Visual–spatial learning impairments are associated with hippocampal PSD-95 protein dysregulation in a mouse model of fragile X syndrome

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Fragile X syndrome is the most common cause of inherited intellectual disability and is caused by the lack of fragile X mental retardation protein (FMRP) expression. In-vitro findings in mice and post-mortem autopsies in humans are characterized by dendritic spine abnormalities in the absence of Fmrp/FMRP. Biochemical and electrophysiological studies have identified postsynaptic density protein (PSD)-95 as having an established role in dendritic morphology as well as a molecular target of Fmrp. How Fmrp affects the expression of PSD-95 following behavioral learning is unknown. In the current study, wild type controls and *Fmr1* knockout mice were trained in a subset of the Hebb–Williams (H–W) mazes. Dorsal hippocampal PSD-95 protein levels relative to a stable cytoskeleton protein (β-tubulin) were measured. We report a significant upregulation of PSD-95 protein levels in wild type mice, whereas training-related protein increases were blunted in *Fmr1* knockout mice. In addition, there was a significant negative correlation between mean total errors on the mazes and PSD-95 protein levels. The coefficient of determination indicated that the mean total errors on the H–W mazes accounted for 35% of the variance in PSD-95 protein levels. These novel findings suggest that reduced PSD-95-associated postsynaptic plasticity may contribute to the learning and memory deficits observed in human fragile X syndrome patients. *NeuroReport* 25:255–261 © 2014 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Fragile X syndrome (FXS) is the most common cause of inherited intellectual disability and occurs because of loss-of-function mutation on the fragile X mental retardation 1 (*FMR1*) gene on the X chromosome resulting in a lack of fragile X mental retardation protein (FMRP) expression (reviewed in [1]). Relevant to the present investigation, FXS patients display poorer performances as compared with developmentally matched participants on a number of different visual–spatial-dependent tasks [2,3].

Fmr1 knockout (KO) mice [4] show several behavioral deficits found in human patients [4,5]. In humans and mice, *FMR1*/*Fmr1* mRNA is highly expressed in a number of brain regions including the hippocampus [6,7], suggesting that FMRP/Fmrp may be important in the underlying functions subserved by this region. Spatial navigation and learning measures are dependent on the hippocampus; however, tests using *Fmr1* KO mice have generated inconsistent results that may be a function of the tasks employed [4,5,8].

The Hebb–Williams (H–W) mazes are a viable test alternative as they are sensitive in detecting hippocampal deficits in spatial navigation and learning [9,10]. We previously reported that human FXS patients and *Fmr1* KO mice had a comparable pattern of deficits whereby they committed more errors than controls on the same H–W mazes. These results suggested commonalities in maze navigational performance and that the spatial learning deficits were attributable to a lack of FMRP/Fmrp [3].

Postsynaptic modifications of AMPA receptors (AMPARs) by long-term potentiation and/or long-term depression are believed to be the neural correlates of learning and memory [11]. One candidate protein that may be involved in both AMPAR regulation and dendritic spine structure is postsynaptic density protein (PSD-95) of 95 kDa. PSD-95 has been implicated as a scaffolding protein, which can indirectly bind to AMPARs [12] and modulate AMPAR synaptic numbers and synaptic strength [13]. Regarding structure, overexpression of PSD-95 enhanced postsynaptic clustering, size, and number of dendritic spines [14], whereas knockdown or mutation of PSD-95 N or C termini impaired spine growth [15].

The murine model of FXS is characterized by dendritic spine abnormalities [16]. Fmrp has an established role in regulating plasticity-associated proteins such as PSD-95 [17]. Regarding PSD-95, increased translational

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levels were observed during basal states in *Fmr1* KO as compared with wild type mice [18], as well as relatively low protein levels following stimulus induction in this genotype [19]. Furthermore, PSD-95 mRNA transcripts were found to selectively deteriorate in the hippocampus but not in the cortex or the cerebellum of *Fmr1* KO mice [20]. These data suggest that some of the cognitive impairments in FXS could be attributable to a lack of dynamic regulation of PSD-95 following synaptic activity because of the lack of Fmrp.

