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Genetic manipulation of STEP reverses behavioral abnormalities in a fragile X syndrome mouse model

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Abstract

Fragile X syndrome (FXS), the most common inherited form of intellectual disability and prevailing known genetic basis of autism, is caused by an expansion in the *Fmr1* gene that prevents transcription and translation of fragile X mental retardation protein (FMRP). FMRP binds to and controls translation of mRNAs downstream of metabotropic glutamate receptor (mGluR) activation. Recent work identified striatal-enriched protein tyrosine phosphatase (STEP) as an FMRP target mRNA. STEP opposes synaptic strengthening and promotes synaptic weakening by dephosphorylating its substrates, including ERK1/2, p38, Fyn, Pyk2, and subunits of NMDA and AMPA receptors. Here we demonstrate that STEP translation is dysregulated in *Fmr1*^{KO} mice, resulting in elevated basal levels of STEP with a concomitant loss of mGluR-dependent STEP translation. We hypothesized that the weakened synaptic strength and behavioral abnormalities reported in FXS may be linked to excess levels of STEP. To test this hypothesis, we reduced or eliminated STEP genetically in *Fmr1*^{KO} mice. In addition to attenuating audiogenic seizures and seizure-induced c-Fos activation in the periaqueductal gray, genetically reducing STEP in *Fmr1*^{KO} mice reversed characteristic social abnormalities, including approach, investigation, novelty-induced hyperactivity and anxiety. Loss of STEP also corrected select non-social anxiety-related behaviors in *Fmr1*^{KO} mice, such as open arm exploration in the elevated plus maze. Our findings indicate that genetically reducing STEP significantly diminishes seizures and restores social and non-social anxiety-related behaviors in *Fmr1*^{KO} mice, suggesting that strategies to inhibit STEP activity may be effective for treating patients with FXS.

Keywords

fragile X syndrome; behavior; audiogenic seizures; social; anxiety; c-Fos; tyrosine phosphatase; STEP

Introduction

Individuals with fragile X syndrome (FXS) exhibit behavioral abnormalities that include intellectual disability, anxiety, hyperactivity, and seizures (Boyle & Kaufmann, 2010). They are also impaired socially, with 30% meeting the criteria for autism (Harris *et al.*, 2008). The majority of cases are attributed to a CGG expansion in the *Fmr1* 5' untranslated region (Kaufmann & Reiss, 1999) that becomes hypermethylated, leading to transcriptional

silencing and ultimately diminished expression of fragile X mental retardation protein (FMRP) (Chen *et al.*, 1995). FMRP binds select mRNAs and suppresses their translation (Siomi *et al.*, 1993, Darnell *et al.*, 2001, Lagerbauer *et al.*, 2001, Zalfa *et al.*, 2003, Darnell *et al.*, 2011). In the absence of FMRP, translation of some of these mRNAs is upregulated (Zalfa *et al.*, 2003, Lu *et al.*, 2004, Hou *et al.*, 2006, Westmark & Malter, 2007, Gross *et al.*, 2010).

Huber and colleagues (2002) report that *Fmr1* knockout (*Fmr1*^{KO}) mice show exaggerated metabotropic glutamate receptor (mGluR)-dependent long-term depression (LTD), suggesting a role for FMRP in regulating protein synthesis-dependent synaptic plasticity. These results mark the inception of the mGluR theory of FXS, linking dysregulation of proteins normally suppressed by FMRP to mGluR-LTD. This theory posits that many FXS phenotypes, including elevated protein synthesis, increased glutamate receptor endocytosis, immature spine development, and behavioral abnormalities, originate in exaggerated mGluR signaling (Bear *et al.*, 2004, Krueger & Bear, 2011). Consequently, genetic and pharmacological approaches that decrease mGluR signaling attenuate several of these abnormalities in *Fmr1*^{KO} mice (Chuang *et al.*, 2005, Yan *et al.*, 2005, Dolen *et al.*, 2007, De Vrij *et al.*, 2008, Osterweil *et al.*, 2010, Hays *et al.*, 2011, Thomas *et al.*, 2011).

Accordingly, another major focus in FXS research has been to discover alternative therapeutic targets by identifying mRNAs regulated by FMRP and/or mGluR-dependent signaling (Krueger & Bear, 2011). One excellent candidate is striatal-enriched protein tyrosine phosphatase (STEP), a brain-enriched phosphatase that regulates proteins associated with the post-synaptic density and downstream signaling, including NMDA receptors (NMDARs), AMPA receptors (AMPA), Fyn, Pyk2, and the MAPK family of proteins ERK1/2 and p38 (Goebel-Goody *et al.*, 2011). STEP has been coined an “LTD protein” because it is translated in response to mGluR activation and promotes mGluR-stimulated AMPAR endocytosis (Luscher & Huber, 2010, Zhang *et al.*, 2008). In addition, STEP opposes synaptic strengthening by promoting NMDAR internalization and inactivating ERK1/2, Fyn, and Pyk2 (Nguyen *et al.*, 2002, Pelkey *et al.*, 2002, Paul *et al.*, 2003, Snyder *et al.*, 2005, Venkitaramani *et al.*, 2011).

Cross-linking studies of FMRP-RNA complexes demonstrate that STEP mRNA binds to FMRP (Darnell *et al.*, 2011). We hypothesized that STEP translation would be dysregulated in *Fmr1*^{KO} mice and that excess STEP levels might contribute to behavioral phenotypes characteristic of *Fmr1*^{KO} mice (Musumeci *et al.*, 2000, Peier *et al.*, 2000, Spencer *et al.*, 2005, Yan *et al.*, 2005, Liu & Smith, 2009, Mines *et al.*, 2010, Spencer *et al.*, 2011). We demonstrate that genetically modulating STEP levels corrects select behavioral abnormalities *Fmr1*^{KO} mice, indicating STEP as viable target in FXS.

Materials and Methods

Subjects

All experimental protocols were approved by the Yale University Institutional Animal Care and Use Committee and strictly adhered to the NIH Guide for the Care and Use of Laboratory Animals. Every effort was made to minimize the pain, discomfort, and quantity of mice used in this study. *STEP*^{KO} mice (Venkitaramani *et al.*, 2009) were crossed with *Fmr1*^{KO} mice (courtesy of Dr. William T. Greenough, University of Illinois Urbana-Champaign) to generate *STEP* heterozygous (^{HT})/*Fmr1*^{HT} female breeders, as well as *STEP*^{HT}/*Fmr1*^{KO} male breeders (Fig. 2a). In all crossings, animals were on the c57Bl/6 background. Breeders from different litters were mated to produce progeny with a selective reduction in *STEP* or *Fmr1*. Unless otherwise noted, mice were weaned and housed in

groups of 2–5 in standard vented-rack cages in a 12:12 hour light:dark cycle with food and water available ad libitum. Mice weaned to solitary housing were excluded from the study.

Genotyping

Primers used to detect the presence or absence of the WT or KO (neomycin) allele on the *Fmr1* gene were as follows: 5' WT primer-GGTTAAAAGTCATCCGTGGCTA-3', 5' KO primer-CTGAGCCCAGAAAGCGAA-3', 5' common primer-ACCACCACTGCCCTTCTGAT-3'. *Fmr1* primers were combined and the following multi-primer PCR reaction used: 94°C for 5 min; 35 cycles of 94°C for 45 sec, 60°C for 1 min, 72°C for 1 min; final extension at 72°C for 10 min. *Fmr1* products were electrophoresed on a 2% agarose gel to detect bands at 500 bp (KO), 350 bp (WT), or both (HT) (Fig. 2b). Primers used to detect the presence or absence of the WT or KO (neomycin) allele on the *STEP* gene were as follows: 5'-CCCTACTCTCATTCTCCCTTCCC-3', 5' KO primer-CCACCAAAGAACGGAGCC-3', 5' common primer-GGCAGCAGATGCTGGTGGC-3'. *STEP* primers were combined and the following multi-primer PCR reaction used: 94°C for 5 min; 36 cycles of 95°C for 45 sec, 59°C for 1 min, 72°C for 1 min; final extension at 72°C for 10 min. *STEP* products were electrophoresed on a 2% agarose gel to detect bands at 400 bp (WT), 200 bp (KO) or both (HT) (Fig. 2b).

Antibodies and reagents

The antibodies, dilutions used, and their sources are listed in Table 1. Normal horse serum, ABC Vectastain Elite Kit, and DAB Peroxidase Substrate Kit were purchased from Vector Laboratories (Burlingame, CA, USA). Heparin was purchased from Hospira (Lake Forest, IL, USA) and HistoClear was obtained from National Diagnostics (Atlanta, GA, USA). (S)-3,5-Dihydroxyphenylglycine hydrate (DHPG) was obtained from Sigma-Aldrich.

