

Reversal of Fragile X Phenotypes by Manipulation of A β PP/A β Levels in *Fmr1*^{KO} Mice

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Abstract

Fragile X syndrome (FXS) is the most common form of inherited intellectual disability and the leading known genetic cause of autism. Fragile X mental retardation protein (FMRP), which is absent or expressed at substantially reduced levels in FXS, binds to and controls the postsynaptic translation of amyloid β -protein precursor (A β PP) mRNA. Cleavage of A β PP can produce β -amyloid (A β), a 39–43 amino acid peptide mis-expressed in Alzheimer's disease (AD) and Down syndrome (DS). A β is over-expressed in the brain of *Fmr1*^{KO} mice, suggesting a pathogenic role in FXS. To determine if genetic reduction of A β PP/A β rescues characteristic FXS phenotypes, we assessed audiogenic seizures (AGS), anxiety, the ratio of mature versus immature dendritic spines and metabotropic glutamate receptor (mGluR)-mediated long-term depression (LTD) in *Fmr1*^{KO} mice after removal of one *App* allele. All of these phenotypes were partially or completely reverted to normal. Plasma A β _{1–42} was significantly reduced in full-mutation FXS males compared to age-matched controls while cortical and hippocampal levels were somewhat increased, suggesting that A β is sequestered in the brain. Evolving therapies directed at reducing A β in AD may be applicable to FXS and A β may serve as a plasma-based biomarker to facilitate disease diagnosis or assess therapeutic efficacy.

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Introduction

FXS is an X chromosome-linked disorder characterized by highly variable intellectual disability, autistic-like behavior and seizures [1]. In the vast majority of cases, FXS results from a >200 trinucleotide (CGG) repeat expansion in the 5'-UTR of the *FMR1* gene [2] leading to transcriptional silencing and loss or reduction of expression of FMRP [3]. FMRP is a multi-functional mRNA binding protein involved in the dendritic transport, localization and translational regulation of several hundred mRNA ligands [4–9]. In the absence of FMRP, dendritic spine morphology and function are abnormal [10]. Thus, FXS is likely caused by the inappropriate, post-synaptic expression of one or more FMRP mRNA targets. We have previously demonstrated that post-synaptic translation of *App* mRNA is regulated by FMRP through a mGluR₅-dependent pathway. In the absence of FMRP, excess A β PP and its catabolites A β _{1–40} and A β _{1–42}, accumulate in the brains of middle-aged *Fmr1*^{KO} mice [11].

There is very limited data regarding the roles of A β PP mRNA, protein and catabolites in persons with FXS. One group showed

elevated *App* mRNA in the cerebral cortex, hippocampus and cerebellar cortex in *Fmr1*^{KO} mice [12], but we have not observed differences in cortical synaptoneuroosomes [11]. Increased A β levels would predict an increased incidence of AD pathology in aged FXS individuals, which has not been observed in neuropathological analyses of a very small number of specimens [13–15]. Likewise, there are no reports of an increased incidence of AD in FXS, but it is difficult to assess age-related dementia in the mentally retarded and very few elderly individuals with FXS have been studied in significant numbers. Recent data suggests that A β PP or its proteolytic derivatives may be aberrantly expressed in children with severe autism [16,17], which is extremely prevalent in FXS (67% of males and 23% of females) [18] and DS (7%) [19]. Thus, the increased production or altered processing of A β PP may contribute to the intellectual disabilities observed in all of these disorders.

Herein, we demonstrate rescue of several *Fmr1*^{KO} phenotypes in model mice by genetic modulation of A β PP/A β levels. Treatment of primary neurons with A β _{1–42} rapidly triggered extracellular-regulated kinase (ERK) signaling and altered the translation of multiple FMRP target mRNAs including *App*. Conversely, anti-A β

antibody decreased dendritic A β PP levels. These data suggest that A β modulates its own production through a positive feedback loop. Finally, we show that plasma A β_{1-42} is significantly reduced while cerebral A β_{1-42} is likely increased in full-mutation FXS males. These data suggest evolving therapies directed at reducing A β in AD may be applicable to FXS and plasma A β_{1-42} may be a biomarker for disease severity and drug efficacy in FXS.

Results

Genetic Modulation of A β PP Levels in *Fmr1*^{KO} Mice Reduces AGS

Compared to WT, *Fmr1*^{KO} mice exhibit AGS [20], altered anxiety [21–23] and dendritic spine dysmorphogenesis [24–26], features shared with patients with FXS [1,27,28]. In order to establish if A β PP or A β directly contributed to FXS pathogenesis, we modulated A β PP and A β expression in *Fmr1*^{KO} mice. Thus, we generated *Fmr1*^{KO}/*App*^{HET} and *Fmr1*^{KO}/*App*^{KO} mice all in a pure C57BL/6 background to evaluate the effects of genetic modulation of A β PP and A β expression. Western blot analyses confirmed that A β PP levels were reduced by 50% in *Fmr1*^{KO}/*App*^{HET} mice (Figure 1A). At 21 days of age, animals were evaluated for AGS. Wild running (WR) and seizures decreased 32% and 54%, respectively, in *Fmr1*^{KO}/*App*^{HET} mice (Figure 1B). These data suggest that seizures are enhanced when A β PP is absent or over-expressed and that A β PP or one of its metabolites significantly contributes to the AGS phenotype seen in *Fmr1*^{KO} mice.

Pharmacological antagonists directed at or genetic reduction of mGluR₅ correct many *Fmr1*^{KO} phenotypes [22,26,29–31]. We compared the efficacy of mGluR₅ antagonists with genetic manipulation of A β PP/A β in reducing AGS in *Fmr1*^{KO} mice. A 30 min pretreatment with 2-methyl-6-(phenylethynyl)pyridine hydrochloride (MPEP) delivered by I.P. injection reduced WR, AGS and deaths by 82%, 75% and 100%, respectively (Figure 1B). An alternative mGluR₅ antagonist, fenobam, reduced WR, AGS and deaths to 0% (data not shown).

Fmr1^{KO}/*App*^{HET} Mice Lack FXS Behavioral, Dendritic Spine and mGluR-LTD Phenotypes

Hyperactivity, social anxiety and autistic-like behaviors are characteristic features of FXS [1]. We first assessed marble burying in these mice as a measure of repetitive behavior [32]. *Fmr1*^{KO} male mice buried significantly fewer marbles than WT ($p = 0.04$) (Figure 2A), which was rescued in male *Fmr1*^{KO}/*App*^{HET} mice (statistically different compared to *Fmr1*^{KO}, $p = 0.03$; not different from WT, $p = 0.95$). Thus, repetitive digging is a normal mouse behavior that can be rescued in *Fmr1*^{KO} male mice by genetically reducing A β PP/A β levels.

Next, we assessed hyperactivity (Figure 2B) and anxiety (Figure 2C, D) in the open field test. *Fmr1*^{KO} exhibit increased center time (reflecting a loss of anxiety) but equivalent locomotion in the open field compared with WT mice [21,22]. *App*^{HET} and *App*^{KO} mice exhibited significantly more anxiety/thigmotaxis than WT mice (Figure 2C). The increased thigmotaxis in the *App*^{HET} is partially due to decreased locomotion compared with WT mice (Figure 2B); however, total distance traveled was equivalent between *App*^{KO} and *App*^{HET} indicating that decreased A β PP/A β levels correlate with increased anxiety. *Fmr1*^{KO}/*App*^{HET} mice exhibited equivalent center time as WT mice and significantly less than *Fmr1*^{KO} mice indicating that thigmotaxis was rescued. Total distance traveled in the open field was not statistically different between *Fmr1*^{KO}/*App*^{HET}, WT and/or *Fmr1*^{KO} mice indicating equivalent locomotion.