In an effort to address inconsistencies in the animal literature and to better understand protein dynamics in an in-vivo learning model of FXS, we examined PSD-95 protein levels using the H–W mazes. We hypothesized that there would be an upregulation of PSD-95 in wild type mice and this response would be reduced in *Fmr1* KO mice. Protein levels from both genotypes were hypothesized to correlate negatively with total errors on the H–W mazes.

Materials and methods

Animals

A total of 36 male, naïve mice of a Friend leukemia virus B (FVB) background strain were obtained from Jackson Laboratories (Bar Harbor, Maine, USA) that included 18 wild type control (FVB.129P2-Pde6b^{+}Tyr$^{c\text{-}ch}$/AntJ, JAX Stock # 004828) and 18 *Fmr1* KO mice (FVB.129P2-Fmr1^{tm1Cgr}/J, JAX Stock # 004624). The *Fmr1* KO mice were bred from homozygote mating pairs and backcrossed for 11 generations. The FVB genetic background was chosen in view of the documented modest visual–spatial abilities. Mice of both genotypes were shipped at 4 weeks of age and were ~12 weeks old when they began experimental procedures. Mice were given 2 weeks acclimation and were housed in groups of four in standard ($27 \times 21 \times 14$ cm) polypropylene cages. Eight days before testing, all mice were housed in individual cages. To ensure high levels of motivation during the study, mice were maintained at ~85–90% of their original body weight and fed a food ration ~30 min after daily testing procedures ended. The study received ethical approval from the University of Ottawa and efforts were made to minimize pain and suffering as outlined by the Canadian Council of Animal Care.

Apparatus

The H–W test apparatus was constructed according to the specifications outlined by the developers [21]. The apparatus, made of black Plexiglass (Plastics of Ottawa Ltd, Ottawa, Ontario, Canada), consisted of a large open area, square in shape ($60 \times 60 \times 10$ cm), with diagonally opposing start and goal box areas ($20 \times 10 \times 10$ cm). The start and goal box areas were equipped with sliding, removable Plexiglass doors to control entry and confinement, covered by clear Plexiglass lids. In the goal box, a recessed food cup (2.5 cm diameter) was placed in the center and baited with a piece of food (Harlan Global Rodent Chow, 20 mg; Harlan Laboratories Inc., Mississauga, Ontario, Canada) during the latter phases of the experiment. The floor of the square open area was delineated by 36 equal squares and these were used for manually placing barriers that defined different maze problems and error zones [21]. The barriers (10 cm high) were constructed with black opaque Plexiglass.

Procedure

wild type control ($n = 12$) and *Fmr1* KO ($n = 12$) mice underwent behavioral testing. During the acclimation period, one wild type mouse was euthanized because of poor health (of an unknown cause) and two KO mice were euthanized because of progressive and continuous seizures resulting in a smaller sample size (control, $n = 11$; *Fmr1* KO, $n = 10$). Twelve additional mice (six wild type; six *Fmr1* KO) were used as an untrained control group. These mice did not participate in maze learning; however, underwent daily handling, food restrictions procedures, and exposure to the H–W mazes similar to those animals running the mazes. The experiment was conducted in three phases: habituation, acquisition, and testing. During the habituation phase, the H–W apparatus was cleared of all barriers and each mouse was allowed 20 min/day on 4 consecutive days to explore. During the last 2 days, the goal box area was baited with a small piece of food (20 mg) and each mouse had ad-libitum access to the food for the duration of the session.

The acquisition phase consisted of training mice on six practice mazes ([3]; Fig. 1). Specifically, each mouse was trained for two sessions per day, the first starting at 08:00 h and the second at 13:00 h. Each session consisted of one different practice maze (five trials per maze) commencing with maze A. A trial was considered complete when the mouse entered the goal area and took a bite of food or 180 s had elapsed. Mice completed all six acquisition mazes in sequence (A–F) as many times as necessary for them to reach criterion; that of two consecutive sessions completed in less than 30 s each.