Synaptoneurosome preparation and DHPG treatments

Synaptoneurosomes (SNs) were obtained using a modified protocol (Hollingsworth *et al.*, 1985). Hippocampi from 4–8 month *STEP*^{WT}/*Fmr1*^{WT} or *STEP*^{WT}/*Fmr1*^{KO} (three mice pooled for n=1) were homogenized with 15 passes in all-glass tissue grinders in ice-cold HEPES buffer containing protease inhibitors (Roche Applied Science, Indianapolis, IN, USA) and the following reagents (in mM): 124 NaCl, 3.2 KCl, 1.06 KH₂PO₄, 26 NaHCO₃, 1.3 MgCl₂, 2.5 CaCl₂, 10 glucose, 10 HEPES (pH 7.4). Homogenates were passed through two 100 µm nylon mesh filters (Millipore) and then one 5 µm nitrocellulose filter (Millipore). Filtrates were spun at 1000 × g for 10 min at 4°C. Supernatants were collected for cytosolic fractions, and the resulting SN pellets were rinsed with HEPES buffer and centrifuged at 1000 × g for 10 min at 4°C. Supernatants were discarded and SNs pellets resuspended in HEPES buffer. After splitting in half, SNs were pre-incubated for 10 min at 37°C. One half was treated with 50 µM DHPG, while the other remained untreated (control), for 10 min at 37°C. After stimulation, SNs were lysed by adding SDS to a final concentration of 1%, sonicated, boiled for 5 min, and frozen at –80°C until further analysis. Protein concentrations were determined using a BCA protein assay kit (Pierce, Rockford, IL, USA) with bovine serum albumin (BSA) as a standard.

Immunoblotting

Proteins (10–40 µg) were resolved by 8 or 10% SDS-PAGE, transferred to polyscreen polyvinylidene difluoride membranes, blocked in 3% (w/v) BSA in Tris-buffered saline (140 mM NaCl, 20 mM Tris, pH 7.6) plus 0.1% Tween 20 (TBST) for 1 hr (RT), and incubated overnight at 4°C with primary antibodies. Following washes, membranes were incubated in secondary antibodies for 1 hr (RT). Immunodetection was accomplished using chemiluminescence (SuperSignal Kits, Pierce) and visualized with a G:BOX imaging station

using the GeneSnap image program (Syngene, Frederick, MD, USA). Blots were quantified using Image J software (version 1.43u; NIH). Protein signals were normalized to the average signal of all lanes on the same blot as described (Osterweil *et al.*, 2010). This value was divided by the normalized GAPDH signal in the same lane and expressed as the mean of the normalized values $\pm$ s.e.m. All biochemical results in the text refer to STEP₆₁, which is the membrane-associated and only isoform of STEP present in cortical and hippocampal tissue (Boulanger *et al.*, 1995, Oyama *et al.*, 1995, Bult *et al.*, 1996).

Behavioral tests

Two cohorts of age-matched, sexually naïve mice were used for testing: cohort 1 for audiogenic seizures and c-Fos analysis, and cohort 2 for the behavioral battery. Each animal in cohort 2 was subject to the same number and order of tasks. The sequence of tasks was as follows: open field, marble burying, elevated plus maze, social choice test, social dominance tube test, and light/dark box. Details of the behavioral parameters analyzed and main effect differences are presented in Tables 2–5. Tests were conducted between the hours of 08:00 and 17:00 and, unless otherwise noted, the room was brightly lit with a modest and constant white background noise (60 dB). With the exception of audiogenic seizures, marble burying, and social dominance tube test, ANY-maze video tracking software (Stoelting, Wood Dale, IL, USA) was utilized with an overhead video camera system to automate behavioral testing and provide unbiased results. Investigators remained blind to the genotypes of mice during testing. All components of the behavioral apparatuses were wiped thoroughly with MB-10 (200 ppm) and 70% ethanol between tests to disinfect and eliminate odor trails between mice.

Audiogenic seizures

For audiogenic seizures (AGS), it was imperative that the mice were born and reared in static cages in a quiet room. Mice were weaned, tagged and tissue obtained for genotyping analysis at least 24 hrs prior to testing. At PND 20–24, male and female mice were habituated for 30 sec to a plexiglass test chamber (40 cm diameter $\times$ 60 cm high encased in sound-proof foam). The audio stimulus consisted of a 130 dB siren (modified personal alarm, PAL-1, Lane Self Defense, Roseville, MN, USA) powered by a DC converter and mounted on the chamber lid. Seizures were graded by two observers across a range of increasing intensity from no response to wild running, clonic/tonic seizures, or status epilepticus with respiratory arrest. The stimulus duration lasted until seizure onset or up to 5 min (whichever was first). No differences were observed between male and female mice in the incidence of AGS (data not shown); therefore the data were pooled together for statistical analysis.

Open field

Total activity and center square exploration of male mice (2–5 months old) were assessed in an empty open field box constructed of white, opaque plexiglass (50 cm long $\times$ 50 cm wide $\times$ 30 cm tall) for 5 min. The center square was designated as the center 25% of the total area.

Marble burying

Male mice (2–5 months old) were assessed for repetitive behavior using a modified version of the marble burying assay (Thomas *et al.*, 2009). After completing open field, mice were singly housed in static cages containing Alpha-Dri bedding (2.5 cm) for 48 hrs. On the test day, total activity was first assessed in home cages without marbles for 5 min. The mouse was briefly removed and 20 black glass marbles (15 mm diameter) were evenly arranged in a 4 $\times$ 5 pattern on the bedding. The mouse was returned to the cage and allowed to freely explore for 20 min. Upon completion, an overhead picture was captured. The percentage of

each visible marble was quantified using ImageJ (version 1.43u; NIH). Images were converted to grayscale, thresholded, and a constant cutoff value established whereby anything darker than that value was considered to be a visible marble (i.e., eliminating shadows). Scales were normalized to the width of the cage. The area of each marble was then measured and compared to the area of a completely visible marble (control). Those with a visible area of 60% of the control marble (i.e., buried at least 40%) were considered buried.

Elevated plus maze

After completing marble burying, male mice (2–5 months old) were rehoused with their littermates in groups of 2–5 in vented-rack cages for at least 48 hrs before conducting elevated plus maze (EPM). The apparatus was comprised of four arms (30 cm long × 7 cm wide) configured in a plus manner situated 40 cm above the floor. The two opposing closed arms were constructed with 15 cm tall walls. This task was performed in dim light (6–7 lux). Mice were individually placed in the center square and allowed to freely explore for 5 min while the investigator left the room. Any mice that fell from the maze were excluded. Entries were defined as 4 paws (or 75% of its body) in the zone. Videos were retroactively scored by an investigator for the number of risk assessing and/or exploratory head dips while in the open arms.

Social choice test

Male mice (5–10 months old) were tested in a modified three-chambered social choice task as described previously (Moy *et al.*, 2009). The three-chambered apparatus consisted of an opaque rectangular plexiglass box (64 cm long × 43 cm wide × 38 cm tall) divided into three compartments (20 cm long × 43 cm wide) by clear plexiglass walls with small, retractable entryways. Each side chamber contained an inverted wire cup (10.5 cm tall × 10.5 cm diameter bottom × 7.6 cm diameter top, 1 cm bar spacing). The task consisted of three 10 min phases: (1) habituation, (2) socialization, and (3) social novelty. During habituation, the test mouse was allowed to freely explore the entire apparatus, with the side chambers containing only cups. During socialization, a stranger mouse was placed under a cup in one side while the other remained empty. The test mouse was allowed to then freely explore the apparatus. Activity in each chamber and in close proximity to either the mouse or cup (front 25% of the mouse 1 inch margin around the cup) was recorded. Socialization index was calculated as the time in the mouse chamber divided by total time in the mouse and cup chambers. During social novelty, a novel stranger mouse was positioned under the remaining empty cup, while the stranger introduced during the socialization phase remained (designated as familiar). The test mouse was allowed to then freely explore the apparatus. Activity in each chamber and in close proximity to either the novel or familiar mouse was recorded. Novelty index was determined as the time in the novel mouse chamber divided by the total time in the novel mouse and familiar mouse chambers. All stranger mice were non-littermate, age-matched mice of varying genotypes that had been previously habituated to being enclosed under the cup for 10 min on the same day of testing.

Social dominance tube test

Male mice (8–10 months old) were tested in a social dominance tube test as described previously (Spencer *et al.*, 2005). In this test, mice were evaluated for their number of ‘wins’ against *STEP^{WT}/Fmr1^{WT}* mice. Immediately prior to the match, mice were singly housed and individually habituated to a clear plastic tube (30.5 cm long × 3.8 cm inner diameter) contained within the open field box for 5 min. Matches began when two opposing mice were placed head first at either end of the tube and released simultaneously. Matches concluded when one mouse completely backed out and retreated from the tube (designated as the

loser). Matches lasting longer than 2 min or where mice crawled over each other were excluded. Each mouse performed 3 matches against non-littermate, age-matched *STEP*^{WT}/*Fmr1*^{WT} mice.

