Reversal of activity-mediated spine dynamics and learning impairment in a mouse model of Fragile X syndrome

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Abstract

Fragile X syndrome (FXS) is characterized by intellectual disability and autistic traits, and results from the silencing of the FMR1 gene coding for a protein implicated in the regulation of protein synthesis at synapses. The lack of functional Fragile X mental retardation protein has been proposed to result in an excessive signaling of synaptic metabotropic glutamate receptors, leading to alterations of synapse maturation and plasticity. It remains, however, unclear how mechanisms of activity-dependent spine dynamics are affected in Fmr knockout (Fmr1-KO) mice and whether they can be reversed. Here we used a repetitive imaging approach in hippocampal slice cultures to investigate properties of structural plasticity and their modulation by signaling pathways. We found that basal spine turnover was significantly reduced in Fmr1-KO mice, but markedly enhanced by activity. Additionally, activity-mediated spine stabilization was lost in Fmr1-KO mice. Application of the metabotropic glutamate receptor antagonist α-Methyl-4-carboxyphenylglycine (MCPG) enhanced basal turnover, improved spine stability, but failed to reinstate [...]
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1Department of Basic Neurosciences, School of Medicine, University of Geneva, Geneva 4 1211, Switzerland
2Center for Psychiatric Neurosciences, Cery, Prilly-Lausanne, Switzerland

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Introduction

The FMR1 gene codes for an RNA-binding protein implicated in the shuttling of mRNAs to dendritic sites where it contributes to the local control of protein synthesis. Studies of the Fmr1 knockout (Fmr1-KO) mouse model of Fragile X syndrome (FXS) strongly suggest that synaptic defects are an important component of the pathological mechanisms responsible for the disease. Alterations of both the structure and function of excitatory synapses have been reported (Comery et al., 1997; Irwin et al., 2000; Huber et al., 2002; Lauterborn et al., 2007; Hu et al., 2008; Auerbach & Bear, 2010). These defects have been proposed to result from an excessive signaling mediated by metabotropic glutamate receptors (Pfeiffer & Huber, 2007) that could affect spine synapse formation and maturation. In vivo experiments have indeed reported alterations of spine dynamics in the cortex and the presence of unstable spines insensitive to modulation by sensory experience (Cruz-Martin et al., 2010; Pan et al., 2010).

Considering that alterations of spine dynamics could represent key mechanisms susceptible to interfere with the development of synaptic networks (Caroni et al., 2012) and thus play a significant role in the pathology of FXS, we investigated whether and how these defects could be regulated in Fmr1-KO mice. Several different approaches have been proposed or even tested over the last years to counteract the deficient phenotypes observed in Fmr1-KO mice. A main strategy has been to interfere with metabotropic glutamate receptor signaling, which has been shown to correct many behav-
Materials and methods

Organotypic cultures and transfection

Hippocampal slice cultures (400 μm) were prepared from Fmr1-KO mice (Mientjes et al., 2006) and wild-type (WT) littermates at postnatal day 5, and cultured for 3 weeks (Stopponi et al., 1991). The experimental protocols were reviewed and approved by the Ethics Committee of the University Medical Center of Geneva, by the Cantonal Veterinary Office, Geneva, Switzerland, and were carried out in conformity with the guidelines laid down by the NIH in the USA regarding the care and use of animals for experimental procedures. Transfection was performed with the gene gun method (Helios Gene Gun; Bio-Rad) at 8–10 days in vitro using different plasmid constructs (pCDNA3.1-EGFP and pCX-mRFP1; ratio of plasmid: 10 μg for 5 mg of 1.6 μm gold microcarriers). The shRNA construct was kindly provided by I. Caillé (Université Pierre et Marie Curie, Paris, France), and its efficacy demonstrated both in in vitro and in vivo experiments (Scotto-Lomassese et al., 2011). A negative control small interference sequence was obtained from Qiagen Allstars. (S)-MCPG (Tocris Bioscience) was used at 100 μM. BpV (Enzo Life Sciences and Calbiochem/Millipore) was used at 15 nM in culture medium, and 30 μg/100 g for intraperitoneal (i.p.) injections.