Following acquisition, mice were given a selection of the standard test mazes ([3]; Fig. 1B) on the basis of the same procedures used during acquisition. Mice were tested on a different maze in each session (five trials per maze) in the same order (i.e. 2, 4, 5, 8, 9, 11, and 12) until all seven were completed, spanning 3.5 days/animal. The dependent measures of interest were latency and number of errors. Latency was recorded from the moment the barrier in the start box was raised until the animal took its first bite of food. An error was registered each time a mouse crossed its two front paws into an error zone ([3]; Fig. 1B). Data from the testing phase were recorded using an overhead Sony camcorder and Media Cruise software (Thomson Canopus Co. Ltd, Kobe, Japan) on a standard desktop computer. The experimenter was blind to the genotype of the mice and never visible to the mice during the runs.

Western blot

Immediately after finishing the H–W mazes, mice were euthanized (100 µl intraperitoneal injection of euthasol),

their brains were removed and tissue blocks were cut using a stainless steel brain matrix ($1 \times 1.5 \times 0.75$ inches). Both dorsal hippocampi were dissected according to the mouse atlas of Paxinos and Franklin [22] and frozen on dry ice. Briefly, hippocampi were then homogenized over ice in a homogenate buffer/protease inhibitor cocktail (Sigma Aldrich, Oakville, Ontario, Canada). The homogenates were centrifuged, protein content was quantified using a standard BSA kit (Pierce, Rockford, Illinois, USA), and samples were frozen at −80°C until further analysis. Proteins were loaded at a concentration of 300 µg/ml and samples in quadruplicate (12 µg/lane) were resolved by SDS-PAGE. Proteins were then transferred to pure nitrocellulose membranes and blocked for 1 h in 5% skim milk and 10 M PBS solution at room temperature. Antibody specificity was determined before commencing western blot analyses on experimental animals by confirming a single band of binding of the proteins of interest at the appropriate molecular weight: 95 kDa (PSD-95) and 55 kDA (β-tubulin). Optimal concentrations of primary/secondary antibody were then confirmed by serial dilutions. Membranes were then incubated in 5% skim milk and Tris-buffered saline with Tween 20 (TBST) (20 mM Tris/HCl, 137 mM NaCl, 0.4% Tween 20, pH 7.6) solution with monoclonal anti-PSD-95 antibody (1 : 2000; Millipore Corporation, Burlington, Ontario, Canada) and monoclonal anti-β-tubulin antibody (1 : 10,000; Sigma Aldrich) at 4°C overnight. After 3×10 min washes in TBST, fluorescent Alexa 680-linked antibody (1 : 10 000; Molecular Probes, Burlington, Ontario, Canada) and IR 800 antibody (1 : 10 000; LI-COR Biosciences, Lincoln, Nebraska, USA) in 5% skim milk and TBST solution were applied for 1 h at 4°C. After 3×10 min washes in TBST, western blots were scanned using the Odyssey infrared system (LI-COR Biosciences) in 700 and 800 nm channels in a single scan at 169 µm resolution. Simultaneous detection of two fluorescent antibodies (i.e. Alexa 680 and IR 800) were allowed for the measurement of PSD-95 and β-tubulin proteins within each sample. The density of each protein band of interest was measured, background subtracted, and normalized to β-tubulin by the LI-COR analysis software. Similar western blot procedures were carried out on the untrained control group who were euthanized after 15.5 days, the average time it took for the maze learning mice to complete all phases of the H–W mazes.