Light/dark box exploration

Male mice (8–10 months old) were examined in a partitioned light/dark box (38 cm long × 38 cm wide × 36 cm tall) composed of two equally-sized adjoining plexiglass chambers where one side was black and the other was clear. The room was brightly lit so that the clear side was fully illuminated. Mice were individually placed in the illuminated chamber and allowed to freely explore for 10 min. The total number of entries in the light and dark sides and overall activity within the illuminated side was recorded. Entries were defined as 4 paws (or 75% of its body) in the zone.

c-Fos immunohistochemistry

Thirty minutes after exposure to the audio stimulus, mice were anesthetized by sodium pentobarbital (i.p. 100 mg/kg) and perfused transcardially with 0.1 M sodium phosphate buffer (PB; pH 7.4) containing 5% sucrose and 1 unit/ml heparin followed by 4% paraformaldehyde in PB. Brains were post-fixed in the same solution for 12 hrs, then cryoprotected by equilibration in 20% and 30% sucrose in PB, embedded in a 0.9% saline solution containing 20% BSA and 0.5% gelatin, polymerized with 4.6% glutaraldehyde, and frozen. Fifty μ m thick coronal free-floating sections were obtained and stored at -20°C in cryoprotectant solution (30% glycerol and 30% ethylene glycol in 0.1M PB). Unless otherwise noted, all subsequent steps were completed under constant agitation at RT. Every sixth section from the anterior hippocampus to the posterior inferior colliculus was selected, resulting in a stained series at 300 μ m intervals. Sections were rinsed in phosphate buffered saline (PBS), submerged in 3% H_2O_2 in PBS for 20 min to quench endogenous peroxidase activity, blocked in 3% normal horse serum in PBS with 0.3% Triton X-100 (blocking buffer) for 30 min, and incubated for 72 hrs in blocking buffer at 4°C containing anti-c-Fos antibody (1:500). Sections were washed with PBS plus 0.3% Triton X-100 and incubated for 1 hr in blocking buffer containing biotinylated horse anti-goat IgG secondary antibody (1:300). Following washes, sections were incubated in ABC Vectastain Elite Kit for 60 min, washed, and reacted for 3 min in Tris buffer (pH 7.5) containing diaminobenzidine (DAB), nickel substrate, and hydrogen peroxide according to manufacturer's recommendations (DAB Peroxidase Substrate Kit). Sections were then washed in PBS, mounted, air dried, and nuclei were stained with methyl green. Sections were then dehydrated in ethanol, defatted in Histoclear, and mounted in DPX.

Quantification of c-Fos staining

Three sections containing the periaqueductal gray (PAG) were selected at intervals of 300 μ m, based on the Paxinos mouse brain atlas, corresponding approximately to stereotaxic coordinate Bregma -3.80 mm. The PAG region within the brain sections was projected at a 200X magnification onto paper using a microprojector (Ken-a-Vision 1000, Ken-A-Vision Manufacturing Company, Inc., Kansas City, MO, USA) and the locations of all c-Fos+ neurons were plotted. An investigator who was blind to genotype and AGS response quantified the total c-Fos+ neurons in the PAG from three sections per animal.

Statistics

All data were presented as means $\pm$ s.e.m. (with the exception of audiogenic seizures and social dominance tube test) and significance set at $P = 0.05$ as determined by SPSS software (IBM, New York, NY, USA). A 2×2 factorial ANOVA [*Fmr1* genotype (WT, KO) $\times$ treatment (control, DHPG)] was used to determine main effect differences in protein levels.

A one-tailed, unpaired Student's t-test was used to calculate treatment differences in SNs compared to *STEP*^{WT}/*Fmr1*^{WT} mice because changes in one direction only were expected based on previous results (Zhang *et al.*, 2008). Two-tailed Chi² Fisher's exact tests were conducted to determine significant differences in audiogenic seizures (genotype vs. *STEP*^{WT}/*Fmr1*^{WT} or genotype vs. *STEP*^{WT}/*Fmr1*^{KO}) and the social dominance tube test (genotype vs. *STEP*^{WT}/*Fmr1*^{WT}). A one-way ANOVA (genotype + response) with LSD post hoc comparisons was used to calculate differences in the number of c-Fos+ cells. For all other behavioral tests, a 3×2 factorial ANOVA [*STEP* genotype (WT, HT, KO) × *Fmr1* genotype (WT, KO)] was performed to determine main effects. Post hoc comparisons were made using LSD following confirmation of a significant main effect of *STEP*. Where indicated, a planned contrast one-way ANOVA (*STEP* genotype) was performed when mice were *Fmr1*^{KO} to determine if genetically reducing *STEP* significantly altered behaviors of *Fmr1*^{KO} mice.

Results

Loss of functional FMRP in mice increased basal *STEP* protein levels and prevented rapid mGluR-dependent *STEP* synthesis in synaptoneurosomes

A recent study utilized high-throughput sequencing of RNA isolated by crosslinking immunoprecipitation (HITS-CLIP) to rigorously identify endogenous mRNA targets of FMRP in neurons (Darnell *et al.*, 2011). This work revealed a highly significant association of *STEP* mRNA (*Ptpn5*) with FMRP across 7 independent experiments ($p=9.97E-5$), including use of two different FMRP antibodies and multiple biological replicates. One function of FMRP is to suppress translation (Siomi *et al.*, 1993, Darnell *et al.*, 2001, Lagerbauer *et al.*, 2001, Zalfa *et al.*, 2003, Darnell *et al.*, 2011). Accordingly, we predicted that *STEP* expression would be upregulated at baseline in *STEP*^{WT}/*Fmr1*^{KO} mice. We also assessed whether mGluR-stimulated translation of *STEP* was disrupted. Hippocampal synaptoneurosomes (SNs) from 4–8 month old *STEP*^{WT}/*Fmr1*^{WT} and *STEP*^{WT}/*Fmr1*^{KO} mice were prepared and stimulated with the mGluR agonist DHPG (50 μM, 10 min) and analyzed by immunoblotting (Fig. 1). We first verified enrichment of PSD-95 in the SNs fraction, and confirmed that, while expressed in both fractions, *STEP* was most enriched in the SN fraction (Fig. 1a). No significant main effects of genotype or drug treatment were observed in GAPDH expression (Fig. 1b).

Main effect analysis showed that *STEP* expression was significantly greater in SNs from *STEP*^{WT}/*Fmr1*^{KO} than *STEP*^{WT}/*Fmr1*^{WT} mice ($F_{(1, 8)} = 6.177$, $P = 0.038$) (Fig. 1c). As reported previously (Zhang *et al.*, 2008), we found that DHPG treatment significantly increased *STEP* expression in *STEP*^{WT}/*Fmr1*^{WT} SNs ($P = 0.048$), but not in *STEP*^{WT}/*Fmr1*^{KO} SNs ($P = 0.454$) (Fig. 1c). No significant differences were observed between unstimulated cytosolic fractions from *STEP*^{WT}/*Fmr1*^{WT} and *STEP*^{WT}/*Fmr1*^{KO} mice (Fig. 1d) ($P = 0.207$), demonstrating that removal of FMRP selectively increases basal *STEP* expression in SNs. This increase was not limited to the hippocampus, because *STEP* levels were also significantly higher in whole brain extracts from *STEP*^{WT}/*Fmr1*^{KO} mice as compared to *STEP*^{WT}/*Fmr1*^{WT} ($P = 0.04$, data not shown). These findings indicated that in *Fmr1*^{KO} mice, basal *STEP* expression was enhanced while mGluR-induced translation of *STEP* was impaired.

As a positive control, we found that PSD-95 expression was significantly higher in SNs from *STEP*^{WT}/*Fmr1*^{KO} than *STEP*^{WT}/*Fmr1*^{WT} mice ($F_{(1, 8)} = 20.715$, $P = 0.002$) (Fig. 1e), consistent with exaggerated basal translation of PSD-95. Previous studies reported that DHPG stimulation significantly increases PSD-95 expression in *STEP*^{WT}/*Fmr1*^{WT}, but not *STEP*^{WT}/*Fmr1*^{KO}, neurons (Todd *et al.*, 2003, Muddashetty *et al.*, 2007). Similarly, we observed a modest, but not statistically significant, DHPG-induced increase in PSD-95

expression in $STEP^{WT}/Fmr1^{WT}$ SNs ($P = 0.089$), which was absent in $STEP^{WT}/Fmr1^{KO}$ SNs ($P = 0.217$).

Genetic reduction of STEP decreased audiogenic seizures and reduced seizure-induced c-Fos activation in the periaqueductal gray of $Fmr1^{KO}$ mice

We hypothesized that excessive STEP levels contribute to the manifestation of abnormal behavioral phenotypes normally present in $Fmr1^{KO}$ mice. To test this prediction, we genetically reduced or eliminated STEP in $Fmr1^{KO}$ mice (Fig. 2a). Examination of both $STEP^{HT}/Fmr1^{KO}$ and $STEP^{KO}/Fmr1^{KO}$ afforded the opportunity to increase therapeutic relevance by testing whether a 50% or 100% reduction in STEP protein (Venkitaramani *et al.*, 2009) corrects behavioral phenotypes. We also examined $STEP^{HT}/Fmr1^{WT}$ and $STEP^{KO}/Fmr1^{WT}$ mice because their performance in the tasks used in this study had not yet been evaluated.

We first tested whether genetically reducing STEP decreased audiogenic seizures (AGS) in $Fmr1^{KO}$ mice. As expected, none of the $STEP^{WT}/Fmr1^{WT}$ mice had seizure activity in response to the audio stimulus, and neither did $STEP^{HT}/Fmr1^{WT}$ or $STEP^{KO}/Fmr1^{WT}$ mice (Fig. 3a, Table 2). Consistent with other reports (Musumeci *et al.*, 2000, Yan *et al.*, 2005, Dolen *et al.*, 2007), 76.2% of $STEP^{WT}/Fmr1^{KO}$ mice showed seizure activity ($P = 0.0001$, vs. $STEP^{WT}/Fmr1^{WT}$ mice). This value dropped to 53.3% in $STEP^{HT}/Fmr1^{KO}$ mice and 43.5% in $STEP^{KO}/Fmr1^{KO}$ mice (Fig. 3a, Table 2). Of relevance, the incidence of AGS was significantly lower in $STEP^{KO}/Fmr1^{KO}$ mice compared with $STEP^{WT}/Fmr1^{KO}$ mice ($P = 0.036$), demonstrating that STEP deficiency significantly reduced AGS in $Fmr1^{KO}$ mice.