Confocal imaging and electrophysiology

Four days after transfection, slice cultures were imaged repetitively using an Olympus Fluoview confocal microscope as described (Dubos et al., 2012). Secondary or tertiary dendritic segments (35–45 μm) on apical dendrites of CA1 pyramidal neurons in the stratum radiatum were visualized at 0, 5, 24, 48 and 72 h. Z-stack images were analysed using Osirix software and cross-checked with another experimenter. All protrusions were included in the analysis, and stability was assessed as the fraction of spines still present at later times. Spines were considered to enlarge if the spine head width increased by more than 0.1 μm between the 0 and 5 h time points. All data are given with n being the number of dendritic segments analysed considering one dendritic segment per neuron and one neuron per slice culture. When appropriate, we also give the total number of spines analysed in the pool of experiments.

For electrophysiological recordings, hippocampal cultures or acute slices (4–6-week-old mice) were maintained in an interface chamber as described (De Roo et al., 2008b), perfused in a medium with the following composition (in mM): NaCl, 124; KCl, 1.6; MgCl₂, 1.5; CaCl₂, 2.5; KH₂PO₄, 1.2; NaHCO₃, 24; glucose, 10; ascorbic acid, 2; bubbled with 95% O₂ and 5% CO₂. Theta-burst stimulation (TBS) consisted of five trains at 5 Hz, each composed of four pulses at 100 Hz, repeated three times at 10-s intervals. Field excitatory postsynaptic potentials and amplitudes were measured using IGOR software.

Western blot

The effect of BpV on AKT phosphorylation on the S473 site was tested in hippocampal slice cultures maintained 12–14 days in vitro and treated with 15 nM BpV, as well as on 4–6-week-old mice injected i.p. with BpV [30 μg/100 g in saline (200 μL)]. The tissue was lysed on ice 30 min or 1–4 h after treatments for slices and animals, respectively, using a solution containing (in mM): NaCl, 150; Tris-HCl, 50, pH 7.4; EDTA, 2; dithiothreitol, 1; 1% Triton X-100, 10% glycerol, complete protease inhibitor tablet (Roche), completed with phoshpatase inhibitors (in mM): sodium orthovanadate, 1; para-nitrophenylphosphate, 20; β-glycerophosphate, 20; okadaic acid, 50 ng/mL. Lysates were sonicated, and 30 μg of total protein put on Nupage 4–12% Bis-Tris gel (Invitrogen). Gels were run in 3-(N-morpholino)propanesulfonic acid sodium dodecyl sulfate buffer (Invitrogen) and transferred to nitrocellulose membranes. Phospho-AKT immunoblotting was performed using 1: 3000 dilution of the primary rabbit monoclonal antibody against phosphoSer473 (Cell Signaling Technology). Densitometric analysis of the pAKT band of the AKT blots was measured in two independent experiments.

Animals and Morris water maze

Twenty male adult Fmr1-KO mice (FVB.129P2-Pde6b<sup>Tyr<sup>c-ch</sup>Fmr1<sup>10IC177FJ⟩</sup> and 20 male adult WT controls (FVB.129P2-Pde6b<sup>Tyr<sup>c-ch</sup>/AntJ) were purchased from Jackson Laboratories. Mice were individually identified and housed according to the Swiss legislation on animal protection. One Fmr1-KO and one WT mice were used for pilot tests; the final sample (19 Fmr1-KO and 19 WT) was divided into two groups: nine mice were treated with BpV and 10 received the vehicle. The mice were trained in a Morris water maze with four daily trials during four consecutive days. At day 5, a probe trial was run in the absence of the escape platform. Immediately after, mice received either an i.p. injection of BpV [30 μg/100 g in saline (200 μL)] or an i.p. injection of 200 μL saline. One hour later, all mice started a novel, four-trial training session with the platform in the opposite quadrant (reversal learning). Reversal training continued for two more days; treatment with BpV or saline was repeated accordingly. At day 8, a second probe test was run. Swim paths were videorecorded and analysed by a videotracking system (EthoVision, Noldus, the Netherlands). Variables assessed were escape latencies, times spent in target quadrants and platform place proximity indices (PIs), calculated by measuring the average distances to the platform [(PI probe1 – PI probe2)/(PI probe1 + PI probe2)×100].

Statistics

Data are represented as mean ± SEM. Statistical analyses were carried out using Student’s t-tests unless otherwise mentioned.