Immunohistochemistry

To visualize PSD-95 antibody staining in CA1 region of the hippocampus, one mouse from each genotype that completed the H–W mazes was prepared for immunohistochemistry analysis. Briefly, immediately following maze learning, mice were administered a 100 µl intraperitoneal injection of euthasol before intracardiac perfusions consisting of brains being flushed with 20 ml of saline followed with 20 ml of 4% paraformaldehyde, 0.2% picric acid in 0.16 M sodium phosphate buffer, pH 7.1. After a 3.5-h postfixation at 4°C, brains were transferred to 10% sucrose in 0.1 M sodium phosphate buffer, pH 7.1 for cryoprotection. Fourteen micron sagittal sections were then cut using a cryostat (Leica Microsystems Inc., Concord, Ontario, Canada) and stored at −80°C. Antibody specificity was confirmed by visualizing section staining consisting of primary/secondary antibody, secondary antibody only, or antifade only on a Zeiss Axioplan fluorescence microscope (Carl Zeiss, Toronto, Ontario, Canada). Optimal concentrations of primary/secondary antibody were then confirmed by serial dilutions. Tissue sections were later washed briefly in .01 M PBS and incubated in monoclonal anti-PSD-95 antibody (1 : 100; Cell Signaling Technology, Boston, Massachusetts, USA) diluted with 0.3% Triton X-100 containing 4% normal donkey serum in PBS for 3 h at room temperature. After 3×5 min washes in PBS, sections were incubated in Alexa Fluor 488 secondary (1 : 1000; Cell Signaling Technology) with 0.3% Triton X-100 containing 4% normal donkey serum in PBS for 30 min at 37°C. After the secondary antibody, a NeuroTrace (530/615) red fluorescent Nissl stain (1 : 50; Molecular Probes, Life Technologies, Burlington, Ontario, Canada) was applied according to the manufacturer's protocol. Sections were then cover slipped using a standard antifade medium (Fisher Scientific, Ottawa, Ontario, Canada). Fluorescence staining of the CA1 region of the hippocampus was then visualized with a Zeiss LSM 510 AxioImager (Carl Zeiss). M1 confocal microscope had a magnification of $\times 40$, according to the stereotaxic coordinates outlined by Paxinos and Franklin [22]. Single optical planes were captured.

Statistical analyses

The behavioral data (latency and errors) were previously published and are presented in the study of MacLeod *et al.* [3]. Briefly, whereas latency to complete the H–W mazes did not differ between wild type and *Fmr1* KO runners, significantly fewer errors were made by Fmrp-intact mice ([3]; Figs 4 and 5). To examine whether KO mice were able to perform the basic, nonspecific behaviors necessary for maze navigation, an independent samples *t*-test was conducted with genotype as an independent variable and days to reach criterion as a dependent variable. Because wild type as compared with KO mice spent significantly more days in the acquisition mazes (see Results section), this nuisance variable was used as a covariate in the subsequent analyses of PSD-95 levels of H–W maze runners.

To test the hypothesis that hippocampal PSD-95 is upregulated only in the wild type runners following successful completion of test mazes, a 2×2 analysis of variance (ANOVA) was carried out (IBM SPSS Statistics, version 19; IBM, Markham, Ontario, Canada) with genotype (wild type; *Fmr1* KO mice) and task (H–W mazes; untrained control) as the independent variables and the protein ratio of PSD-95 normalized to a control protein, β-tubulin, as the dependent variable.

An additional 2×2 ANOVA was carried out to determine whether levels of β-tubulin significantly differed within the independent variables.

To examine the potential relationship between PSD-95 levels and errors committed on the test mazes, three separate bivariate correlational analyses (Pearson's r) were conducted. These analyses used relative PSD-95 protein levels (normalized to β-tubulin) and mean total errors on the H–W mazes, defined as aggregate errors divided by the total number of learning trials (Maze × Trials = 35). Specific correlations focused on the relationship between PSD-95 protein levels and mean errors from: (a) H–W maze runners of both genotypes, (b) wild type runners, and (c) *Fmr1* KO runners. As a control, correlations were also performed between β-tubulin protein levels and mean errors from 1, 2, and 3 as listed above.

Results

Whereas western blots were used to quantify hippocampal PSD-95 protein expression levels, confocal images of the hippocampal distribution of PSD-95 in the CA1 region in wild type and *Fmr1* KO mice, as shown in Fig. 1, are provided for visual depiction purposes only.

Fmr1 KO mice reached criterion in the acquisition phase significantly faster than wild type controls ($t = 3.01$, $P = 0.006$). The mean time to complete the acquisition phase was 5.35 days for the *Fmr1* KO group, 95% confidence interval (4.00, 6.86) and 10.5 days for wild type mice, 95% confidence interval (7.61, 13.21). Despite these differences, the one-way analysis of covariance was significant for the genotype [$F(1, 18) = 7.19$, $P = 0.01$, partial $\eta^2 = 0.29$], indicating higher PSD-95 levels in wild type runners relative to KO runners, when statistically controlling for number of days required to reach acquisition criterion.