AGS are mediated through brainstem circuits involving the periaqueductal gray (PAG) (Faingold, 1999, Faingold & Randall, 1999, N'gouemo & Faingold, 1999). To evaluate possible genotypic differences in neuronal activity induced by the audio stimulus, we examined expression of c-Fos in the PAG shortly after stimulation (Fig. 3, b–h). In the absence of seizure-related behaviors, no differences in the number of c-Fos+ PAG cells were found (Fig. 3h). However, following a tonic/clonic seizure, c-Fos+ cells increased significantly in the PAG, regardless of genotype ($P = 0.0001$) (Fig. 3, b–h). $STEP^{KO}/Fmr1^{KO}$ mice consistently showed fewer seizure-induced PAG c-Fos+ cells than $STEP^{WT}/Fmr1^{KO}$ mice ($P = 0.007$) (Fig. 3, c–d, f–h), indicating that genetic elimination of STEP in $Fmr1^{KO}$ mice attenuated neuronal activation in the PAG when seizures were present.

Social abnormalities of $Fmr1^{KO}$ mice in the social choice task were altered following genetic reduction of STEP

We next investigated whether genetically reducing STEP altered socialization in $Fmr1^{KO}$ mice. One way socialization is evaluated is using a three-chambered social choice task (Crawley, 2007, Moy *et al.*, 2009). There are three phases to this task: (1) habituation, (2) socialization, and (3) social novelty. A 3×2 factorial ANOVA was conducted to examine main effects of $Fmr1$ and $STEP$ genotype on various parameters in these phases (Figs. 4–5, Table 2).

Mice are typically social beings that prefer to spend time sniffing and interacting with other mice (Murcia *et al.*, 2005). During the socialization phase, no main effects of either $Fmr1$ or $STEP$ were observed for the socialization index ($Fmr1$: $F_{(1, 46)} = 2.940$, $P = 0.093$; $STEP$: $F_{(2, 46)} = 0.595$, $P = 0.556$) (Table 3), indicating similar preference for the mouse. We also analyzed main effects of $Fmr1$ and $STEP$ in close proximity to the mouse or the cup (Fig. 4, b–e; Table 3). $Fmr1$ and $STEP$ did not impact the time spent in close proximity to the stranger mouse ($Fmr1$: $F_{(1, 46)} = 3.887$, $P = 0.055$; $STEP$: $F_{(2, 46)} = 0.810$, $P = 0.451$) (Fig.

4c, Table 3) or the cup (*Fmr1*: $F_{(1, 46)} = 0.083$, $P = 0.774$; *STEP*: $F_{(2, 46)} = 0.394$, $P = 0.677$) (Table 3).

Social approach during the socialization phase was determined by the number of entries in close proximity to the stranger mouse, a method providing similar measurements to number of nose contacts (Mines *et al.*, 2010). Main effect analysis indicated that *Fmr1*^{KO} mice enter the area in close proximity to the stranger mouse more times than *Fmr1*^{WT} mice ($F_{(1, 46)} = 13.044$, $P = 0.001$) (Fig. 4d, Table 3). When comparing *Fmr1*^{KO} mice with varying levels of *STEP*, we found that *STEP*^{HT}/*Fmr1*^{KO} mice had significantly fewer visits with the stranger mouse than *STEP*^{WT}/*Fmr1*^{KO} mice ($P = 0.008$, planned contrast) (Fig. 4d). These results demonstrated that genetically reducing *STEP* significantly decreased the number of visits in close proximity, thereby reversing this *Fmr1*^{KO} phenotype.

We also found a significant main effect of *STEP* genotype on the duration of each visit in close proximity to the mouse, where *STEP*^{HT} and *STEP*^{KO} mice spent significantly more time per entry than *STEP*^{WT} mice ($F_{(2, 46)} = 4.50$, $P = 0.016$) (Fig. 4e, Table 3). These results revealed that *STEP*^{HT} and *STEP*^{KO} mice maintained each social interaction longer. Altogether, data from the socialization phase indicated that *STEP*^{WT}/*Fmr1*^{KO} mice visited the stranger mouse more frequently, and genetically reducing *STEP* reversed this phenotype. Decreasing *STEP* levels in *Fmr1*^{KO} mice also increased the duration of each visit, possibly enhancing the length of the interaction.

No significant differences in the total distance traveled were observed among genotypes in the socialization phase, thus excluding the possibility that other aspects were driven by locomotion differences (*Fmr1*: $F_{(1, 46)} = 1.906$, $P = 0.174$; *STEP*: $F_{(2, 46)} = 1.689$, $P = 0.196$) (Fig. 4b, Table 3). Moreover, we found that neither *Fmr1* nor *STEP* had an impact on parameters with the cup alone or chamber during the socialization phase (Table 3). These findings indicated the observed differences were specific for the social aspect of the task.

We subsequently analyzed main effects of *Fmr1* and *STEP* during the social novelty phase, where a novel mouse is introduced to the task (Fig. 5a). Striking main effect differences were observed in locomotion during this phase for both *Fmr1* and *STEP* (Fig. 5b, Table 3). *Fmr1*^{KO} mice traveled significantly more than *Fmr1*^{WT} mice ($F_{(1, 46)} = 6.790$, $P = 0.012$), indicating that *Fmr1*^{KO} mice become hyperactive in the presence of the novel mouse and/or when two mice are in the apparatus. In contrast, *STEP*^{HT} and *STEP*^{KO} mice traveled significantly less compared to *STEP*^{WT} mice ($F_{(2, 46)} = 5.871$, $P = 0.005$), suggesting decreased activity. The net result was that genetically reducing *STEP* reversed the social novelty-induced hyperactivity normally observed in *Fmr1*^{KO} mice.

No significant main effects of *Fmr1* or *STEP* were observed for the novelty index (*Fmr1*: $F_{(1, 46)} = 0.004$, $P = 0.949$; *STEP*: $F_{(2, 46)} = 0.129$, $P = 0.879$) (Table 3), demonstrating similar preference for the novel chamber over the familiar chamber. Nonetheless, main effect analysis revealed that *Fmr1*^{KO} mice spent significantly more time ($F_{(1, 46)} = 4.996$, $P = 0.030$) (Fig. 5c, Table 3) and had more entries ($F_{(1, 46)} = 4.549$, $P = 0.038$) (Fig. 5d, Table 3) in close proximity to the novel mouse than *Fmr1*^{WT} mice. One likely explanation for these results was that the hyperactivity of *Fmr1*^{KO} mice was causing them to spend more time and have more visits to the novel mouse. Neither *Fmr1* nor *STEP* genotype impacted the duration of each entry for either (Fig. 5e, Table 3). For all parameters close to the novel mouse, no significant main effects of *STEP* were observed (Fig. 5, c–e; Table 3), demonstrating that genetically reducing *STEP* in *Fmr1*^{KO} mice did not affect behaviors in close proximity to the novel mouse during this phase.

Genetically reducing STEP mice decreased social anxiety in *Fmr1*^{KO} in a social dominance tube test

As an additional measure of social anxiety, we utilized the social dominance tube test (Lindzey *et al.*, 1961, Spencer *et al.*, 2005) (Fig. 6). As published elsewhere (Spencer *et al.*, 2005), *STEP*^{WT}/*Fmr1*^{KO} mice won only 34.5% of the time (10/29 matches) ($P = 0.035$). The percentage of wins increased to 56.6% in *STEP*^{HT}/*Fmr1*^{KO} mice (13/23 matches), demonstrating that *STEP*^{HT}/*Fmr1*^{KO} and *STEP*^{WT}/*Fmr1*^{WT} mice retreat from the tube at a similar frequency ($P = 0.556$). Taken further, *STEP*^{KO}/*Fmr1*^{KO} mice won 76.9% of the time (10/13 matches) ($P = 0.017$). These findings revealed that genetically reducing STEP in *Fmr1*^{KO} mice increased the number of wins in the social dominance tube test.

Given that the number of wins was greater for *STEP*^{KO}/*Fmr1*^{KO} mice than *STEP*^{WT}/*Fmr1*^{WT} mice, we next investigated whether loss of the *STEP* gene alone caused the mice to win more often. We found that *STEP*^{KO}/*Fmr1*^{WT} mice did not win significantly more than *STEP*^{WT}/*Fmr1*^{WT} (61.1% wins; 11/18 matches) ($P = 0.318$; data not shown), demonstrating that loss of STEP alone was not causing *STEP*^{KO}/*Fmr1*^{KO} mice to win more often. Altogether, these findings supported the hypothesis that genetically reducing STEP decreased social anxiety in *Fmr1*^{KO} mice.