Results

Decreased spine turnover in Fmr1-deficient hippocampal neurons

Spine dynamics was analysed using a repetitive imaging approach applied to transfected CA1 hippocampal neurons in slice cultures prepared from Fmr1-KO mice or WT littermates. Under basal conditions in WT slice cultures, a substantial fraction of spines are generated and eliminated every day (De Roo et al., 2008a). In Fmr1-KO mice slices, this basic spine turnover was significantly reduced, and affected both newly formed and lost spines (Fig. 1A–E; new spines: 9.8 ± 1.2%, n = 10, 452 spines, Fmr1-KO vs. 16.5 ± 0.8%, n = 11, 348 spines, WT, P < 0.01; lost spines: 9.5 ± 1.5%, Fmr1-KO vs. 16.2 ± 2%, WT, P < 0.05). To further confirm this result, we then also transfected CA1 neurons with EGFP and either a small hairpin construct directed against Fmr1 (shFmr1) or with a control, non-silencing sequence (shCtrl). The efficacy of the shFmr1 construct has been demonstrated to reduce Fragile X mental retardation protein expression levels both under in vitro and in vivo conditions (Scotto-Lomassese et al., 2011). Acute transfection of slice cultures with this shFmr1 construct reproduced the changes observed in Fmr1-KO mice, and reduced both the fraction...
in a marked increase in spine formation and elimination over the next 24 h (Fig. 2A, C and D; new spines: 42.4 ± 7.9%, n = 5, 240 spines analysed, P < 0.01; lost spines: 43.7 ± 8.8%, n = 5, P < 0.01), consistent with previous observations (De Roo et al., 2008b). Additionally, analyses of spine stability and comparisons between spines that did and did not enlarge 5 h after TBS (Fig. 2E) showed that enlarged spines were also more likely to persist over the next 48 h than spines that did not enlarge (Fig. 2F; 87.5 ± 12.5%, n = 4 vs. 55.5 ± 5.0%, n = 4, P < 0.01). This is consistent with the notion that application of TBS and induction of plasticity can lead to a selective stabilization of stimulated synapses (De Roo et al., 2008b). In Fmr1-KO mice, spine turnover was reduced under basal conditions (Fig. 2B), but showed a fourfold increase following TBS, reaching similar levels as those observed in WT mice (Fig. 4C and D: new spines: 42 ± 6.4%, n = 5, 199 spines, P < 0.001; lost spines: 37.8 ± 5.5%, n = 5, P < 0.001). Thus, the sensitivity of spine turnover to activity was strongly enhanced in Fmr1-KO slices. Furthermore, the selective stabilization of the spines that enlarged following TBS was lost in Fmr1-KO slices: both spines that did and did not enlarge showed the same low 48 h stability (Fig. 2F; 56.5 ± 15.3%, n = 4 vs. 53.0 ± 6.1%, n = 4). The proportion of spine that enlarged was, however, not sig-

**Defects in activity-dependent spine dynamics in hippocampal neurons of Fmr1-KO mice**

We then assessed the effects of activity on spine turnover. Application of TBS, a protocol that induces LTP in slices cultures, resulted of newly formed spines per 24 h (10.4 ± 1.9%, n = 9, 204 spines, shFmr1 vs. 17.6 ± 1.7%, n = 7, 219 spines, shCtrl, P < 0.05) and the fraction of eliminated spines (13.0 ± 1.5%, shFmr1 vs. 21.0 ± 4.3%, shCtrl, P = 0.07). No differences in spine density could be detected between these conditions (Fig. 1F). Finally, to further characterize these deficits, we also carried out a morphological analysis of spine size and spine length in Fmr1-KO and shFmr1-transfected cells. The results illustrated in Fig. 1G revealed a significant increase in mean spine length in both Fmr1-KO and shFmr1-transfected neurons, associated with a significant decrease in mean spine width (Fig. 1G; n = 452 and 348 spines analysed; **P < 0.01, ***P < 0.001). Note that the mean spine length in CA1 pyramidal neurons transfected with the scrambled, control shRNA was also significantly shorter than in non-transfected cells (P < 0.001). We do not know the reasons for these differences, but previous work has shown that RNA interference approaches could have off-target non-specific effects on dendritic spines (Alvarez et al., 2006). These results suggest the existence of an increased number of immature spines in Fmr1-KO that is consistent with previous observations (Comery et al., 1997; Grossman et al., 2006).