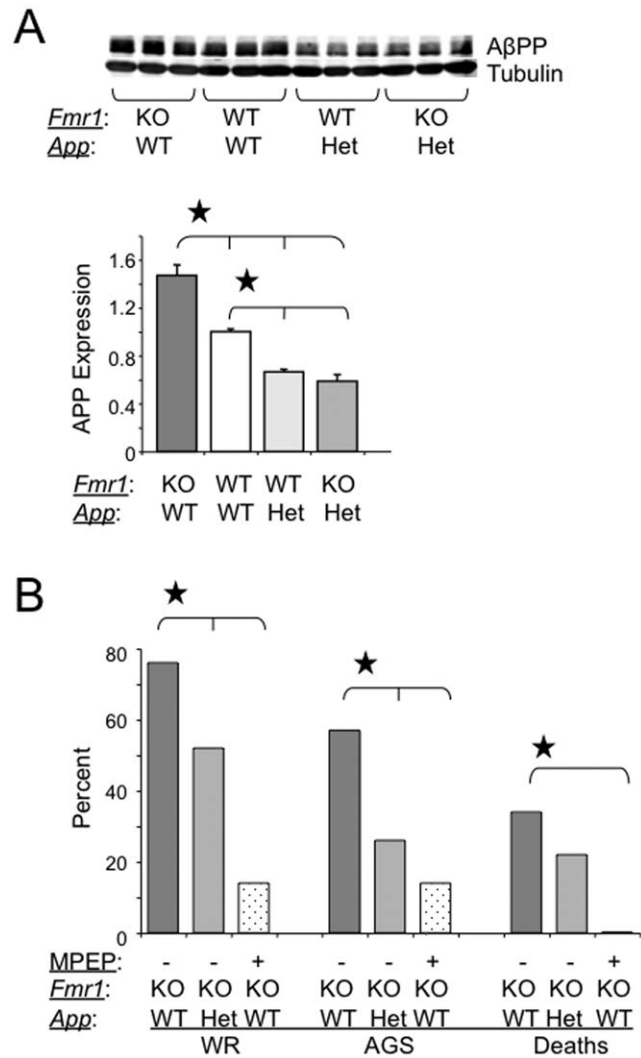


Figure 1. AGS are rescued by genetic manipulation of *App* or mGluR₅ blockade. (A) western blot analyses of A β PP levels in *Fmr1*^{KO}, WT, *App*^{HET} and *Fmr1*^{KO}/*App*^{HET} mice (n=3 male mice per strain, 1 month old). Statistics: one-way ANOVA $p < 0.0001$, $F = 53.95$. Stars (★) denote statistically different levels by Student T-test analyses and by Bonferroni's multiple comparison test ($p < 0.05$). Error bars represent SEM. (B) Assessment of WR, AGS and death rates in *Fmr1*^{KO} mice (age P21, n=58) after genetic manipulation of A β PP levels (n=23) or treatment with MPEP (n=14). Stars (★) denote rates that are statistically different from untreated mice ($p < 0.5$) by Fisher exact tests. doi:10.1371/journal.pone.0026549.g001

Pathological examination of brains from FXS patients has shown an increased density of long and tortuous dendritic spines suggesting a delay in spine maturation [15,33]. We assessed dendritic spine length in primary cultured neurons and found a statistically significant 1.6-fold increase in protrusion length in the *Fmr1*^{KO} cells compared to WT (Figure 3A, B) in agreement with the literature [24–26]. A 4 hr treatment with mGluR₅ antagonists (MPEP or fenobam) rescued the spine/filopodia ratio in *Fmr1*^{KO} mice to WT levels [26]. Likewise, we observed that both dendritic spine length and the percentage of filopodia in primary cultured *Fmr1*^{KO} neurons were reverted to WT levels within 15 min of MPEP treatment (Figure 3B). Average protrusion length was reduced by 11% in the *Fmr1*^{KO}/*App*^{HET} cells (statistically significant $p < 0.03$ compared to *Fmr1*^{KO}), and the percentage of filopodia (immature spines) versus mature spines was completely reverted to

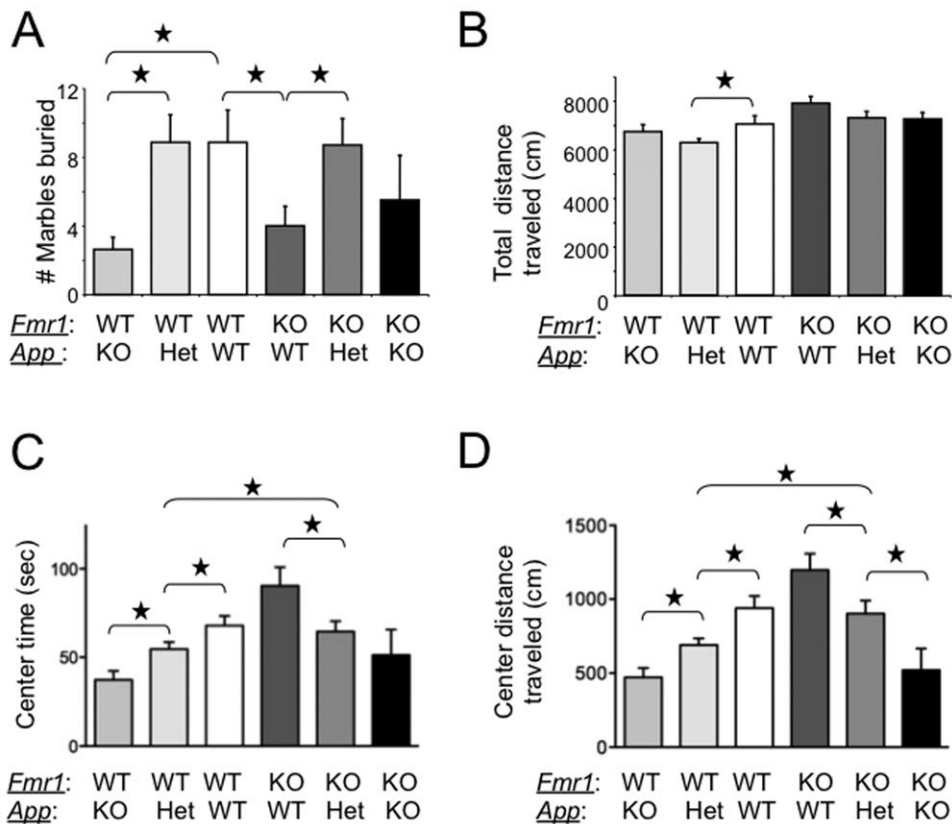


Figure 2. Rescue of behavioral phenotypes in adult *Fmr1*^{KO} mice by genetic manipulation of *App*. (A) male *App*^{KO} (n = 8), *App*^{HET} (n = 14), WT (n = 7), *Fmr1*^{KO} (n = 8), *Fmr1*^{KO}/*App*^{HET} (n = 10) and *Fmr1*^{KO}/*App*^{KO} (n = 4) mice (8–10 weeks old) were assessed for marble burying activity. Statistics: one-way ANOVA $p < 0.022$, $F = 2.95$. (B, C and D) Mice were assessed for anxiety levels and locomotion in the open field. Both genders were included as the males and females exhibited equivalent locomotion and anxiety in the open field [*App*^{KO} (n = 11), *App*^{HET} (n = 23), WT (n = 15), *Fmr1*^{KO} (n = 14), *Fmr1*^{KO}/*App*^{HET} (n = 18) and *Fmr1*^{KO}/*App*^{KO} (n = 7)]. (B) Total distance (cm) traveled is plotted against mouse strain. Statistics: one-way ANOVA $p < 0.0005$, $F = 4.59$. (C) Time (sec) spent in the center of the arena is plotted versus mouse strain. Statistics: one-way ANOVA $p < 0.0001$, $F = 5.98$. (D) Distance (cm) traveled in the center of the arena is plotted versus mouse strain. Statistics: one-way ANOVA $p < 0.0001$, $F = 9.17$. Stars (★) denote statistically different levels by Student T-test analyses ($p < 0.5$). All error bars represent SEM. doi:10.1371/journal.pone.0026549.g002

WT levels. Similarly, minocycline treatment of *Fmr1*^{KO} neurons has been reported to rescue the percentage of mature spines to WT levels albeit without a significant change in dendritic protrusion length [24].