Loss of STEP normalized select non-social anxiety-related phenotypes in *Fmr1*^{KO} mice

To address whether genetically reducing STEP also reversed non-social anxiety-related phenotypes in *Fmr1*^{KO} mice, we first evaluated main effect differences in the elevated plus maze (EPM) (Fig. 7, Table 4). In agreement with studies showing reduced anxiety in *Fmr1*^{KO} mice (Peier *et al.*, 2000, Qin *et al.*, 2002, Spencer *et al.*, 2005, Qin & Smith, 2008, Mines *et al.*, 2010, Yuskaitis *et al.*, 2010, Spencer *et al.*, 2011, Liu *et al.*, 2011, but see also Restivo *et al.*, 2005, Bilousova *et al.*, 2009), main effect analysis revealed that *Fmr1*^{KO} mice entered the open arms more times ($F_{(1, 53)} = 4.720$, $P = 0.034$) (Fig. 7a, Table 4) and spent a greater percentage of total arm time in the open arms ($F_{(1, 53)} = 4.134$, $P = 0.047$) (Table 4) than *Fmr1*^{WT} mice. Conversely, main effect analysis showed that *STEP*^{KO} mice entered the open arms significantly less than *STEP*^{WT} or *STEP*^{HT} mice, suggesting that *STEP*^{KO} mice have greater anxiety in the EPM ($F_{(2, 53)} = 3.560$, $P = 0.035$) (Fig. 7a, Table 4). The net result was that genetically eliminating STEP normalized the effect of *Fmr1* genotype on open arm exploration. While the percentage of open arm time showed a similar pattern for a main effect of STEP, these results did not reach statistical significance ($F_{(2, 53)} = 0.725$, $P = 0.489$) (Table 4).

We observed that neither *Fmr1* nor *STEP* altered the number of total arm entries (*Fmr1*: $F_{(1, 53)} = 1.290$, $P = 0.261$; *STEP*: $F_{(2, 53)} = 2.147$, $P = 0.127$) (Fig. 7b, Table 4), total distance traveled (*Fmr1*: $F_{(1, 53)} = 0.044$, $P = 0.835$; *STEP*: $F_{(2, 53)} = 1.075$, $P = 0.349$) (Table 4), or number of closed arm entries (*Fmr1*: $F_{(1, 53)} = 0.136$, $P = 0.713$; *STEP*: $F_{(2, 53)} = 1.207$, $P = 0.307$) (Table 4) in the EPM. These findings demonstrated that the observed main effects of *Fmr1* and *STEP* on open arm entries were specific for exploration of the open arms and were not accounted for by differences in overall locomotion.

Time spent in the center square of the EPM has been interpreted as decision making time and/or motivation for exploratory behavior (Cruz *et al.*, 1994, Hilber & Chapillon, 2005, Rodgers & Johnson, 1995). Duration therefore is thought to reflect the animal's choice to either approach or avoid conflict and/or their motivation to explore novel environments (Lister, 1990). We discovered that *Fmr1*^{KO} mice spend a greater percentage of total time in the center square ($F_{(1, 53)} = 7.646$, $P = 0.008$) and spend more time per entry ($F_{(1, 53)} = 7.337$, $P = 0.009$) than *Fmr1*^{WT} mice (Fig. 7, c–d; Table 4). Moreover, *Fmr1*^{KO} mice performed more exploratory and/or risk assessing head dips than those that were *Fmr1*^{WT}

($F_{(1, 49)} = 7.044$, $P = 0.011$) (Fig. 7e, Table 4), providing additional support for increased exploratory behavior. In all of these parameters (Fig. 7c–e), *STEP* genotype did not have a significant effect on these parameters (Fig. 7, Table 4), suggesting that the reversal of open arm exploration in *STEP^{KO}/Fmr1^{KO}* mice (Fig. 7a) occurred without changes in behavioral indicators of decision making and/or exploration.

Corroborating our EPM results, there was a significant main effect of *Fmr1* in the light/dark box, where the time ($F_{(1, 49)} = 11.225$, $P = 0.002$) and total distance traveled ($F_{(1, 49)} = 6.431$, $P = 0.014$) in the illuminated side was increased (Fig. 8, a–b; Table 4), suggesting reduced anxiety. Although no significant main effects of *STEP* were observed for these parameters, *STEP^{HT}/Fmr1^{KO}* mice tended to spend less time and travel less distance in the light side than *STEP^{WT}/Fmr1^{KO}* mice (Fig. 8, a–b), but these differences did not reach statistical significance ($P = 0.061$ and $P = 0.072$, respectively, planned contrast).

However, a significant main effect of *STEP* was observed for the number of total light and dark entries ($F_{(2, 49)} = 6.247$, $P = 0.004$), a value comparable to number of transitions (Fig. 8c, Table 4). Specifically, *STEP^{HT}* and *STEP^{KO}* mice had significantly fewer total entries than *STEP^{WT}* mice, suggesting increased anxiety following genetic reduction of *STEP*. Although light side exploration and total number of light/dark entries (i.e., transitions) are often highly correlated (Crawley & Goodwin, 1980), we saw no significant main effect of *Fmr1* on the total number of entries ($F_{(1, 49)} = 2.216$, $P = 0.134$) (Fig. 8c, Table 4).

Corroborating these results, center square exploration in the open field task indicated decreased anxiety in *Fmr1^{KO}* mice (Table 5). Main effect analysis demonstrated that *Fmr1^{KO}* mice had greater number of center entries ($F_{(1, 73)} = 4.975$, $P = 0.029$), increased distance traveled in center ($F_{(1, 73)} = 5.580$, $P = 0.021$), and increased center/total distance traveled ratio ($F_{(1, 73)} = 3.866$, $P = 0.05$). In line with previous reports that *Fmr1^{KO}* mice are more hyperactive in open field tests (Peier *et al.*, 2000, Spencer *et al.*, 2011), we found that the total number of center/border entries were significantly higher in *Fmr1^{KO}* mice than those that were *Fmr1^{WT}* ($F_{(1, 73)} = 4.654$, $P = 0.034$); however, the total distance traveled was not different ($F_{(1, 73)} = 1.416$, $P = 0.238$) (Table 5). Discrepancies in this measure could be accounted for based on methodological differences, as we assessed open field activity for only 5 min and other studies have used 30 min tests (Peier *et al.*, 2000, Spencer *et al.*, 2011). Of note, there were no significant main effects of *STEP* genotype on any parameter in the open field task (Table 5), demonstrating that genetically reducing *STEP* did not reverse behaviors in this particular task.

Perseverative-related behaviors did not explain the observed behavioral abnormalities associated with *Fmr1^{KO}* mice

To determine if the abnormalities we observed in *Fmr1^{KO}* mice were due to increased perseverative and/or repetitive behaviors, we next examined marble burying behaviors. While marble burying was initially characterized as a measure of anxiety (Njung'e & Handley, 1991), more recent studies indicate that it is a reflection of repetitive and/or perseverative behaviors (Thomas *et al.*, 2009). In an effort to reduce inter-investigator variability and subjectivity, we devised a new method to quantitate the percentage of each marble buried. Nonetheless, we observed no significant main effects of *Fmr1* or *STEP* on the number of marbles buried (*Fmr1*: $F_{(1, 68)} = 0.294$, $P = 0.590$; *STEP*: $F_{(2, 68)} = 1.549$, $P = 0.220$) (Table 5). These findings revealed that observed behavioral abnormalities in social and non-social anxiety-related domains in *Fmr1^{KO}* mice were not due to increased perseverative and/or repetitive behaviors.

Discussion

Much FXS research is currently focused on discovering new target proteins whose translation is controlled by GluRs and FMRP and dysregulated in *Fmr1^{KO}* mice (Bear *et al.*, 2004, Krueger & Bear, 2011). Here we describe and validate STEP as a novel target. We establish increased basal STEP expression and abolished mGluR-induced STEP translation in *Fmr1^{KO}* mice. Genetic modulation of STEP corrects select behavioral abnormalities normally present in *Fmr1^{KO}* mice. Specifically, *Fmr1^{KO}* mice null for STEP exhibit fewer AGS and show a corresponding reduction in PAG neuronal activity following seizures. Moreover, most social and non-social anxiety-related abnormalities associated with *Fmr1^{KO}* mice are reversed by genetically reducing STEP. Altogether, these findings highlight the benefit of reducing STEP in *Fmr1^{KO}* mice and suggest that developing STEP inhibitors may be worthy of clinical consideration for FXS.

FMRP suppresses mRNA translation by stalling ribosomal translocation (Muddashetty *et al.*, 2007, Darnell *et al.*, 2011). Accordingly, the expression of some proteins encoded by FMRP target mRNAs is upregulated in *Fmr1^{KO}* mice (Zalfa *et al.*, 2003, Lu *et al.*, 2004, Hou *et al.*, 2006, Westmark & Malter, 2007, Gross *et al.*, 2010). Given that STEP has been identified as a FMRP target (Darnell *et al.*, 2011), we predicted that its translation would be dysregulated in *Fmr1^{KO}* mice. We establish that STEP levels are basally elevated in synaptoneurosomal, but not cytosolic, fractions, suggesting a selective increase in dendrites. Additionally, mGluR-stimulated translation of STEP is absent in *Fmr1^{KO}* mice, consistent with impaired mGluR-induced translation of FMRP targets (Zalfa *et al.*, 2003, Lu *et al.*, 2004, Hou *et al.*, 2006, Muddashetty *et al.*, 2007, Westmark & Malter, 2007, Park *et al.*, 2008). This disruption is likely caused by improper recruitment of mRNAs to active polyribosomes in *Fmr1^{KO}* mice (Weiler *et al.*, 2004, Muddashetty *et al.*, 2007).