**Fig. 1.** Alteration of spine dynamics in the Fmr1-KO mouse model of Fragile X syndrome (FXS). (A–C) Illustrations of 24-h spine turnover in transfected pyramidal neurons of (A) WT and (B) Fmr1-KO slice cultures, and of a WT pyramidal neuron transfected with shFmr1 (C; bar: 2 μm). (D) Decrease in basal spine growth mechanisms detected over 24 h in Fmr1-KO and shFmr1-transfected neurons (n = 7–11). (E) Same, but for disappearing spines. (F) Absence of changes in spine density observed under the same conditions. (G) Increase in spine length and decrease in spine head width observed in Fmr1-KO and shFmr1-transfected cells (n = 452, 348; *P < 0.05, **P < 0.01, ***P < 0.001).

**Fig. 2.** Alteration of activity-dependent spine dynamics in Fmr1-KO mice. (A and B) Illustration of the changes in spine dynamics induced by theta-burst stimulation (TBS) in slice cultures of WT and Fmr1-KO mice (bar: 2 μm). (C) Increase in spine growth detected over 24 h and induced by TBS in slice cultures of WT and Fmr1-KO mice (n = 5). (D) Same, but for lost spines. (E) Illustration of the 48-h stability of spines that enlarged as a result of TBS in slice cultures from WT (left) and Fmr1-KO (right) mice (bar: 2 μm). (F) Stability over 48 h of spines that enlarged and did not enlarge as a result of TBS in WT and Fmr1-KO mice (n = 5; two-way ANOVA; *P < 0.05, **P < 0.01, ***P < 0.001).
significantly different between Fmr1-KO and WT slices (21.8 ± 2.8% vs. 16.4 ± 1.9%). Thus, Fmr1-KO mice showed an increased sensitivity of spine dynamics to activity and a loss of activity-mediated spine stabilization.

**Partial reversal of spine dynamics deficits in Fmr1-KO mice by a metabotropic glutamate receptor antagonist**

We then investigated whether these defects could be reversed, and tested the effects of the metabotropic glutamate receptor antagonist MCPG (100 μM) on spine dynamics. In WT slice cultures, MCPG had no detectable effects on basal spine formation or elimination mechanisms (Fig. 3A and B). However, it prevented the increase in spine turnover triggered by TBS (Fig. 3A and B; new spines: 23.8 ± 3.3%, n = 6, 233 spines vs. 42.4 ± 7.9%, n = 5, 240 spines, P < 0.05; lost spines: 20.8 ± 2.4% vs. 43.7 ± 8.8%, P < 0.05). It also interfered with the mechanisms of activity-dependent spine stabilization. In WT mice, MCPG significantly reduced the differential stabilization of enlarged vs. non-enlarged spines following TBS (Fig. 3C; circles: ns: non-significant difference, two-way ANOVA), consistent with evidence suggesting an involvement of metabotropic receptors in LTP induction mechanisms (Anwyl, 2009). In Fmr1-KO mice, MCPG had three main effects. First, it restored the level of basal turnover by enhancing both newly formed and lost spines per 24 h (Fig. 3A and B: new spines: 17.0 ± 3.2% vs. 9.3 ± 1.3%, n = 6, 8, P < 0.05; lost spines: 15.2 ± 2.5% vs. 9.5 ± 1.5%, n = 6, 8, P < 0.05), consistent with the idea that the decreased turnover observed in Fmr1-KO mice could be due to an over-activation of metabotropic receptors (Pfeiffer & Huber, 2007). Second, it reduced the increased sensitivity of spine turnover to activity: new spine formation still increased following TBS, but to a lesser extent (Fig. 3A; 27.2 ± 3.6%, n = 5 cells, 237 spines vs. 33.8 ± 2.2%, n = 7, 274 spines, P < 0.05). However, the changes