The results of the 2×2 ANOVA conducted to evaluate the effects of H–W maze learning on PSD-95 protein levels as measured by optical density of western blots in wild type and *Fmr1* KO mice indicated a significant main effect for genotype [$F(1, 29) = 14.31$, $P = 0.001$, partial $\eta^2 = 0.33$] and for task [$F(1, 29) = 10.01$, $P = 0.004$, partial $\eta^2 = 0.26$]. There was also a significant interaction between genotype and task [$F(1, 29) = 4.07$, $P = 0.05$, partial $\eta^2 = 0.12$]. With respect to β-tubulin protein levels, there was no main effect of genotype [$F(1, 29) = 2.44$, $P = 0.13$, partial $\eta^2 = 0.08$] or task [$F(1, 29) = 0.05$, $P = 0.82$, partial $\eta^2 = 0.002$], and the genotype by task interaction was not significant [$F(1, 29) = 0.10$, $P = 0.75$, partial $\eta^2 = 0.003$ (Fig. 2)].

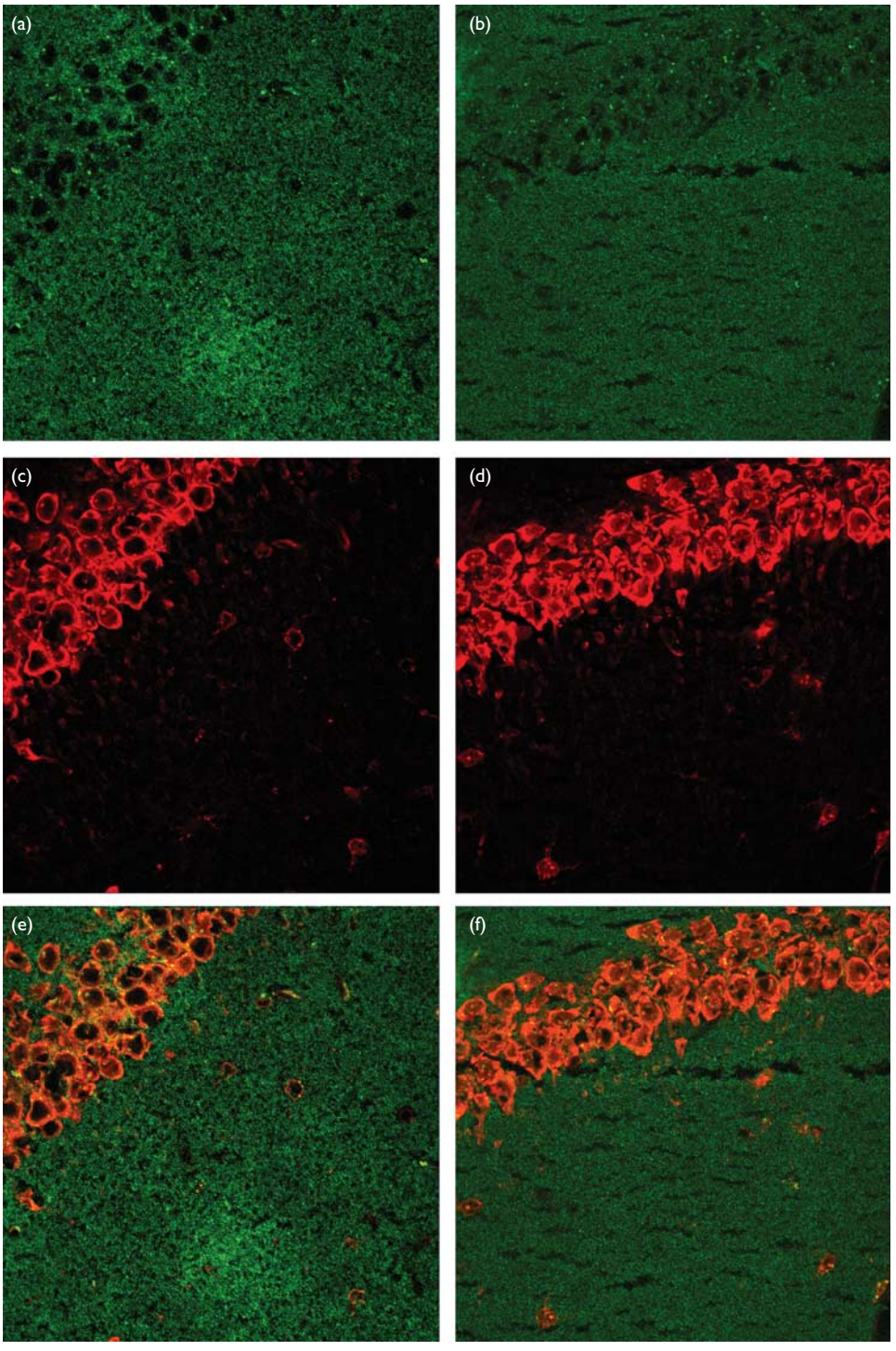
Bonferroni corrections were made to the α level of 0.05 before performing simple main effect analyses resulting in 0.0125 (0.05/4 = 0.0125). Simple main effects of task within genotype indicated that wild type mice that ran the H–W mazes had significantly higher PSD-95 levels than wild type mice in the untrained control group [$F(1, 29) = 13.71$, $P = 0.001$, partial $\eta^2 = 0.32$]. This difference was not found in *Fmr1* KO mice when runners were compared with nonrunners [$F(1, 29) = 0.66$, $P = 0.42$, partial $\eta^2 = 0.02$]. Examination of genotype within each task (H–W runners; naïve control group) revealed that wild type runners had significantly higher PSD-95 levels than *Fmr1* KO runners following completion of the mazes [$F(1, 29) = 23.10$, $P = 0.0001$, partial $\eta^2 = 0.44$]. By comparison, there were no PSD-95 protein differences between wild type and KO mice in the naive, untrained control group [$F(1, 29) = 1.23$, $P = 0.28$, partial $\eta^2 = 0.04$]. Thus, PSD-95 protein upregulation occurs in wild type mice that ran the H–W mazes and this response is blunted in *Fmr1* KO mice (Fig. 2).