Fmr1^{KO} mice exhibit enhanced hippocampal mGluR-long term depression (LTD), which requires rapid protein synthesis [34,35]. We assessed hippocampal mGluR-LTD by field recordings in 3-month-old male WT, *Fmr1*^{KO}, *App*^{HET} and *Fmr1*^{KO}/*App*^{HET} mice. mGluR-dependent synaptic depression was enhanced in the CA1 in *Fmr1*^{KO} slices, equivalent in WT and *App*^{HET} and reduced in *Fmr1*^{KO}/*App*^{HET} (statistically significant $p < 0.0002$ comparing *Fmr1*^{KO}/*App*^{HET} and *Fmr1*^{KO}) (Figure 4). The time course of CA1 fEPSP slopes after DHPG treatment reveals a significant difference between *Fmr1*^{KO} animals relative to wild type, *App*^{HET} and *Fmr1*^{KO}/*App*^{HET} mice. Input/output relationships and paired-pulse facilitation (ppf) were analyzed to assess the basal synaptic properties of the different groups (Figure S1), and did not show significant changes between experimental groups indicating unaltered synaptic transmission in the *Fmr1*^{KO} hippocampus.

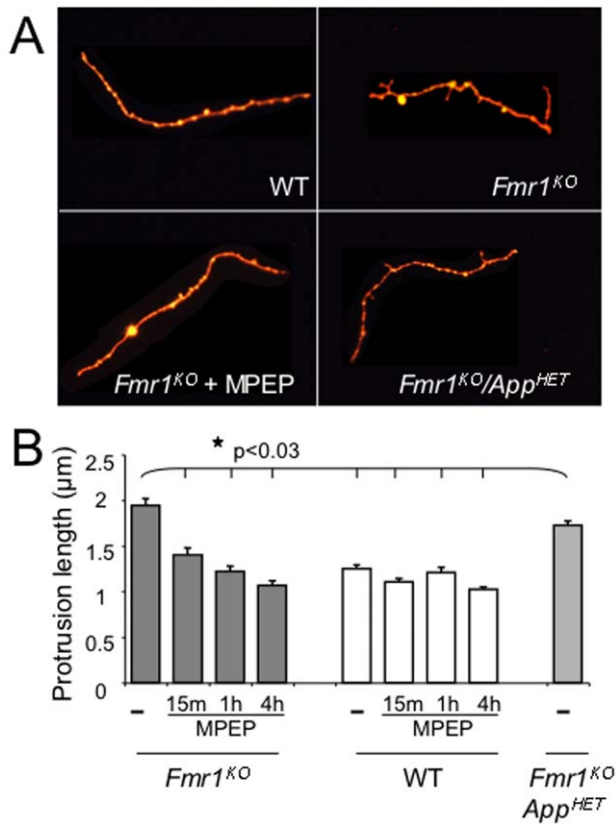
A β _{1–42} Alters Dendritic Expression of FMRP Targets

To begin to understand how excessive production and/or processing of A β PP mediates cell signaling events, we examined dendritic expression of select FMRP targets after treating WT

primary neurons with soluble A β _{1–42}. There was a >2-fold increase in the expression of A β PP, no change in Arc, 40% increase in Map1B, 85% increase in RhoB and 50% decrease in PSD95 (Figure 5A). The A β _{1–42}-mediated increase in dendritic A β PP could be blocked with MPEP or anisomycin indicating that it was mGluR₅- and translation-dependent (Figure 5B). Conversely, reduction of A β in the cell culture media by treatment with anti-A β antibody through a transwell reduced dendritic A β PP expression by 31% (Figure 5C). We utilized transwells to avoid direct contact between the antibody and the cells as direct application of anti-A β to the culture media dramatically increased A β PP expression presumably due to cell signaling events initiated by anti-A β binding to cell surface receptors (data not shown). MPEP and A β _{1–42} altered phosphorylated levels of ERK (Figure S2), suggesting that the mitogen activated protein kinase pathway is as an intermediate in A β -mediated A β PP translation.

A β _{1–42} Levels are Abnormal in Humans with FXS

We are unaware of any published data evaluating A β PP or its products in the blood of adult FXS patients. We found that plasma A β PP/A β PP α and A β _{1–40} levels were comparable in adult FXS patients and controls (Figure 6A, B). These results are distinct from those previously observed in children [16] and suggest that A β PP expression and processing decrease with age. Indeed, A β PP α levels



% Filopodia	64	59	57	55	52	53	48	45	51
% Spines	36	41	43	45	48	47	52	55	49

Figure 3. Dendritic spine morphology is partially rescued in *Fmr1^{KO}/App^{HET}* or MPEP treated *Fmr1^{KO}* mice. (A) Representative fluorescent images of primary cultured neurons prepared from WT (upper left), *Fmr1^{KO}* (upper right and lower left) and *Fmr1^{KO}/App^{HET}* (lower right) embryos stained with Dil and visualized by fluorescent microscopy (100× objective). The arrows denote dendritic spines. (B) The lengths of dendritic protrusions were quantitated with Stereo- Investigator software and plotted against mouse strain/treatment. The percentage of filopodia versus spines for each condition is given below the histogram. Statistics: one-way ANOVA comparison of the three genotypes (untreated) $p < 0.0001$, $F = 27.18$. All genotypes are statistically different from each other by Student T-Test and Bonferroni's multiple comparison tests. Two-way ANOVA comparison of WT versus *Fmr1^{KO}* ± MPEP: $p < 0.0001$, $F = 12.89$ (interaction), $F = 35.01$ (genotype) and $F = 27.62$ (MPEP). The untreated and 15 min MPEP treated WT spines are statistically different from the corresponding *Fmr1^{KO}* spines by the Bonferroni multiple comparison test ($p < 0.5$). Stars (★) denote statistically different spine lengths by Student T-test analyses ($p < 0.5$). Error bars indicate SEM [*Fmr1^{KO}*: untreated ($n = 746$ spines), 15 min MPEP ($n = 263$), 1 hr MPEP ($n = 300$), 4 hr MPEP ($n = 293$); WT: untreated ($n = 994$), 15 min MPEP ($n = 535$), 1 hr MPEP ($n = 373$), 4 hr MPEP ($n = 1221$); *Fmr1^{KO}/App^{HET}* ($n = 2469$)]. doi:10.1371/journal.pone.0026549.g003

are higher in children 7 years and younger than those 10 years and older [16]. While plasma $A\beta_{1-40}$ levels were unchanged between FXS and controls, $A\beta_{1-42}$ was significantly lower in the FXS group (2.1-fold decrease, $p < 0.004$) (Figure 6B). As seen in other amyloidogenic diseases, the $A\beta_{1-42}/A\beta_{1-40}$ ratio (1.4:1) in blood plasma was substantially reduced compared to controls (3.4:1) suggesting decreased clearance from the brain. There was no statistically significant increases in cell-associated AβPP, secreted AβPPα nor *APP_{695/751/770}* mRNA in peripheral blood mononu-