**Reversal of spine dynamics deficits in Fmr1-KO mice by PTEN inhibition**

As the reduced turnover and loss of activity-dependent stability observed in Fmr1-KO mice suggested a deficit in LTP-mediated signaling mechanisms, we then investigated whether enhancing PI3K signaling could also reverse these alterations as previously shown for other LTP-associated deficits (Lauterborn et al., 2007; Hu et al., 2008). To do this, we used BpV, a PTEN inhibitor that is selective when used at low nanomolar concentrations, and has been shown to increase PI3K activity and AKT phosphorylation even under in vivo conditions (Schmid et al., 2004; Jurado et al., 2010; Mao et al., 2013). We first verified that 15 nM of BpV applied to slice cultures or 30 μg/100 g given intraperitoneally under in vivo conditions was able to increase AKT phosphorylation, assessed using a phospho-specific antibody against the S473 site of AKT. Figure 4A illustrates the increase in AKT phosphorylation of S473 obtained 1 h after injection of BpV (30 μg/100 g) intraperitoneally in WT and Fmr1-KO mice. The experiments were also reproduced in slice cultures, and S473 phosphorylation assessed 30 min after addition of BpV to the culture medium. All quantitative data normalized to the optical densities measured in WT tissue are shown in Fig. 4B (n = 3–9). The results indicate that BpV at the concentrations used promoted phosphorylation of AKT on its S473 site both under in vitro and in vivo conditions, and in WT and Fmr1-KO mice. Note that our data also indicate a reduced phosphorylation of the S473 site of AKT in Fmr1-KO mice as compared with WT animals.

We then examined the effects of BpV on spine turnover in WT and Fmr1-KO mice. In WT slice cultures, BpV significantly increased the level of basal turnover, enhancing spine formation mechanisms to the level observed following TBS, occluding further effects of TBS (Fig. 5A; BpV: 41.8 ± 4.6%, n = 5, 168 spines; TBS: 42.4 ± 7.9%, n = 5, 133 spines). BpV also increased spine elimination in a similar manner (Fig. 5B). BpV, however, did not alter mechanisms of activity-mediated spine stabilization, and the differential survival of enlarged and non-enlarged spines observed after TBS was not modified (Fig. 5C; stable spines at 48 h: 77.6 ± 8.0%, n = 5 vs. 60.0 ± 6.5%, n = 5, P < 0.05; two-way ANOVA).

In Fmr1-KO mice, BpV reversed all the defects of spine dynamics. BpV increased basal turnover both regarding new spine formation and spine elimination (Fig. 5A; KO, new spines: 9.2 ± 3.7%, n = 10, 348 spines; KO + BpV, new spines: 22.8 ± 2.7%, n = 6, 277 spines, P < 0.01; Fig. 5B; KO, lost spines: 9.5 ± 1.5%; KO + BpV: 19.4 ± 2.8%, P < 0.01). Furthermore, a sensitivity to activity was preserved and the level of turnover could still be increased by TBS application (Fig. 5A and B; KO + BpV + TBS, new spines: 33.8 ± 2.2%, n = 11, 369 spines, P < 0.01;
KO + BpV + TBS, lost spines: 29.0 ± 3.0%, P < 0.05). Most important, BpV also reversed the loss of activity-mediated spine stabilization observed in Fmr1-KO mice: enlarged spines were now differentially stabilized when compared with non-enlarged spines (Fig. 5D; 82.4 ± 5.1% vs. 62.8 ± 5.4%, n = 9, P < 0.01, two-way anova, Bonferroni post hoc test).

Reversal of LTP and learning deficits in Fmr1-KO mice

We then wondered whether BpV treatment could also have functional implications. For this, we first tested the effects of BpV on LTP induction mechanisms on acute hippocampal slices of young adult mice (4–6-week-old mice). As shown in Fig. 6A, and consistent with other studies (Lauterborn et al., 2007; Meredith et al., 2007; Hu et al., 2008), we found that LTP was decreased in Fmr1-KO mice (Fig. 6A and B; 15.5 ± 3.0% vs. 37.1 ± 5.8%, n = 6–8, P < 0.01). Application of BpV (15 nM) had no detectable effects on basal transmission, but partially reversed the decrease in LTP measured at 30 min (28.9 ± 0.9% vs. 15.5 ± 3.0%, n = 6–8, P < 0.01), without significantly affecting LTP in WT slices. This result thus suggested that BpV could be beneficial for LTP-dependent learning in Fmr1-KO mice. We thus tested whether and how BpV affected adult Fmr1-KO mice behavior in a Morris water maze task. Four groups of mice, WT (n = 19) and Fmr1-KO mice (n = 19), with (n = 9) and without (n = 10) treatment of BpV were compared. Mice underwent 4 days of training and were then submitted to a probe trial without platform. After this, they were retested for reversal learning over three consecutive days with BpV [30 µg/100 g in saline (200 µL)] or saline (200 µL) injected before each test. Finally they were submitted to a final probe trial. Before any treatment, Fmr1-KO and WT mice learned equally well to localize the platform position (Fig. 6C). In contrast, Fmr1-KO mice were significantly less proficient than WT in the reversal task (Fig. 6, open circles in D and E; P < 0.001, repeated-measures ANOVA). Comparison of BpV-treated and vehicle-injected WT mice showed no detectable differences. However, BpV significantly improved the learning curve of Fmr1-KO mice in the reversal task (F_{6,117} = 2.9, P < 0.05, repeated-measures ANOVA; Fig. 6E). BpV treatment also improved the new target quadrature preference of Fmr1-KO mice in the second probe trial (KO: 21.9 ± 2.7 s; WT: 20.3 ± 2.0 s; KO: 17.6 ± 2.2 s, P < 0.01, ANOVA). Furthermore, analysis of the PI reflecting the average distance to the target platform in the second probe trial showed that the searching precision improved slightly for both WT mice and BpV-treated Fmr1-KO mice, while vehicle-treated Fmr1-KO mice clearly worsened their performance (Fig. 6F; P < 0.05). Together these results provide strong support to the rescuing potential of BpV in this learning task.