The first correlational analysis revealed that for H–W maze runners of both genotypes, there was a significant, negative correlation between PSD-95 protein levels and mean total errors on the H–W mazes [$r(19) = -0.59$, $P = 0.002$, $r^2 = 0.35$ (Fig. 3)]. Two further analyses within genotype were also completed and revealed correlations that trended toward significance (after Bonferroni adjustments to α, 0.05/3 = 0.02). Specifically, in wild type mice, there was a negative correlation between PSD-95 protein levels and mean total errors [$r(9) = -0.44$, $P = 0.09$, $r^2 = 0.18$]. In *Fmr1* KO mice, there was a negative correlation between PSD-95 protein levels and mean total errors [$r(8) = -0.54$, $P = 0.05$, $r^2 = 0.29$]. As a control, β-tubulin protein levels were also correlated with mean total maze errors. For runners of both genotypes, there was no correlation between β-tubulin protein levels and mean total errors on the H–W mazes [$r(19) = -0.078$, $P = 0.37$, $r^2 = 0.006$]. Within genotype, there was no correlation between β-tubulin protein levels and mean total errors for wild type runners [$r(9) = -0.20$, $P = 0.27$, $r^2 = 0.04$], nor for *Fmr1* KO mice [$r(8) = 0.31$, $P = 0.19$, $r^2 = 0.09$]. Thus, these results highlight a relationship of covariance, specific to PSD-95, between hippocampal protein levels and mean errors on the H–W mazes.