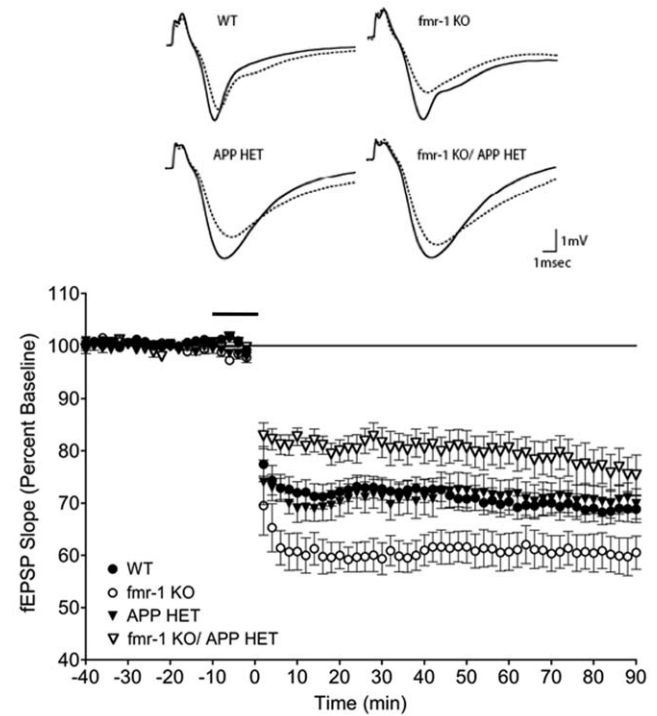


Figure 4. Rescue of mGluR-dependent synaptic depression in the CA1 in *Fmr1^{KO}/App^{HET}* mice. (Top) Representative fEPSP taken before the addition of DHPG (solid lines) and at the end of the recordings (dotted lines). (Bottom) Time course of CA1 fEPSP slope after incubation with DHPG (50 μM, 10 min) in hippocampal slices from WT (black circles) ($n = 20$), *Fmr1^{KO}* (white circles) ($n = 13$), *App^{HET}* (black triangles) ($n = 15$) and *Fmr1^{KO}/App^{HET}* (white triangles) ($n = 10$) male mice (3 months old). WT, *App^{HET}* and *Fmr1^{KO}/App^{HET}* were all statistically different from *Fmr1^{KO}* by two-way ANOVA/Bonferroni multiple comparison tests. WT versus *App^{HET}*, $p = 0.92$; WT versus *Fmr1^{KO}*, $p < 0.006$; WT versus *Fmr1^{KO}/App^{HET}*, $p < 0.02$; *App^{HET}* versus *Fmr1^{KO}/App^{HET}*, $p < 0.04$; *App^{HET}* versus *Fmr1^{KO}*, $p = 0.02$; *Fmr1^{KO}* versus *Fmr1^{KO}/App^{HET}*, $p < 0.0002$. Error bars indicate SEM. doi:10.1371/journal.pone.0026549.g004

clear cells (PBMC) samples from FXS and control donors (Figure 6A and data not shown).

We next assessed $A\beta_{PP}/A\beta_{PP\alpha}$, $A\beta_{1-40}$ and $A\beta_{1-42}$ in hippocampal and neocortical control and FXS autopsy brain tissue. The data represents analyses of four FXS and three control brains. There is a strong trend toward increased soluble $A\beta_{1-40}$ in lysates of FXS brain samples (Figure 6D) while $A\beta_{PP}/A\beta_{PP\alpha}$ levels are reciprocally decreased (Figure 6C) in both the neocortex and hippocampus. Despite the small sample size due to the unavailability of tissue, the 1.7-fold decrease in $A\beta_{PP}/A\beta_{PP\alpha}$ in neocortex approaches statistical significance. These data suggest that the brain may act as a sink for Aβ and that lower blood plasma levels may indicate increased brain deposition. These results are similar to those in *Fmr1^{KO}* mice, which exhibited elevated Aβ in the brain [11]. Murine Aβ levels in blood plasma were below the ELISA detection limit and could not be determined.

These data strongly support the hypothesis that modest over-expression of AβPP and/or Aβ, in the context of the *Fmr1^{KO}*, is necessary for many of the pathological phenotypes including AGS, anxiety, dendritic dysmorphogenesis and mGluR-LTD observed in the mice and that these effects are likely mediated by mGluR₅ signaling. Furthermore, these data suggest a positive feedback loop whereby extracellular Aβ stimulates dendritic translation of AβPP

