult female FVB.129-*Fmr1* KO mice also suffer measurable responses to the AGS test compared to the control WT group, detecting significant wild running, induction of seizures and final death, compared to WT control, which were not affected (Table 1).

Female FVB.129-*Fmr1* KO mice show impaired cue fear memory

FXS patients often present cognitive disabilities, with females having lower rates of learning problems and/or cognitive defects. Herein, we used conditioning fear learning to examine whether female FVB.129-*Fmr1* KO mice display memory dysfunction. As an integral part to the paradigm used

Table 1 Susceptibility of female FVB.129 and FVB.129-*Fmr1* knockout to audiogenic seizures(AGS). Data are presented before and after treatment with NSC23766 (5 mg/kg bw, 13 days).

Femal	Wild running (%)	Seizure (%)	Death (%)
FVB.129WT (n = 10)	0	0	0
FVB.129-*Fmr1* KO(n = 13)	69.23	30.77	15.38
FVB.129WT-NSC(n = 7)	0	0	0
FVB.129-*Fmr1* KO-NSC(n = 7)	28. 57	28. 57	0

Female FVB.129 and FVB.129-*Fmr1* knockout mice were treated with either a saline solution or NSC23766 (5 mg/kg bw for 13 days). Mice were tested for AGS induction (118 dB for 5 min) 2 h after the last injection.

here, we first determine whether sensory-perceptual function was altered in these female mice by placing them on a 55°C hot plate and measuring their latency to react to discomfort caused by heat. We considered jumping, hind paw licking or hind paw flicking as an indicator of sensory detection and discomfort. Wild type (WT) and FXS (*Fmr1* KO) mice showed similar response to heat discomfort, indicating that the loss of FMRP has not affected their sensitivity to perceive a painful stimulus (data not shown).

Next, we assessed whether female FVB-*Fmr1* KO mice have an alteration in learning and memory. Contextual fear conditioning was used to examine the hippocampal function in these FXS female mice, to test fear response and contextual memory. The freezing behavior of mice in response to fear to a foot shock is an indicator of learning and memory (LeDoux, 1996). Fear response recorded during the training phase indicated that KO female mice have a similar freezing response than control mice (WT), signifying that they are capable of learning at the same rate and efficiency as the WT controls (Fig. 4A, WT vs. KO). Twenty-four hours after training (long-term memory), mice were re-introduced into the same chamber to determine their recall skills to their previous training. FXS mice showed similar freezing behavior when returned to the chamber as compared to the control groups, indicating that long-term memory is not impaired following contextual training (data not shown). Interestingly, we also tested these animals in the cued/tone memory test as described before. It is believed that the brain circuitry mediating cued fear conditioning to a tone involves the amygdala, and that the one mediating contextual fear conditioning is dependent on the hippocampus and other brain regions. FXS mice (KO) mice showed impaired freezing to the administered sound as compared to controls (WT) when tested 24 h after training (Fig. 4B, WT vs. KO; $*p < 0.05$). These results suggest that FVB.129-*Fmr1* KO female mice display normal learning and contextual long-term memory but impaired cued memory compared to FVB.129 female WT controls.

Rescue of altered behavioral phenotypes in female FVB.129-*Fmr1* KO mice by regulating Rac1-GTP

Previous research in our laboratory has demonstrated that Rac1 is related and essential for plasticity in the brain and neuronal development and maintenance (Bongmba et al., 2011; Martinez and Tejada-Simon, 2011). Moreover, we have