Discussion

We examined the spatial navigation and learning abilities in wild type and *Fmr1* KO mice to better understand the protein changes that accompany learning of a visual–spatial navigation measure, the H–W mazes. FXS patients as well as *Fmr1* KO mice completing the H–W mazes were previously shown to have comparable maze navigational performances such that they committed more errors than controls, results that were attributable to a lack of FMRP/Fmrp [3]. We add to this literature by demonstrating that runners completing the H–W mazes exhibited an upregulation of an important scaffolding protein normally under the control of Fmrp in the hippocampi of wild type mice and that this response was blunted in *Fmr1* KO mice who lack Fmrp. Moreover, protein upregulation was specific to PSD-95, as evidenced by stable levels of a control protein (β-tubulin)

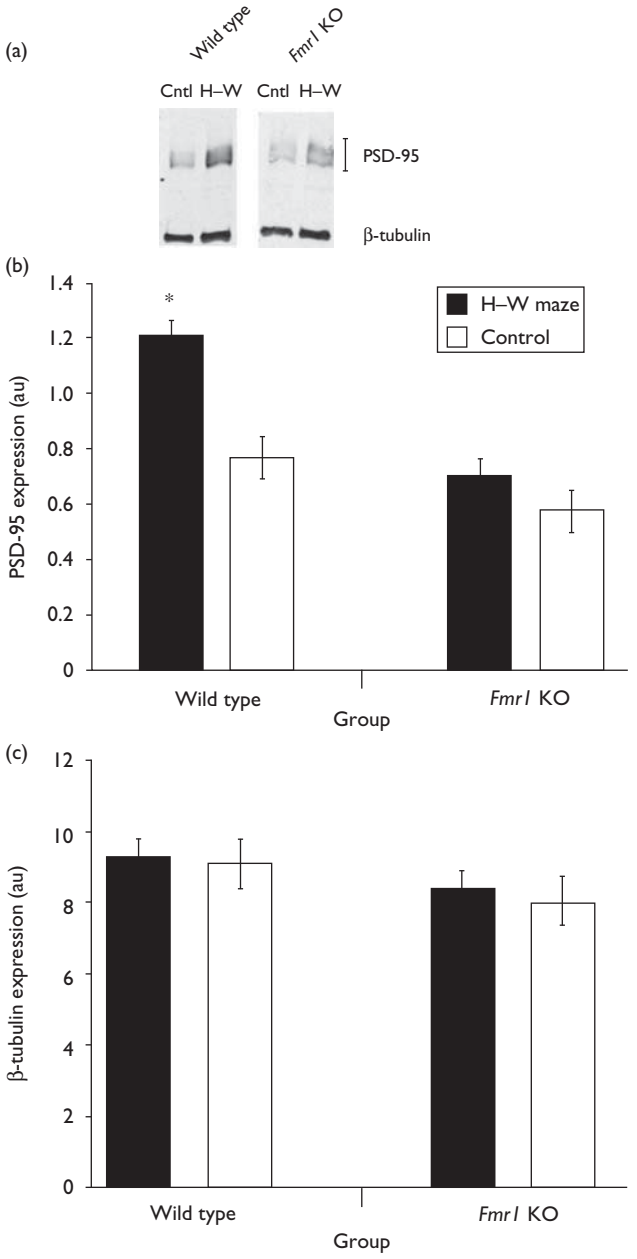
Fig. 1



× 40 confocal image of the CA1 region of the dorsal hippocampus. (a, b) depicts a representative raw image of postsynaptic density protein-95 punctate staining, (c, d) of Nissl fluorescent staining, and (e, f) a merge of both stainings in wild type ($n=1$; left) and *Fmr1* knockout ($n=1$; right) mice.

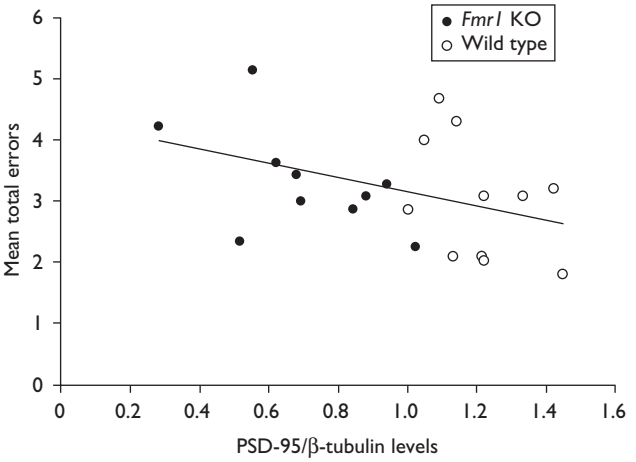
across genotypes and condition. Given the commonalities in visual–spatial learning impairments in humans and mice [3], our results suggest that human FXS patients may display poorer performance on visual–spatial-dependent tasks as a result of dysregulation of PSD-95 protein levels. Further evidence that strict regulation of PSD-95