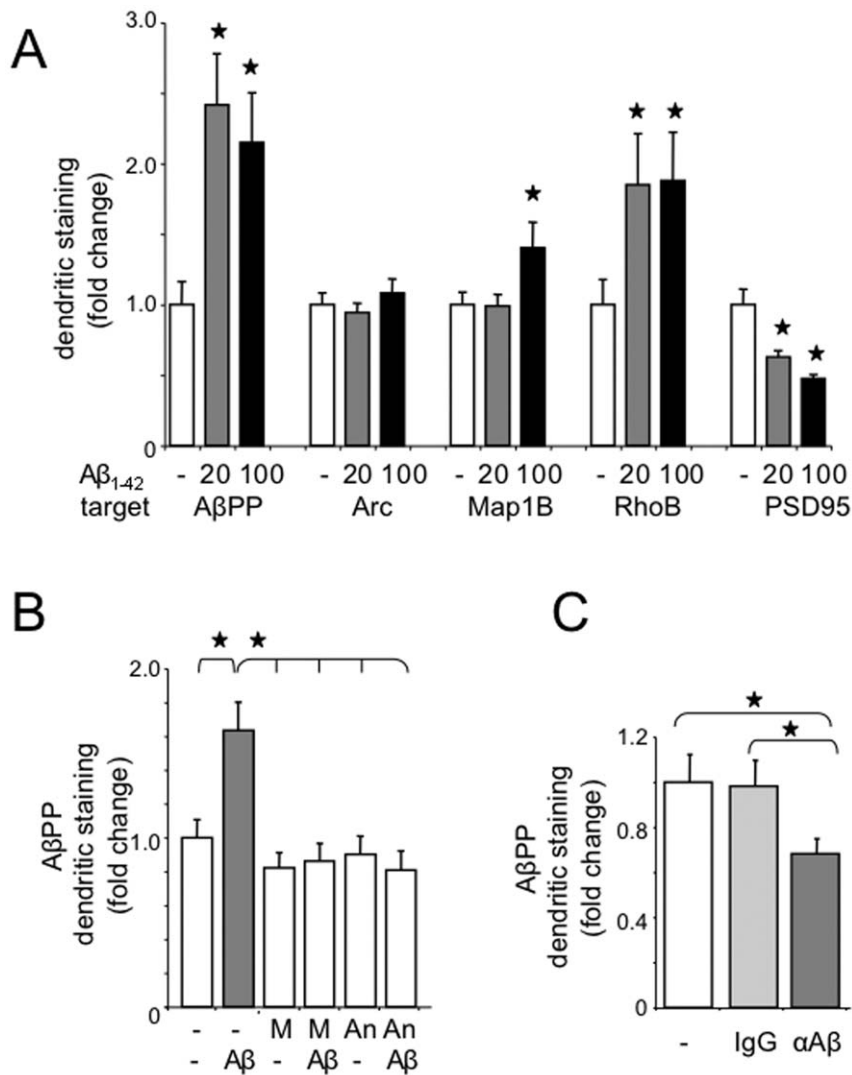


Figure 5. A β_{1-42} alters dendritic protein levels. (A) WT neuronal cells treated with vehicle, 20 or 100 nM A β_{1-42} for 1 hr followed by fixation and staining for A β PP, Arc/Arg, Map1B, RhoB and PSD95 and analyses by confocal fluorescent microscopy. (B) WT neuronal cells pre-treated with vehicle, 2.5 μ M MPEP or 40 μ M anisomycin for 15 min prior to treatment with 20 nM A β_{1-42} for 1 hr and fixation and staining for A β PP. Statistics: one-way ANOVA $p < 0.0001$, $F = 7.04$. All treatments are statistically different from the 20 nM A β_{1-42} treatment by the Bonferroni multiple comparison test ($p < 0.05$). A minimum of 1793 particles were analyzed per treatment cohort. (C) WT neuronal cells treated with mouse IgG or anti-A β antibody in transwells for 3 days prior to fixation and staining for A β PP. Statistics: one-way ANOVA $p < 0.05$, $F = 3.03$. A minimum of 2,644 particles were analyzed per treatment cohort. Stars (\star) denote statistical differences by Student T-test analyses ($p < 0.5$). Error bars indicate SEM. doi:10.1371/journal.pone.0026549.g005

through a mGluR $_5$ signaling pathway providing more target for amyloidogenic processing and the generation of additional A β .

Discussion

FXS is the most common form of inherited mental retardation and autism. It is caused by the loss of FMRP, an mRNA binding protein, which localizes to dendrites and regulates protein synthesis. Considerable effort has focused on characterizing the FMRP mRNA ligands and signaling pathways, particularly mGluR $_5$ [36], that contribute to FXS phenotypes. We have demonstrated that *App* mRNA is an FMRP mRNA ligand whose translation is regulated through mGluR $_5$ [11].

A β PP plays a critical physiological role in synapse formation and maintenance [37,38] with expression increasing during neuronal differentiation, maximal during synaptogenesis and decline when mature connections are completed [39–42].

Maximal A β PP expression coincides with the critical period of sensory development in rodents (postnatal weeks 2–6) [43]. FMRP is also developmentally regulated in the neonatal brain where it peaks at the end of the first postnatal week and declines thereafter [44]. A β PP is processed by α -, β - and/or γ -secretases to produce soluble N-terminal domains of A β PP (sA β PP α and sA β PP β), A β and C-terminal fragments. A β , which is over-expressed in AD and DS [45], is strongly implicated in impaired synaptic function and synapse loss observed early in the development of AD [46,47], and we propose that their dysregulated production in FXS contributes to disease pathology. From conception, DS individuals over-express *APP* mRNA, A β PP and extracellular amyloid [48,49]. Like FXS, DS patients show mental retardation, craniofacial abnormalities and dendritic dysmorphogenesis [1,50]. Thus, increased A β PP and/or A β PP proteolytic products could provide a common effector at the molecular level for the neuroanatomic and behavioral phenotypes observed in all of these disorders [51].

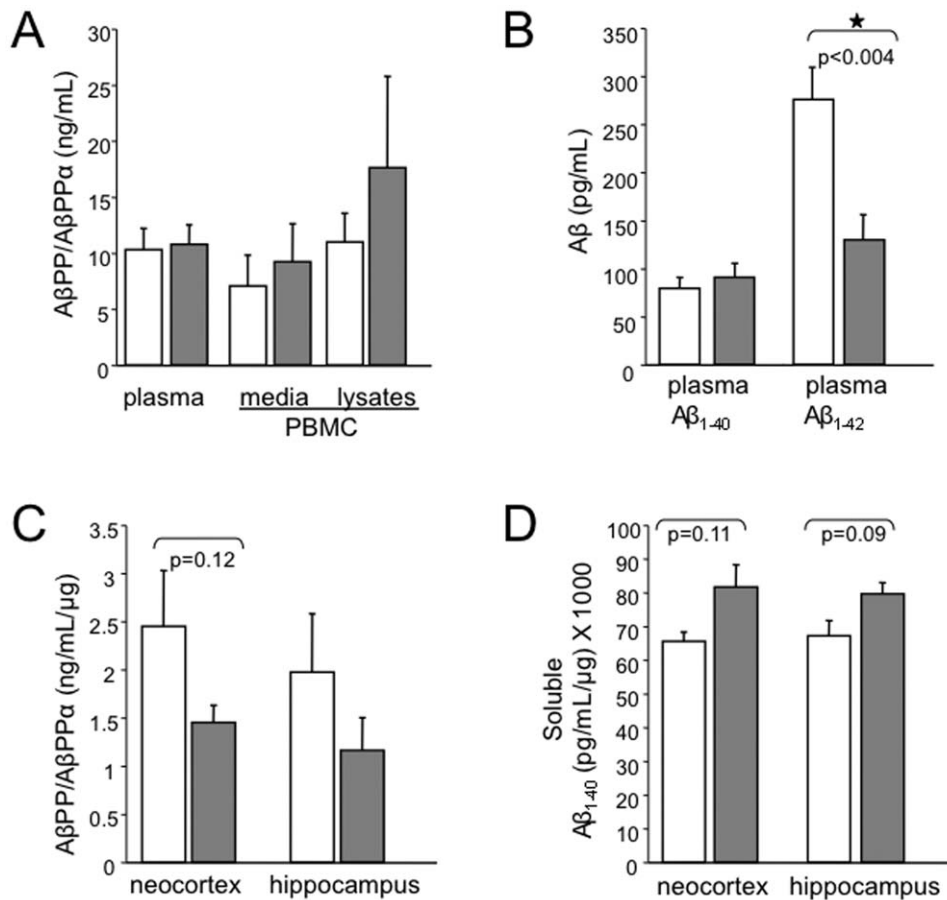


Figure 6. AβPP/Aβ are abnormal in blood and brain from FXS patients. (A) AβPPα levels in control (n = 7) and FXS (n = 10) plasma and PBMC culture media (n = 7 controls and 4 FXS) and AβPP in PBMC (n = 7 controls and 5 FXS). For the plasma and PBMC culture media data, AβPPα is expressed as ng/mL and for the PBMC lysate measurements, AβPP is expressed as ng/mL/μg lysate. (B) Aβ₁₋₄₀ and Aβ₁₋₄₂ levels in control (n = 7) and FXS (n = 10) plasma. For Aβ₁₋₄₂, p < 0.004 as determined by Student T-Test analyses. (C) AβPP/AβPPα in control (n = 3) and FXS (n = 4) neocortex and hippocampus. Controls were gender- and age-matched to FXS donors by the University of Maryland Brain Bank. All of the donors were Caucasian males and their ages ranged from 21–85 years old. (D) Soluble Aβ₁₋₄₀ levels in control (n = 3) and FXS (n = 4) neocortex and hippocampus. White bars = control samples and gray bars = FXS. Error bars represent SEM. doi:10.1371/journal.pone.0026549.g006

We found that the peripheral concentration of Aβ₁₋₄₂ and the Aβ₁₋₄₂/Aβ₁₋₄₀ ratio were significantly decreased in full-mutation FXS males compared to control donors. A reduced Aβ₁₋₄₂/Aβ₁₋₄₀ ratio is an independent risk factor for AD irrespective of the total Aβ load [52–58]. Plasma Aβ₁₋₄₂ levels are increased in patients with mild cognitive impairment, but drop to control levels by the time of AD diagnosis [59]. In DS, elevated plasma Aβ₁₋₄₂ is associated with earlier onset of AD [60] and the Aβ₁₋₄₂/Aβ₁₋₄₀ blood plasma ratio is lower than in controls [61]. Thus, our finding of a reduced Aβ₁₋₄₂/Aβ₁₋₄₀ ratio in FXS patients compared to control plasma is consistent with other amyloidogenic diseases.