We hypothesized that increased STEP levels contribute to synaptic deficits and behavioral abnormalities in *Fmr1^{KO}* mice. STEP is found in dendrites and has emerged as critical modulator of synaptic plasticity and key player in several neuropsychiatric disorders (Boulanger *et al.*, 1995, Pelkey *et al.*, 2002, Zhang *et al.*, 2008, Zhang *et al.*, 2010, Goebel-Goody *et al.*, 2011). Substrates include NMDARs, AMPARs, Fyn, Pyk2, ERK1/2 and p38 (Nguyen *et al.*, 2002, Pelkey *et al.*, 2002, Munoz *et al.*, 2003, Paul *et al.*, 2003, Snyder *et al.*, 2005, Zhang *et al.*, 2008, Venkitaramani *et al.*, 2011). STEP-mediated dephosphorylation of Fyn, Pyk2, ERK1/2, and p38 inactivates these enzymes, whereas dephosphorylation of surface NMDARs and AMPARs promotes endocytosis. Thus, STEP appears to oppose synaptic strengthening and promote synaptic weakening. In particular, STEP is translated following mGluR stimulation and is required for mGluR-mediated AMPAR internalization (Zhang *et al.*, 2008), suggesting a role in mediating mGluR-LTD (Moult *et al.*, 2006). Given that STEP is among a handful of “LTD proteins” dysregulated in *Fmr1^{KO}* mice (Zalfa *et al.*, 2003, Lu *et al.*, 2004, Hou *et al.*, 2006, Westmark & Malter, 2007, Park *et al.*, 2008), we propose that the complex interaction of STEP with other affected proteins in FXS leads to behavioral abnormalities.

Using a comprehensive battery of tests, we evaluated whether genetically reducing STEP ameliorates abnormalities in *Fmr1^{KO}* mice. One consistent phenotype of *Fmr1^{KO}* mice is their increased AGS susceptibility (Musumeci *et al.*, 2000, Yan *et al.*, 2005). Motivation for assessing AGS in our study stems from prior work showing a striking seizure resistance in *STEP^{KO}* mice (Briggs *et al.*, 2011). Here we establish that loss of STEP in *Fmr1^{KO}* mice significantly reduces AGS. Notably, genetic reduction of mGluR5 and APP in *Fmr1^{KO}* mice leads to similar magnitude changes in AGS frequency (Dolen *et al.*, 2007) (Westmark *et al.*, 2011), highlighting common signaling pathways that may exist for attenuating AGS. Although the mechanism behind this reduced susceptibility in *STEP^{KO}/Fmr1^{KO}* mice is still

unknown, studies point to altered excitatory/inhibitory balance. Specifically, *Fmr1^{KO}* mice exhibit decreased neocortical inhibition, leading to hyperexcitability (Gibson *et al.*, 2008, Olmos-Serrano *et al.*, 2010, Paluszkiwicz *et al.*, 2011) and increased seizure susceptibility (Musumeci *et al.*, 2000, Yan *et al.*, 2005). In contrast, loss of STEP increases hippocampal inhibitory excitability and decreases excitation, thereby reducing seizure susceptibility (Briggs *et al.*, 2011). Further experiments are required to determine whether normalized excitatory/inhibitory balance contributes to decreased AGS in *STEP^{KO}/Fmr1^{KO}* mice.

Seizure activity can be mapped by examining expression of the immediate early gene, c-Fos, a transcription factor that marks cellular activation (Morgan *et al.*, 1987, Herrera & Robertson, 1996). We report that AGS significantly increase c-Fos activation in the PAG, a modulatory structure in the AGS efferent pathway (Faingold, 1999, Faingold & Randall, 1999, N'gouemo & Faingold, 1999, Ross & Coleman, 2000). Of significance, AGS-induced c-Fos staining is significantly less in *STEP^{KO}/Fmr1^{KO}* mice compared to *STEP^{WT}/Fmr1^{KO}* mice, suggesting attenuated PAG activation. Changes in c-Fos number and/or intensity are associated with different seizure stages and varying degrees of severity (Morgan *et al.*, 1987), indicating that loss of STEP may reduce the severity of AGS in *Fmr1^{KO}* mice. Alternatively, it is possible that patterning and neuronal output of AGS is modified in *STEP^{KO}/Fmr1^{KO}* mice. While it is likely that other subcortical auditory nuclei are affected (Le Gal La Salle & Naquet, 1990), our findings provide compelling evidence that loss of STEP significantly reduces AGS frequency and PAG activation in *Fmr1^{KO}* mice.

Individuals with FXS exhibit socially anxious behaviors with abnormal approach and interaction (Wolff *et al.*, 1989, Cornish *et al.*, 2008, Harris *et al.*, 2008). These impairments are attributed to anxiety and hyperarousal to social stimuli, since the motivation for the interaction is present but is too overwhelming to maintain (Cornish *et al.*, 2008). Aberrant social behaviors are also found in *Fmr1^{KO}* mice (Spencer *et al.*, 2005, Mineur *et al.*, 2006, McNaughton *et al.*, 2008, Liu & Smith, 2009, Moy *et al.*, 2009, Mines *et al.*, 2010, Liu *et al.*, 2011). Using the social choice task and social dominance tube test, we demonstrate increased social anxiety alongside heightened interest and hyperactivity in *Fmr1^{KO}* mice that is reversed by genetically manipulating STEP. Our findings are in accord with *Fmr1^{KO}* mice displaying more active social behaviors in direct interaction tests (Spencer *et al.*, 2005) and spending less time per nose contact with mice in the social choice task (McNaughton *et al.*, 2008, Mines *et al.*, 2010). One speculation is that these abnormalities reflect hyperarousal to social stimuli concomitantly with social anxiety, as seen in FXS humans. Disparities between our work and those finding diminished social approach and novelty preference in *Fmr1^{KO}* mice may be explained by the age of the mice and methodology used to quantify nose contacts (Liu *et al.*, 2011, Liu & Smith, 2009, Moy *et al.*, 2009).

Another well-characterized behavioral phenotype of *Fmr1^{KO}* mice is reduced anxiety in non-social paradigms (Peier *et al.*, 2000, Qin *et al.*, 2002, Spencer *et al.*, 2005, Qin & Smith, 2008, Mines *et al.*, 2010, Yuskaitis *et al.*, 2010, Liu *et al.*, 2011, Spencer *et al.*, 2011). Using three independent non-social anxiety tasks, we confirm that *Fmr1^{KO}* mice are less anxious than *Fmr1^{WT}* mice. An alternative interpretation is that *Fmr1^{KO}* mice show increased exploratory behaviors in novel environments, since EPM center square time and number of head dips are increased and these behaviors appear to rely heavily on exploratory behavior (Rodgers & Johnson, 1995). Whether the abnormality associated with *Fmr1^{KO}* mice is due to reduced anxiety and/or increased exploratory behavior, we find that loss of STEP in *Fmr1^{KO}* mice reverses open arm exploration in the EPM, thereby normalizing this phenotype.

We also report novel social and non-social anxiety-related phenotypes of *STEP^{HT}* and *STEP^{KO}* mice. First, they maintain each social interaction longer in the socialization phase

of the social choice task, so it is interesting to consider that the quality of each interaction is enhanced. Prior studies report that *STEP^{KO}* mice exhibit more dominance behaviors in direct interaction tasks (Venkitaramani *et al.*, 2011), so another explanation might be that increased aggression leads to longer visits. However, our results show that *STEP^{KO}/Fmr1^{WT}* and *STEP^{WT}/Fmr1^{WT}* mice retreat from the social dominance tube test at a similar frequency, reducing the likelihood of this possibility. Second, in both the EPM and light/dark box tasks, there are indications that loss of STEP causes mice to be more anxious in non-social paradigms and/or have decreased exploratory behavior. While the basis for these phenotypes remain unknown, several studies have found altered social, anxiety, and depressive-like behaviors following genetic modulation of some STEP substrates, such as NMDARs and ERK2, (Boyce-Rustay & Holmes, 2006, Satoh *et al.*, 2011).

In conclusion, the present study establishes that genetically reducing STEP in *Fmr1^{KO}* mice, either fully or partially, reduces AGS susceptibility and normalizes select social and non-social anxiety-related behaviors. Our results may therefore suggest a potential therapeutic role of STEP in reversing characteristic phenotypes of FXS. Further investigation is required to uncover the molecular basis and/or downstream pathways responsible for attenuating *Fmr1^{KO}* behaviors when STEP is reduced. Moreover, because STEP is differentially modulated in several neuropsychiatric disorders (Goebel-Goody *et al.*, 2011), future work must address how to inhibit STEP in particular brain regions affected in FXS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Translation of STEP is dysregulated in the absence of FMRP

(a) Characterization of synaptoneurosomes (SN) and cytosolic (Cyto) fractions.

Representative blots with equal amounts of protein demonstrating that PSD-95 is expressed almost exclusively in SNs, and, while expressed in both fractions, STEP is enriched in SNs.