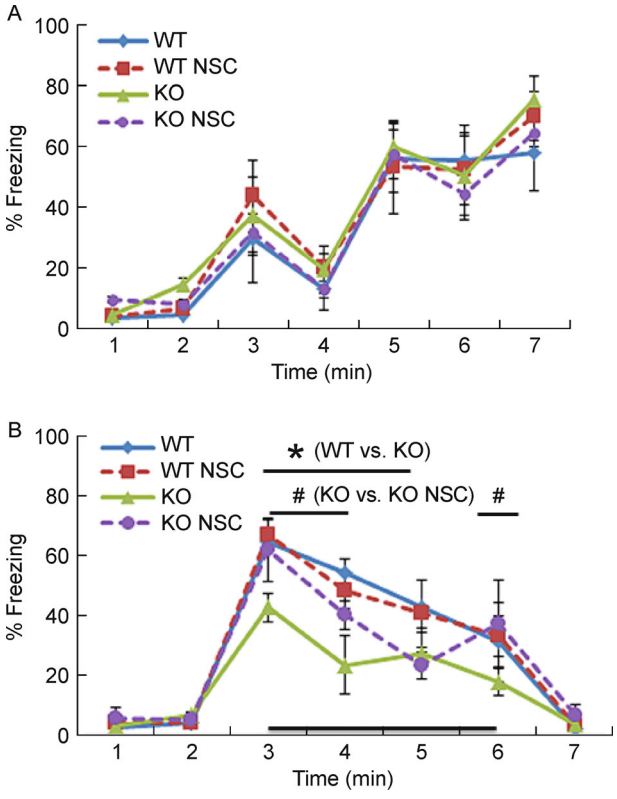

Figure 4 FVB.129-*Fmr1*-knockout female mice present an impairment in cued fear memory, which is rescued by treatment with the Rac1 inhibitor NSC23766. (A) Learning behavior during the training protocol on the conditioning fear paradigm on female FVB.129 (WT) and FVB.129-*Fmr1* knockout mice (KO) untreated or treated with 5mg/kg bw with NSC 23766 intraperitoneally for 10 days. Rac1 inhibitor treatment does not affect learning in female FVB.129 wild type mice. Treatment with NSC 23766 does not have an effect on the learning behavior of FVB.129-*Fmr1* knockout female mice. (B) Recall 24 h after training to the sound administered during learning phase. Rac1 inhibitor treatment does not have an effect on female FVB.129 wild type mice but restores cued fear conditioning deficit in female FVB.129-*Fmr1* knockout mice. WT = FVB.129 female wild type mice injected with 0.9NaCl saline solution for 10 days; WT NSC = FVB.129 female wild type mice injected with NSC 23766 (5 mg/kg bw for 10 consecutive days). KO = FVB.129-*Fmr1* knockout female mice injected with 0.9NaCl saline solution for 10 days; KO NSC = FVB.129-*Fmr1* knockout female mice injected with NSC 23766 (5 mg/kg bw for 10 consecutive days). (* $p < 0.05$ WT vs. KO; # $p < 0.05$ KO vs. KO-NSC; n = 7–8/group).

also shown that FXS mouse male models present excessive levels of the small GTPase Rac1 (Bongmba et al., 2011), most likely leading to the aberrant cytoarchitecture of dendritic spines as well as behavioral deficits. If this excessive level of Rac1 is directly involved in behavior abnormalities, one would expect that normalizing levels of Rac1 will occlude or reverse those abnormalities. To test this, we injected our female FVB.129 control and FVB.129-*Fmr1* experimental animals with NSC 23766, a Rac1 activation inhibitor. We then measured the same behaviors that appeared altered in those same animals, namely, conditioning fear and susceptibility to audiogenic seizures.

In both tests, use of just vehicle solution (0.9NaCl) did not yield changes in either WT or KO mice measured behaviors (data not shown). However, while treatment with Rac1 inhibitor NSC23766 (5 mg/kg bw for 10 days) did not produce behavior alterations in female FVB.129 control mice in either fear conditioning or susceptibility to audiogenic seizures, we did find differences in the female FVB.129-*Fmr1* KO mice when treated with NSC23766 as follows. Levels of performance during training were similar between untreated and NSC23766-treated FVB.129-*Fmr1* KO female mice (Fig. 4A, KO vs. KO NSC). However, treatment with NSC23766 rescued the perceived cue memory impairment (Fig. 4B, KO vs. KO NSC, $\#p < 0.05$), previously observed when comparing WT to KO. Consistent with our hypothesis, FVB.129-*Fmr1* KO female mice exhibited a strong rescue in their memory recall to the cue version of the fear conditioning test when levels of Rac1 were regulated by its inhibitor, reaching now freezing levels comparable to the control female FVB.129 WT mice assessed previously.

Additionally, susceptibility to audiogenic seizures was not altered in the control female FVB.129 WT group regardless of treatment with inhibitor (no wild running, seizures or death was observed, Table 1). Interestingly, treatment with NSC23766 protected FVB.129-*Fmr1* female mice from death after the induction of seizures, upon administering a loud noise for a period of 5 min. While treatment with NSC 23766 decreased but did not totally avoid wild running/seizures, the inhibitor precluded those animals from death (Table 1).

Discussion

The advances in the study of FXS have been possible due to valuable tools such as animal models of the disease lacking the FMRP protein. Most of the therapeutic investigations with those animals have employed only males probably for two main reasons: one, because they are hemizygous for the disease, and two, because in humans, the majority of males suffering from FXS present more severe phenotypes and a larger number suffer from mental retardation (Bailey et al., 1998), in comparison to only half of the FXS females (Rousseau et al., 1994). The *Fmr1*-knockout mice model has been established in many different backgrounds, yielding a wide variety of results in the field even within the same gender. To our knowledge, there are few studies that examined multiple behavioral problems in female *Fmr1* knockout in general, and particularly in a model developed in the FVB.129 background. Herein, we report that female FVB.129-*Fmr1* knockout mice present similar behavioral and functional deficits as their males' counterpart. Additionally, those behavioral deficits resemble problems that have been reported in humans suffering from this syndrome. For example, in humans it has been stated that, in general, females with FXS do not have greater levels of anxiety compared to other females from control groups (Mazzocco et al., 1998; McCauley et al., 2001), being able to clearly manage their syndrome-associated anxieties. This appears to be consistent with our findings in this animal model, where we saw lack of anxiety in the elevated plus maze. Both control and KO female mice spent an equivalent time in the open and closed arms of the maze, presenting also a similar pattern in locomotion and speed, without an indication of hyperactivity.