The effectiveness of drug therapy in FXS is currently assessed exclusively by behavioral testing. Perhaps most importantly, the availability of a plasma biomarker for FXS may permit the monitoring of drug therapy as well as predict disease progression. Other studies have demonstrated reduced cAMP production in platelets [62] and delayed early-phase phosphorylation of ERK in lymphocytes [63] from FXS patients. Our data demonstrates that Aβ₁₋₄₂ is significantly lower in FXS plasma than control subjects, and in conjunction with two studies demonstrating elevated sAβPPα in autistic children [16,17], suggests that both AβPP and Aβ are viable biomarkers for FXS. We did not observe increased sAβPPα in adult FXS plasma suggesting that AβPP expression and

processing decrease with age consistent with prior studies [16]. Previously reported control plasma levels of Aβ range from 130–208 pg/mL for Aβ₁₋₄₀ and 15 pg/mL–85.7 ng/mL for Aβ₁₋₄₂ [53,58,64,65]. Thus, there is wide range of reported plasma Aβ₁₋₄₀ and Aβ₁₋₄₂ levels in the literature and our studies fall within that range. A critical question remains regarding if these catabolites are associated with disease severity or progression. There is a paucity of human FXS brain tissue available for analyses. Our preliminary studies indicate a trend for elevated Aβ in FXS brain, which agrees with data in *Fmr1^{KO}* mice [11].

FXS patients exhibit hyper-reactivity to visual, olfactory, tactile and auditory stimulation [1,66–69]. This hypersensitivity phenotype is manifested as AGS in *Fmr1^{KO}* mice [20]. If Aβ contributes to AGS, then AD and DS mice would also be susceptible to seizures. Consistent with this, Tg2576, FRAXAD and DS mice, which all over-express hAβPP with the Swedish familial mutation and/or mouse AβPP, exhibit AGS [70]. *Fmr1^{KO}/Aβ^{HET}* male mice exhibited a statistically significant decrease in AGS but not in WR whereas MPEP significantly reduced WR in *Fmr1^{KO}* mice. These data suggest that the reduction of AβPP/Aβ in *Fmr1^{KO}* mice is not particularly effective at reducing the induction of AGS, but does retard progression to clonic-tonic seizures. *Fmr1^{KO}* mice also exhibit enhanced mGluR-LTD [34], which is lost in *Fmr1^{KO}/*

App^{HET} mice. The *Fmr1*^{KO} reduces mGluR-LTD in an *App*^{HET} background, but enhances mGluR-LTD in an *App*^{WT} background. The large difference in the maximal depression of synaptic transmission between *Fmr1*^{KO}/*App*^{HET} and *Fmr1*^{KO} in opposite directions from WT mice, which exhibit equivalent mGluR-LTD as *App*^{HET}, suggests that FMRP and A β PP/A β play important and synergistic roles in modulating mGluR-LTD. Thus, the over-expression of A β PP or an A β PP catabolite lowers seizure threshold and enhances mGluR-LTD, and approaches to attain normal synaptic levels of these proteins could prove therapeutic.

MPEP is a potent and highly selective noncompetitive antagonist of mGluR₅ [71,72] that reduces AGS, anxiety phenotypes and dendritic spine protrusion morphology in *Fmr1*^{KO} [22,26]. We have previously demonstrated that mGluR₅ blockade inhibits translation of A β PP in synaptoneurosomes [11] and herein demonstrate reversion of several FXS phenotypes by genetic manipulation to reduce A β PP/A β . Minocycline, a second-generation tetracycline compound, reverts several FXS phenotypes [24], possibly by altered A β fibril formation [73] or A β -induced neuronal death and glial activation [74]. In aggregate, these data suggest that mGluR₅ antagonists and minocycline therapies converge on a similar signaling pathway resulting in decreased A β levels/activity as obligatory for the rescue of FXS phenotypes.

How A β mediates synaptic dysfunction remains unclear. A β promotes AD-like cytoskeletal abnormalities and can promote intracellular accumulation of sA β PP in primary cultured neurons [75]. Soluble oligomers of A β increase LTD in WT hippocampal slices and inhibit long-term potentiation [76–80]. A similar enhancement of mGluR₅-mediated LTD occurs in the hippocampus of *Fmr1*^{KO} mice [34], and MPEP prevents the block in long-term potentiation [81]. A β causes membrane depolarization and calcium influx, activates mGluR₁ [82] and functions as an extracellular scaffold for mGluR₅ [83]. *In vitro*, A β _{1–42} altered the expression of important dendritic proteins regulated by FMRP including A β PP, Map1B and PSD95 with known roles in synaptogenesis and/or dendritic spine morphology [37,38,44, 84]. A previous report utilizing neuronal hybrid cells demonstrated that A β _{1–40} increases A β PP levels also supporting the existence of an A β -driven positive feedback loop [85]. Lower levels of PSD95 are observed in Tg2576 neurons, which constitutively overexpress A β and ADDLs [84]. In our hands, levels of the immediate-early gene Arc were not changed during a 1 hr stimulation with low molecular weight oligomers of A β _{1–42}; however, others have observed that higher molecular weight A β oligomers (10–100 kDa) bind in a punctate pattern to the surface of neurons, colocalize with PSD95 and upregulate Arc [47]. We can block the A β -induced increase in A β PP levels with either MPEP or anisomycin suggesting that an mGluR₅- and translation-dependent pathway is involved. The varied expression of known FMRP targets in response to A β suggests that dendritic translation can be modulated through FMRP-dependent and independent pathways. A β _{1–42} rapidly increases phosphorylated ERK levels suggesting that ERK mediates downstream signaling.

In conclusion, our work demonstrates that A β PP translation is regulated through an mGluR₅/FMRP-mediated pathway. Excessive signaling through mGluR₅ in the absence of FMRP leads to increased A β PP production and processing and we have observed elevated A β PP and A β levels in *Fmr1*^{KO} mice [11]. Genetic reduction of A β PP levels in *Fmr1*^{KO} mice has reverted or partially rescued FXS seizure, behavioral, dendritic spine and mGluR-LTD phenotypes. Furthermore, treatment of primary neurons with A β _{1–42} increased while anti-A β reduced dendritic A β PP expression suggesting that an A β -driven positive feedback loop

drives synthesis/processing of A β PP through a mGluR₅ signaling pathway. Our results have potential implications for the treatment of FXS as plasma A β PP/A β can be readily screened as biomarkers to evaluate potential therapies including mGluR₅ antagonists as well as secretase inhibitors and anti-A β , which are currently undergoing testing for the treatment AD.