(b) GAPDH levels do not vary with *Fmr1* genotype or change in response to DHPG stimulation, so these values were used to normalize other protein signals. (c) Main effect analysis reveals that STEP expression is significantly higher in SNs from *STEP*^{WT}/*Fmr1*^{KO} mice (**P* = 0.038). DHPG treatment significantly increases STEP levels in SNs from *STEP*^{WT}/*Fmr1*^{WT} mice (#*P* = 0.048); however this is completely abolished in *STEP*^{WT}/*Fmr1*^{KO} mice.

Fmr1^{KO} SNs. (d) Expression of STEP in unstimulated Cyto fractions does not differ with *Fmr1* genotype, suggesting that the basal increase in STEP expression occurs selectively in SNs. (e) Main effect analysis reveals that PSD-95 expression is significantly higher in SNs from *STEP*^{WT}/*Fmr1*^{KO} mice (**P* = 0.002). DHPG tends to increase PSD-95 levels in *STEP*^{WT}/*Fmr1*^{WT} SNs, but this was not statistically significant (*P* = 0.089). No DHPG-induced change in PSD-95 expression is observed in *STEP*^{WT}/*Fmr1*^{KO} SNs. Data represent the mean normalized protein signal divided by the normalized GAPDH signal ± s.e.m. for all values (n = 3).

Figure 2. Generation of *Fmr1*^{KO} mice with a selective reduction in *STEP*

(a) Breeding strategy employed in the study. *Fmr1*^{KO} mice were cross-bred with *STEP*^{KO} mice on a pure c57Bl/6 background to produce breeder progeny that were *STEP*^{HT}/*Fmr1*^{HT} (females) and *STEP*^{HT}/*Fmr1*^{KO} (males). Breeders from different litters were then mated to produce offspring with a selective reduction or elimination of *STEP* and *Fmr1*. The six offspring analyzed in this study are listed. (b) Representative gels of PCR reactions screening for the presence or absence of the WT and KO (neomycin) *STEP* and *Fmr1* alleles.

Figure 3. Genetically reducing STEP decreases audiogenic seizures (AGS) and reduces seizure-induced c-Fos activation in the periaqueductal gray (PAG) of *Fmr1*^{KO} mice
 (a) None of the *STEP*^{WT}/*Fmr1*^{WT} (n = 12), *STEP*^{HT}/*Fmr1*^{WT} (n = 13), or *STEP*^{KO}/*Fmr1*^{WT} (n = 5) mice show seizure activity in response to a 130 dB stimulus. On the contrary, 76% of *STEP*^{WT}/*Fmr1*^{KO} (n = 21) exhibit a response. Genetically reducing STEP decreases AGS since only 53.3% of *STEP*^{HT}/*Fmr1*^{KO} (n = 30) and 43.5% of *STEP*^{KO}/*Fmr1*^{KO} (n = 23) mice have a response (*indicates significantly different from *STEP*^{WT}/*Fmr1*^{WT}, #; indicates significantly different from *STEP*^{WT}/*Fmr1*^{KO}). Data represent the mean percentage of mice showing seizure incidence (wild running, tonic/clonic seizure). (b–g) Comparison of c-Fos immunoreactivity in the PAG among different genotypes and seizure responses 30 min after exposure to the audio stimulus. Scale equals 200 microns in (b–d) and 10 microns in (e–g).

(b, e) Only a sparse number of c-Fos+ neurons are found in the PAG of *STEP^{WT}/Fmr1^{WT}* mice when no seizure response is observed. (c, f) *STEP^{WT}/Fmr1^{KO}* mice that have seizures show robust c-Fos staining in the PAG. (d, g) The number of seizure-induced c-Fos+ neurons is consistently less in *STEP^{KO}/Fmr1^{KO}* mice compared to *STEP^{WT}/Fmr1^{KO}* mice. (h) Number of c-Fos+ cells in the PAG among genotypes in the absence or presence of a seizure. When no response is observed, there are no significant differences between *STEP^{WT}/Fmr1^{WT}* (n = 5), *STEP^{WT}/Fmr1^{KO}* (n = 4), or *STEP^{KO}/Fmr1^{KO}* (n = 6) mice. Following seizures, the number of c-Fos+ PAG neurons is significantly increased regardless of genotype (**P* = 0.0001); however this number is significantly less in *STEP^{KO}/Fmr1^{KO}* (n = 6) compared to *STEP^{WT}/Fmr1^{KO}* (n = 5) mice (#*P* = 0.01). Data represent the mean c-Fos + cells in the PAG ± s.e.m.

Figure 4. Abnormalities in social approach are reversed in *Fmr1*^{KO} mice following genetic reduction of STEP

(a) Schematic of apparatus during the socialization phase of the social choice task. No significant differences among genotypes are detected in total distance traveled (b) or time in close proximity to the stranger mouse (c). (d) Main effect analysis reveals that *Fmr1*^{KO} mice enter the area in close proximity to the stranger mouse more times than *Fmr1*^{WT} (***P* = 0.001). *STEP*^{HT}/*Fmr1*^{KO} mice have significantly fewer visits than *STEP*^{WT}/*Fmr1*^{KO} mice ([#]*P* = 0.008), indicating that decreasing STEP levels reverses this phenotype. (e) A significant main effect of *STEP* genotype was observed for the duration of each entry in close proximity to the stranger mouse (**P* = 0.016). Specifically, *STEP*^{HT} and *STEP*^{KO} mice spend more time per visit in close proximity to the stranger mouse than *STEP*^{WT} mice. Data represent the mean for each measure ± s.e.m. [*STEP*^{WT}/*Fmr1*^{WT} (*n* = 8), *STEP*^{HT}/*Fmr1*^{WT} (*n* = 11), *STEP*^{KO}/*Fmr1*^{WT} (*n* = 6), *STEP*^{WT}/*Fmr1*^{KO} (*n* = 13), *STEP*^{HT}/*Fmr1*^{KO} (*n* = 8), and *STEP*^{KO}/*Fmr1*^{KO} (*n* = 6)].

Figure 5. Genetically decreasing STEP levels ameliorates social novelty-induced hyperactivity in *Fmr1*^{KO} mice

(a) Schematic of apparatus during the social novelty phase of the social choice task. (a) Main effect analysis reveals that *Fmr1*^{KO} mice travel significantly greater distance than *Fmr1*^{WT} mice during the social novelty phase (***P* = 0.012). In contrast, *STEP*^{HT} and *STEP*^{KO} mice travel significantly less distance compared to *STEP*^{WT} mice (**P* = 0.005). The net result is that genetically reducing STEP ameliorates the effect of *Fmr1* genotype on locomotion. Significant main effects of *Fmr1* are observed for both the total time (c) and number of entries (d) in close proximity to the novel mouse (c, ***P* = 0.030; d, ***P* = 0.038), which may be explained, in part, by the overall increased locomotion in *Fmr1*^{KO} mice (b). (e) No significant differences among genotypes are detected in the duration of each visit in close proximity to the novel mouse. Data represent the mean for each measure ± s.e.m. [*STEP*^{WT}/*Fmr1*^{WT} (n = 8), *STEP*^{HT}/*Fmr1*^{WT} (n = 11), *STEP*^{KO}/*Fmr1*^{WT} (n = 6), *STEP*^{WT}/*Fmr1*^{KO} (n = 13), *STEP*^{HT}/*Fmr1*^{KO} (n = 8), and *STEP*^{KO}/*Fmr1*^{KO} (n = 6)].

Figure 6. Social anxiety in *Fmr1^{KO}* mice is reversed following genetic reduction of STEP in a social dominance tube test

The percentage of wins by *STEP^{WT}/Fmr1^{KO}* (n = 10), *STEP^{HT}/Fmr1^{KO}* (n = 8), and *STEP^{KO}/Fmr1^{KO}* (n = 6) against *STEP^{WT}/Fmr1^{WT}* mice is presented. Each mouse was tested against three *STEP^{WT}/Fmr1^{WT}* mice. Genetically reducing STEP in *Fmr1^{KO}* mice increases the number of wins against *STEP^{WT}/Fmr1^{WT}*, indicating that social anxiety in this task is corrected. Data represent the mean percentage of wins (*indicates significantly different from *STEP^{WT}/Fmr1^{WT}*).

Figure 7. Genetically reducing STEP in *Fmr1*^{KO} mice reverses open arm exploration in the elevated plus maze

(a) Main effect analysis reveals that *Fmr1*^{KO} mice enter the open arms more times than *Fmr1*^{WT} mice (***P* = 0.034), indicative of decreased anxiety. In contrast, *STEP*^{KO} mice enter the open arms significantly less than *STEP*^{WT} or *STEP*^{HT} mice (**P* = 0.035), suggesting increased anxiety. The net result is that genetically reducing *STEP* normalizes the effect of *Fmr1* genotype on open arm exploration. (b) No significant differences among genotypes are detected in the number of total arm entries, indicating similar locomotion in this task. Significant main effects of *Fmr1* are found for the percentage of total time in the center square (c), duration of each center square entry (d), and number of head dips in the open arms (e) (c, ***P* = 0.008; d, ***P* = 0.009; e, ***P* = 0.011), suggesting that *Fmr1*^{KO} mice have increased exploratory behaviors compared to *Fmr1*^{WT} mice. In these measures (c–e), there were no main effects of *STEP* genotype. Data represent the mean for each measure ± s.e.m. [*STEP*^{WT}/*Fmr1*^{WT} (n = 10), *STEP*^{HT}/*Fmr1*^{WT} (n = 11), *STEP*^{KO}/*Fmr1*^{WT} (n = 10), *STEP*^{WT}/*Fmr1*^{KO} (n = 13), *STEP*^{HT}/*Fmr1*^{KO} (n = 8), and *STEP*^{KO}/*Fmr1*^{KO} (n = 7)].