As stated before, FXS patients and mouse models display significant cognitive impairments. We used conditioning fear learning to examine whether female FVB.129-*Fmr1* KO mice also displayed defective memory. Our study suggests that these females are capable of learning, and no impairment was observed in their contextual memory. Conversely, other studies in C57BL/6-*Fmr1* knockouts have shown contextual fear memory impairment in females (Ding et al., 2014), but only when the protocol involved milder shocks than the ones provided in our training tests. Interestingly, our female knockout mice presented a deficit underlying memory formation when subjected to an auditory cue, which might be related to the differential sensitivity in auditory skills measured by the startle and PPI test between the knockout group and the controls.

Measuring auditory skills, several laboratories have shown increased PPI and reduced startle response in male *Fmr1* KO mice (Nielsen et al., 2002; Frankland et al., 2004; Pietropaolo et al., 2011), very similar to the results reported in our study for female FVB.129-*Fmr1* KO mice. It has been well established that patients with FXS have a higher susceptibility to seizures, and this phenotype has been also widely confirmed for *Fmr1* KO mice regardless of the background. The degree of AGS susceptibility in female FVB.129-*Fmr1* knockout is very high, and we have observed that it is exhibited in adult mice, which is consistent with previous reports (Veeraragavan et al., 2011; Goebel-Goody et al., 2012). Susceptibility to AGS is probably considered the most constant phenotype in *Fmr1* knockout mice (Musumeci et al., 2000; Chen and Toth, 2001; Yan et al., 2004). FXS is linked to cholinergic super-sensitivity, and many reports suggest a role for muscarinic receptors in AGS because they modulate sensorimotor gating in rodents (Veeraragavan et al., 2011). It has been proposed that NSC23766 might be a competitive antagonist of muscarinic receptors (Levay et al., 2013). As such, relieve of AGS severity after NSC 23766 treatment

could be related, not only to Rac1 inhibition, but to the blockage of muscarinic receptors in female FVB.129-*Fmr1* KO mice.

Previous studies in our laboratory have shown that deleting the small GTPase Rac1 postnatally translates in impaired synaptic plasticity, behavioral deficits, and decreased density of dendritic spines (Bongmba et al., 2011; Tejada-Simon and Bongmba, 2012). Interestingly, studies in a FXS animal model (Bongmba et al., 2011) as well as in FXS patients (Fatemi et al., 2013) have revealed that lack of FMRP translates in excess levels of Rac1. We hypothesize that this elevated translation of Rac1 might be responsible for the altered plasticity, aberrant phenotypic behavior and increased density of dendritic spines reported for this disease. Thus, the approach taken by us in this study has been to pharmacologically reduce the activation of Rac1 in female FVB.129-*Fmr1* KO mice, which also have elevated Rac1. This strategy rescued FXS-associated phenotypes on several levels, from plasticity to memory. Taken together, our study suggests that leveling a critical molecular protein such as Rac1 contributes to normalizing FXS-associated defect in neuronal and behavioral function.

It remains interesting to determine whether another of the strong phenotypes in FXS mice, altered spine density, is somehow different in these females. In the hippocampus, dendritic spine density appears to be regulated by estrogens (Gould et al., 1990; Woolley et al., 1990; Woolley and McEwen, 1992, 1993). Moreover, learning tasks stimulate the synthesis of this female hormone in this brain region, where levels have been reported to be considerably much elevated than those found in plasma (Hojo et al., 2009; Kato et al., 2013). Numerous studies have examined the role of hippocampally-synthesized estrogen on hippocampal spine morphology and physiology. Studies are on the way to determine any cytoarchitectural differences in these females. Moreover, we acknowledge that overall possible effects of estrous cycle on these reported results remains to be determined.

In summary, although preliminary, these findings provide evidence that female *Fmr1* knockout mice present similar protein dysfunctions than male, as well as behavioral deficits. Thus, we believe preclinical studies can be pursued using both males and females. This represents an additional advantage, as the amount of laboratory animals needed to perform a specific research can be reduced, complying with the guidelines for good animal care and use, decreasing the number of animals employed in biomedical research. Because the normalization of exaggerated levels of Rac1 protein rescues aberrant phenotypes, this opens the possibility of new targets to address behavioral and functional symptoms associated to this disease.

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Compliance with ethics guidelines

All institutional and national guidelines for the care and use of laboratory animals were followed. Our experiments were executed in agreement with the Public Health Service policies and the Animal Welfare Act, with animal used protocols approved by the University of Houston Institutional Animal Care and Use Committee (IACUC). Stacy Nguy and Maria V. Tejada-Simon, both declare that they have no conflict of interest.

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