Materials and Methods

Ethics Statement

Adequate measures were taken to minimize pain or discomfort to the mice, and all husbandry, seizure and euthanasia procedures were performed in accordance with NIH and an approved University of Wisconsin-Madison animal care protocol administered through their Research Animal Resources Center (approval #G00468). Males with FXS and age-matched controls were recruited from the FXS Clinic at Rush University Medical Center (RUMC) in Chicago, IL. The study was approved by the RUMC Institutional Review Board and all donors or their legal guardians signed the appropriate consent forms for study participation.

Mouse Husbandry

WT and *Fmr1*^{KO} mice (C57BL/6 background) were bred and housed as previously described [11]. *Fmr1*^{KO} females [86] were crossed with *App*^{KO} males [87] (Jackson Laboratories #004133, C57BL/6 background) to generate *Fmr1*^{HET}/*App*^{HET} females and *Fmr1*^{KO}/*App*^{HET} males that were crossed to generate *Fmr1*^{KO}/*App*^{KO} mice. For the behavioral testing, littermate controls were generated by crossing *Fmr1*^{KO}/*App*^{HET} females with *Fmr1*^{KO}/*App*^{HET} males to generate *Fmr1*^{KO}/*App*^{WT}, *Fmr1*^{KO}/*App*^{HET} and *Fmr1*^{KO}/*App*^{KO} progeny and by crossing *Fmr1*^{WT}/*App*^{HET} females with *Fmr1*^{WT}/*App*^{HET} males to generate *Fmr1*^{WT}/*App*^{WT}, *Fmr1*^{WT}/*App*^{HET} and *Fmr1*^{WT}/*App*^{KO} progeny. Genotypes were determined by PCR analysis of DNA extracted from tail biopsies. The WT, HET or KO state of the *App* gene was determined by genotyping with primer 1: 5'-CTG CTG CAG GTG GCT CTG CA-3', primer 2: 5'-CAG CTC TAT ACA AGC AAA CAA G-3', and primer 3: 5'-CCA TTG CTC AGC GGT GCT GTC CAT-3' to generate a 250 base pair WT allele with primers 1 and 2 and a 470 base pair targeted KO allele with primers 2 and 3.

Assessment of A β PP by Western Blot Analyses

Left hemispheres from WT, *Fmr1*^{KO}, *App*^{HET} and *Fmr1*^{KO}/*App*^{HET} mice (1 month old males; n=3 per genotype) were homogenized in protein extraction buffer [10 mM Tris (pH 7.6), 2 mM EDTA, 150 mM NaCl, 1% Triton X-100, 0.25% NP-40, 1 \times protease inhibitor cocktail (Research Products International Corp., Mount Prospect, IL, USA, catalog #P50600)], mixed for 30 min at 4°C and spun at 12,000 rpm for 10 min at 4°C. The protein concentrations of the supernatants were determined by BCA assay (Thermo Fisher/Pierce, Rockford, IL, USA). Lysates (18.75 μ g per lane) were separated by 12% SDS-PAGE, transferred to nitrocellulose and western blotted as previously described [11] with anti-A β PP antibody (Life Technologies Corporation, Carlsbad, CA, USA, catalog #51-2700; diluted 1:250) and anti-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA, catalog #sc-8035; diluted 1:250). A β PP signals were normalized to tubulin and plotted as a percentage compared to WT levels. Error bars represent the SEM of three mice.

Audiogenic Seizures

All mouse strains were tested at postnatal day 21, the peak of AGS sensitivity in C57BL/6. The experimental apparatus consisted of a clear, Plexiglas box (13"L \times 8"W \times 7"H) with the

sound source located inside the box (LOUD KEY™ jogger's alarm). Mice were weighed and then placed individually into the center of the chamber and exposed to a siren that generated noise at 118 dB for 5 min. Loud, acoustic stimulation causes WR within 20–30 sec followed by erratic leaping, clonic convulsions and tonic hind limb extension by 40–50 sec followed by respiratory arrest and death [32]. The percentage of mice exhibiting WR, AGS and death were scored versus gender and genotype and assessed for statistical significance by the Fisher exact test. The *Fmr1^{KO}/App^{HET}* mice used in the AGS studies were offspring generated by crossing *Fmr1^{KO}* females with *Fmr1^{KO}/App^{KO}* males to avoid effects due to maternal genotype in comparing *Fmr1^{KO}* and *Fmr1^{KO}/App^{HET}*. MPEP was a kind gift from FRAXA Research Foundation (Newburyport, MA) and was dissolved at 1 mg/mL in DPBS before I.P. injection at 30 mg/kg body weight 30 min prior to AGS testing at age P21.

Marble Burying

Mice were acclimated to the behavioral testing room for at least 15 min prior to transfer to a clean cage containing corn cob bedding and 20 black marbles arranged in a rectangular 4×5 grid over 2/3 of the cage. The mouse was placed into the cage at the end that did not contain marbles and allowed to explore the new cage with the marbles for 30 min after which the mouse was returned to its homecage and the number of visible marbles (more than half not buried) counted. Background white noise in the room was set to 70 dB. There is variability in this assay with some laboratories observing that *Fmr1^{KO}* mice bury more marbles than WT (R. Paylor, personal communication); however, differences in behavioral outcomes can vary between facilities due to genetic background or altered environmental factors such as housing conditions and diet.

Open Field

Mice were acclimated to the behavioral testing room for at least 30 min before placement into the center of a clear, Plexiglas chamber measuring 14.5″L×14.5″W×10″H. Mouse movement was monitored in the chamber for 15 minutes with LimeLight2 software interfaced with an overhead camera. The open field arena was arranged into a 16 square grid in the camera window with 4 squares in the center and 12 squares around the perimeter. Time spent in the center 4 squares (measure of anxiety) and total distance traveled (measure of locomotion) were compared between genotypes.

DiI Labeling and Analyses of Dendritic Spines

Primary mouse neurons were prepared from embryonic (age E15–17) dissected brains from timed pregnant WT and *Fmr1^{KO}* female mice as previously described [11]. To generate *Fmr1^{KO}/App^{HET}* cells, *Fmr1^{KO}* females were mated with *Fmr1^{KO}/App^{KO}* males. Cells were cultured for 15 days on poly(D)-lysine coated glass coverslips inside of 12-well tissue culture dishes, fixed with 4% paraformaldehyde and stained with lipophilic DiI dye (Life Technologies Corporation, Carlsbad, CA, USA). For the staining, the wells were aspirated and sprinkled with DiI crystals and a small amount of DBPS was added to the edge of the wells to prevent dehydration of the cells. Cells were stained for 10 min, copiously washed with DPBS to remove all crystals and fixed to slides with ProLong Gold Antifade (Life Technologies Corporation, Carlsbad, CA, USA). Slides were allowed to dry for at least 3 days to allow complete migration of the DiI into dendritic spines. Dendritic spines were imaged on a Zeiss Axioplan 2 Imaging Photomicroscope equipped with a MBF Biosciences automated XYZ stage and MicroFire A/R camera. Images were taken using

the 100× objective (Zeiss FLUAR 100×/1.30 oil) and Zeiss Immersol™ 518F oil at ambient temperature. Spine length was quantitated with StereoInvestigator v9 software. Contours were drawn around the protrusions and the feret max (length) and feret min (widest width) of the contours were calculated. A minimum of 2–6 coverslips were analyzed per neuronal cell prep and images of neurons were taken from multiple areas of those coverslips. Data is representative of multiple batches of neuronal cells. A minimum of 746 spines were quantitated per genotype. The feret width was divided by feret max and protrusions having a ratio less than 0.5 were classified as filopodia and those with a ratio greater than or equal to 0.5 were classified as spines. For MPEP treatments, 2.5 μ M MPEP was added to the neurons for the indicated times followed by washing the cells with DPBS and fixation and staining as previously described. A minimum of 263 protrusions were quantitated per MPEP treatment.