Figure 8. Effects of STEP and *Fmr1* on light/dark box exploration

Main effect analysis reveals that *Fmr1*^{KO} mice spent more time (a) and travel greater distance (b) than *Fmr1*^{WT} mice in the illuminated side of the light/dark box (a, ***P* = 0.002; b, ***P* = 0.014), suggesting decreased anxiety. While the time (a) and distance traveled (b) for *STEP*^{HT}/*Fmr1*^{KO} mice tends to be less than *STEP*^{WT}/*Fmr1*^{KO} mice, these results did not reach statistical significance (a, *P* = 0.061; b, *P* = 0.072). (c) A significant main effect of STEP is observed for the total number of light/dark side entries (**P* = 0.004). Specifically, *STEP*^{HT} and *STEP*^{KO} mice have fewer total entries than *STEP*^{WT} mice, providing further support that reducing STEP increases anxiety. Data represent the mean for each measure ± s.e.m. [*STEP*^{WT}/*Fmr1*^{WT} (*n* = 8), *STEP*^{HT}/*Fmr1*^{WT} (*n* = 10), *STEP*^{KO}/*Fmr1*^{WT} (*n* = 7), *STEP*^{WT}/*Fmr1*^{KO} (*n* = 11), *STEP*^{HT}/*Fmr1*^{KO} (*n* = 10), and *STEP*^{KO}/*Fmr1*^{KO} (*n* = 9)].

Table 1

List of primary and secondary antibodies used in immunoblotting and immunohistochemistry.

Antibody	Host	Dilution	Source
Anti-STEP (23E5)	Mouse	1:1000	Boulanger <i>et al.</i> , 1995
Anti-PSD-95	Rabbit	1:5000	Millipore
Anti-GAPDH	Mouse	1:40000	Millipore
Anti-rabbit	Goat	1:10000	Bio-Rad Laboratories
Anti-mouse	Goat	1:5000	Bio-Rad Laboratories
Anti-c-Fos	Goat	1:500	Santa Cruz Biotechnology, Inc.
Anti-Goat (biotinylated)	Horse	1:300	Vector Laboratories

Table 2

Breakdown of AGS responses following genetic reduction of *Fmr1* and/or *STEP*.

Genotype	% Incidence	Wild Running	Seizure (clonic/tonic)
<i>STEP^{WT}/Fmr1^{WT}</i>	0%	0/12	0/12
<i>STEP^{HT}/Fmr1^{WT}</i>	0%	0/13	0/13
<i>STEP^{KO}/Fmr1^{WT}</i>	0%	0/5	0/5
<i>STEP^{WT}/Fmr1^{KO}</i>	76.2% *	9/21	7/21
<i>STEP^{HT}/Fmr1^{KO}</i>	53.3% *	2/30	14/30
<i>STEP^{KO}/Fmr1^{KO}</i>	43.5% *#	3/23	7/23

Table 3Summary of differences in the social choice task following genetic reduction of *Fmr1* and/or *STEP*.

Phase and Parameter	<i>Fmr1</i> main effect	<i>STEP</i> main effect
<u>Habituation</u>		
Total distance traveled	n.s. ($P = 0.534$)	*$P = 0.05$; KO < WT = HT
<u>Socialization (Mouse vs. Cup)</u>		
Total distance traveled	n.s. ($P = 0.174$)	n.s. ($P = 0.196$)
Socialization index	n.s. ($P = 0.093$)	n.s. ($P = 0.556$)
Mouse: close proximity time	n.s. ($P = 0.055$)	n.s. ($P = 0.451$)
Mouse: # of close proximity entries	*$P = 0.005$; KO > WT	n.s. ($P = 0.149$)
Mouse: time per entry in close proximity	n.s. ($P = 0.195$)	*$P = 0.02$; HT > KO > WT
Mouse: chamber time	n.s. ($P = 0.066$)	n.s. ($P = 0.521$)
Cup: close proximity time	n.s. ($P = 0.774$)	n.s. ($P = 0.677$)
Cup: # of close proximity entries	n.s. ($P = 0.192$)	n.s. ($P = 0.161$)
Cup: time per entry in close proximity	n.s. ($P = 0.058$)	n.s. ($P = 0.877$)
Cup: chamber time	n.s. ($P = 0.177$)	n.s. ($P = 0.720$)
<u>Social Novelty (Familiar Mouse vs. Novel Mouse)</u>		
Total distance traveled	*$P = 0.02$; KO > WT	*$P = 0.005$; KO < HT < WT
Novelty index	n.s. ($P = 0.949$)	n.s. ($P = 0.879$)
Novel: close proximity time	*$P = 0.05$; KO > WT	n.s. ($P = 0.953$)
Novel: # of close proximity entries	*$P = 0.05$; KO > WT	n.s. ($P = 0.553$)
Novel: time per entry in close proximity	n.s. ($P = 0.517$)	n.s. ($P = 0.385$)
Novel: chamber time	n.s. ($P = 0.821$)	n.s. ($P = 0.978$)
Familiar: close proximity time	n.s. ($P = 0.240$)	n.s. ($P = 0.996$)
Familiar: # of close proximity entries	n.s. ($P = 0.154$)	*$P = 0.05$; KO = HT < WT
Familiar: time per entry in close proximity	n.s. ($P = 0.607$)	n.s. ($P = 0.086$)
Familiar: chamber time	n.s. ($P = 0.998$)	n.s. ($P = 0.832$)

Significance was set at $P = 0.05$ (*), and main effect differences highlighted in bold. Parameters that were not statistically significant are denoted as n.s.

Table 4

Summary of differences in the elevated plus maze and light/dark box following genetic reduction of *Fmr1* and/or *STEP*.

Task and Parameter	Fmr1 main effect	STEP main effect
<u><i>Elevated Plus Maze</i></u>		
Total distance traveled	n.s. ($P = 0.835$)	n.s. ($P = 0.349$)
# of total arm entries	n.s. ($P = 0.261$)	n.s. ($P = 0.127$)
Open arms: # of entries	*$P = 0.05$; KO > WT	*$P = 0.05$; KO < HT = WT
Open arms: % time	*$P = 0.05$; KO > WT	n.s. ($P = 0.489$)
Closed arms: # of entries	n.s. ($P = 0.713$)	n.s. ($P = 0.307$)
Closed arms: % time	*$P = 0.05$; KO < WT	n.s. ($P = 0.489$)
Center square: # of entries	n.s. ($P = 0.220$)	n.s. ($P = 0.130$)
Center square: % time	*$P = 0.01$; KO > WT	n.s. ($P = 0.367$)
Center square: time per entry	*$P = 0.01$; KO > WT	n.s. ($P = 0.465$)
# of head dips to/on open arms	*$P = 0.02$; KO > WT	n.s. ($P = 0.145$)
# of stretch attend/flat back postures to/on open arms	n.s. ($P = 0.299$)	n.s. ($P = 0.768$)
<u><i>Light/Dark Box</i></u>		
# of total entries in light/dark sides	n.s. ($P = 0.143$)	*$P = 0.01$; KO = HT < WT
Light side: time	*$P = 0.005$; KO > WT	n.s. ($P = 0.335$)
Light side: distance traveled	*$P = 0.02$; KO > WT	n.s. ($P = 0.139$)
Light side: latency to first exit	n.s. ($P = 0.397$)	n.s. ($P = 0.147$)
Dark side: time	*$P = 0.005$; KO < WT	n.s. ($P = 0.336$)

Significance was set at $P = 0.05$ (*), and main effect differences highlighted in bold. Parameters that were not statistically significant are denoted as n.s.

Table 5

Summary of differences in open field and marble burying tasks following genetic reduction of *Fmr1* and/or *STEP*.

Task and Parameter	<i>Fmr1</i> main effect	<i>STEP</i> main effect
<u>Open Field</u>		
# of center entries	*P 0.05; KO > WT	n.s. (<i>P</i> = 0.285)
# of total entries (center + border)	*P 0.05; KO > WT	n.s. (<i>P</i> = 0.248)
Center time	n.s. (<i>P</i> = 0.237)	n.s. (<i>P</i> = 0.962)
Distance traveled in center	*P 0.05; KO > WT	n.s. (<i>P</i> = 0.485)
Total distance traveled	n.s. (<i>P</i> = 0.238)	n.s. (<i>P</i> = 0.565)
Ratio of center distance/total distance	*P 0.05; KO > WT	n.s. (<i>P</i> = 0.672)
<u>Marble Burying</u>		
No marbles: total distance traveled	n.s. (<i>P</i> = 0.800)	n.s. (<i>P</i> = 0.962)
With marbles: total distance traveled	n.s. (<i>P</i> = 0.078)	n.s. (<i>P</i> = 0.499)
# of marbles buried > 40%	n.s. (<i>P</i> = 0.590)	n.s. (<i>P</i> = 0.220)

Significance was set at *P* = 0.05 (*), and main effect differences highlighted in bold. Parameters that were not statistically significant are denoted as n.s.