Fig. 2



Postsynaptic density protein-95 (PSD-95) expression is increased in wild type but not *Fmr1* knockout (KO) mice following a learning paradigm. (a) Representative western blots from dorsal hippocampi of wild type and KO mice for protein expression of PSD-95 and β-tubulin. PSD-95 is found around the expected molecular weight of 95 kDa and β-tubulin at 55 kDa. Control represents animals in the untrained group, whereas Hebb–Williams (H–W) refers to trained animals from each genotype. (b) Quantification of protein levels for PSD-95 normalized to β-tubulin, and (c) β-tubulin in wild type ($n=17$) and KO ($n=16$) mice. Error bars represent the SEM; *$P<0.025$.

Fig. 3



Levels of hippocampal postsynaptic density protein-95 (PSD-95) are correlated with behavioral performance. Negative correlation between mean maze errors on the Hebb–Williams mazes and PSD-95 protein levels were normalized to β-tubulin in wild type ($n=11$) and knockout (KO; $n=10$) mice. A significant negative correlation was observed [$r(19)=-0.59$, $P=0.003$, $r^2=0.35$].

may contribute to normal cognitive functioning in humans is supported by findings of decreased PSD-95 expression in conditions with neurocognitive impairments such as Alzheimer's disease, schizophrenia, and mood disorder [23,24].

One of our goals was to further validate the H–W mazes as a behavioral assay for reliably documenting visual–spatial learning deficits in *Fmr1* KO mice. This would in turn allow for future studies to evaluate the effectiveness of pharmacological or behavioral interventions intended to mitigate symptoms of FXS. Interestingly, *Fmr1* KO mice reached criterion in the acquisition phase significantly faster than wild type controls. This was likely because of the combination of the ease of the acquisition mazes (e.g. requiring a single response to correctly reach the goal box) and the known pattern of hyperactivity in *Fmr1* KO mice of the FVB background strain [25], which makes them more likely to achieve the less than 30-s latency criterion faster than their wild type counterparts. Although KO mice reached criterion earlier, the pattern of errors on acquisition mazes was similar between genotypes. The acquisition data, taken together with the finding that *Fmr1* KO mice commit more errors than controls [3], suggest that when presented with more challenging visual–spatial tasks such as the test mazes, *Fmr1* KO mice evidence a poorer learning strategy. Moreover, impaired performance of *Fmr1* KO mice on the test mazes alongside superior performance on the acquisition mazes serves to strengthen the notion that observed deficits and concomitant blunted PSD-95 expression are meaningful rather than being attributable to a generalized impairment of the KO mice in being able to perform the basic, nonspecific, behaviors necessary for maze navigation.

We observed a significant negative correlation between mean maze errors and PSD-95 expression. That is, mice that committed fewer mean errors on the mazes exhibited greater PSD-95 protein levels and vice versa. In an attempt to further characterize this relationship, we performed

additional correlation analyses within each genotype, which were found to trend toward significance, and may have achieved significance with larger sample sizes.

The deficits of PSD-95 protein upregulation in *Fmr1* KO mice is consistent with a lack of and an inability to dynamically regulate protein synthesis during maze learning. It has been suggested that pharmacological treatments stabilizing basal protein translation levels may ameliorate some of the core symptoms in FXS by restoring normal protein synaptic synthesis, thereby allowing for improved regulation during periods of synaptic plasticity [26]. However, the identification of specific proteins responsible for the morphological changes in FXS has to date remained speculative. Additional studies investigating the use of pharmacological agents are needed to ascertain whether spatial navigation and learning deficits and protein correlates such as PSD-95 are amenable to the treatment in *Fmr1* KO mice. Such studies may provide valuable insight into the neurobiological basis of and treatment for the FXS phenotype.

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Conflicts of interest

There are no conflicts of interest.

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