Hippocampal Slice Preparation and mGluR-LTD Field Recordings

WT, *Fmr1^{KO}*, *App^{HET}* and *Fmr1^{KO}/App^{HET}* mice were rapidly decapitated by cervical dislocation (3-month old males). Hippocampal slices were prepared and electrophysiology performed as previously described [88]. After a 50 min stable baseline, slices were bathed in 50 μ M S-DHPG for 10 min to induce mGluR-LTD. A 2 min trace of an average of four 30 sec traces was recorded over 100 min post-drug application and the slope of the fEPSP was measured and graphed as a function of time.

Treatment, Staining and Immunofluorescence Analyses of Primary Neurons

For inhibitor treatments, cells were pretreated with vehicle, 2.5 μ M MPEP or 40 μ M anisomycin for 15 min prior to culture for 1 hr with vehicle or 20 nM A β _{1–42}. A β _{1–42} (CalBiochem, catalog #171596) was prepared as previously described to generate oligomers, but not high molecular weight or fibrillar aggregates [89,90]. For antibody treatments, 10 μ g mouse IgG (Sigma #I5381) or anti-A β (Santa Cruz, catalog #28365LS) were added to 0.5 mL culture media inside transwells (Corning, catalog #3460, 0.4 μ m pore size) that were situated above neuron-coated glass coverslips in 12-well tissue culture dishes. Each well contained 1 mL culture media and the transwell contained an additional 0.5 mL of culture media. Neurons were cultured for 3 days with the indicated antibodies prior to fixation and staining. Cells were stained overnight with: anti-22C11 against the amino-terminus of A β PP (Chemicon, catalog #MAB348, 1:2000), anti-phosphoERK (Santa Cruz, catalog #sc-23759, 1:100), anti-Arc/Arg (Santa Cruz, catalog #sc-17839, 1:100), anti-Map1B (Santa Cruz, catalog #58784, 1:100), anti-RhoB (Santa Cruz, catalog #sc-180, 1:100) and anti-PSD95 (Santa Cruz, catalog #sc-71935, 1:100) followed by visualization with appropriate goat anti-mouse or anti-rabbit rhodamine-conjugated secondary antibodies (Invitrogen, 1:500 for 30 min in the dark). Images were acquired with a Nikon C1 Laser Scanning Microscope (Nikon Eclipse E600 upright microscope) using the 543 Diode (1 mw Mellet Griot) laser, the Nikon Plan Apo 60×/1.40 oil objective with Zeiss Immersol™ 518F oil at ambient temperature, and Nikon EZ-C1, v3.91 software (Nikon Corp, Tokyo, Japan). Stained protein levels in the puncta of 4–7 dendrites per sample were quantitated with IMAGE J software using the Analyze Particles function (Rasband, W.S., Image J, U.S. National Institutes of Health, Bethesda, Maryland, USA, <http://rsb.info.nih.gov/ij/>, 1997–2006). Statistical significance was determined by one-way ANOVA and Student T-test analyses.

Blood Collection

Males with FXS and age-matched controls were recruited from the FXS Clinic at Rush University Medical Center (RUMC) in Chicago, IL. All FXS subjects (ages 9–32 years old) were positive by DNA analyses for a fully methylated expansion mutation in the *FMR1* gene. Controls (age 23–33) were normal volunteers working at RUMC and had no history of cognitive or mental health disorders. The age and medications of the donors are listed in Table S1. Blood was drawn from donors into lithium heparin-coated blood collection tubes and spun at 1,500 rpm. The plasma supernatant was removed and frozen at -80°C . The anticoagulated blood was mailed by overnight delivery from RUMC to the University of Wisconsin-Madison where PBMC were isolated within 24 hr.

Assessment of A β PP/A β PP α , A β _{1–40} and A β _{1–42} by ELISA

Plasma was thawed and clarified at 12,000 rpm for 10 min at 4°C prior to ELISA assays for A β PP/A β PP α , A β _{1–40} and A β _{1–42} per the manufacturer's instructions (BioSource #KHB0051, KHB3482, KHB3442) with the following modifications for the A β assays: (1) the sample volume was doubled from 50 μL to 100 μL , (2) the incubation time was extended from 3 hr to overnight at 4°C , and (3) after the overnight incubation, the samples were removed from the antibody-coated wells prior to addition of the detection antibody. PBMC were isolated as previously described [91] and cultured for 24 hr prior to harvesting the cells and culture media for ELISA analyses. Hippocampus and neocortex (pre and post central gyri) samples were sectioned from left cerebral hemispheres of control and FXS brain autopsy tissue. Detergent-soluble lysates were prepared as previously described [11] for analyses of A β PP and A β _{1–40}.

Statistical Analyses

One-way ANOVA was performed using GraphPad Prism version 5.0 d for Mac OS X (GraphPad Software, San Diego, CA) to compare the means of three or more unmatched groups for the behavioral and dendritic spine analyses. Student T-test analyses were used to quantitate statistical significance for the western blotting, marble burying, open field, dendritic spine protrusion length, immunofluorescence and ELISA data. Fisher exact tests were used to quantitate statistical significance for the AGS data. Two-way ANOVA with Bonferroni posthoc tests was used to quantitate statistical significance for the mGluR-LTD analyses.

Supporting Information

Figure S1 Assessment of hippocampal slice health. Hippocampal slices from WT (black circles), *Fmr1*^{KO} (white

circles), *App*^{HET} (black triangles) and *Fmr1*^{KO}/*App*^{HET} (white triangles) mice exhibit equivalent synaptic transmission as determined by the input/output relationship (A) and ppf (B). i/o was also measured at the end of the recordings to assess slice health and showed similar i/o relationships to those shown in (A) (data not shown). (B) fEPSP size as percent of first stimulus versus interstimulus interval (msec). Data were analyzed by two-way ANOVA/Bonferroni multiple comparison analyses. There were no statistically significant differences in the i/o relationships. The WT ppfs were statistically different ($p < 0.05$) from *App*^{HET} (many time points), WT versus *Fmr1*^{KO} (50 msec time point); WT versus *Fmr1*^{KO}/*App*^{HET} (first four time points). There were no other statistically significant differences in ppfs for the remaining datasets.

(TIFF)

Figure S2 MPEP and A β _{1–42} alter dendritic phosphoERK levels. (A) WT and *Fmr1*^{KO} neurons were treated with 10 μM MPEP [one-way ANOVA $p < 0.05$, $F = 2.1$], and (B) WT neurons were treated with 20 nM A β _{1–42} for the indicated times prior to fixation and staining with anti-phosphoERK [one-way ANOVA $p < 0.0001$, $F = 13$]. Stars (★) denote statistically different results by Student T-test analyses ($p < 0.05$).

(TIFF)

Table S1 Age and medications of donors.

(XLS)

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Author Contributions

Conceived and designed the experiments: CJW MT EMB-K JSM. Performed the experiments: CJW PRW KJO BCR CMH MSS SHA KMS LAS. Analyzed the data: CJW KJO CB EMB-K JSM. Contributed reagents/materials/analysis tools: CJW PRW MT JSM. Wrote the paper: CJW EMB-K JSM.

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