Discussion

Multiple lines of evidence indicate that FXS results from a dysregulation of mechanisms of protein synthesis at synapses that could be linked to an exaggerated signaling through group I metabotropic glutamate receptors (Osterweil et al., 2012). Accordingly, the main stream strategy currently used to try to reverse the deficits associated with the disease includes the development of selective antagonists of these receptors (Dolen et al., 2007; Krueger & Bear, 2011; Michalson et al., 2012). A few other studies, however, also suggest that interfering with other signaling pathways could be beneficial for reversing behavioral phenotypes in Fmr1-KO mice (Hayashi et al., 2007; Lauterborn et al., 2007; Dolan et al., 2013; Osterweil et al., 2013). Here we focused on spine dynamics alterations in the hippo-
campus, and provide evidence that promoting PI3K signaling reverses the spine dynamics deficits and improves LTP and cognitive functions in this model.

First, our study extends to the hippocampus previous in vivo analyses indicating that properties of structural plasticity are affected in the somatosensory cortex of Fmr1-KO mice (Cruz-Martin et al., 2010; Pan et al., 2010). In the hippocampus, we find that the level of turnover of spine synapses is significantly reduced under basal conditions, with a decreased formation and elimination of spines. The effect was observed both in Fmr1-KO mice and following acute knockdown of the Fragile X mental retardation protein using a shRNA approach. Our results, however, do not suggest an increase in spine elimination as proposed by some studies (Pfeiffer et al., 2010). On the other hand, we find that spine dynamics show an increased sensitivity to activity, and furthermore that the mechanisms of spine stabilization induced by activity are deficient. This result is consistent with recent observations indicating that signaling pathways and molecules important for LTP stability are defective in Fmr1-KO mice (Chen et al., 2010; Seese et al., 2012). Thus, activity strongly stimulates the formation of unstable synapses, a result that is in line with the conclusion of an in vivo study carried out in the somatosensory cortex of adult mice (Pan et al., 2010). In this and another in vivo study, however, the level of basal turnover was enhanced and not reduced (Cruz-Martin et al., 2010). Spine turnover was, however, poorly sensitive to sensory experience, suggesting that it was already maximally activated. It might thus be that the discrepancies observed in basal level of spine turnover could reflect differences in the levels of neuronal activity. This might also explain why under dissociated culture conditions, where spontaneous activity is usually high, spine elimination could be enhanced (Pfeiffer et al., 2010). All together, these different studies suggest that an important component of the synaptic alterations present in Fmr1-KO mice is a deficit in the mechanisms of activity-dependent synapse stabilization (Chen et al., 2010; Seese et al., 2012), resulting in the formation of an excessive number of unstable spines. A similar conclusion was reached in analyses of PAK3 KO mice, another mouse model of intellectual disability, where spine dynamics is also significantly altered and associated with an increased formation of unstable spines (Dubos et al., 2012).

A second interesting result of this study is the possibility to differentially reverse these spine dynamics deficits by MCPG, a group I metabotropic glutamate receptor antagonist, and by BpV, a PTEN inhibitor that enhances PI3K signaling (Schmid et al., 2004; Jurado et al., 2010; Mao et al., 2013). MCPG restored a control level of basal turnover, it also improved global spine stability in Fmr1-KO mice, but did not reverse the deficit in activity-mediated spine stabilization. This suggests a differential contribution of metabotropic glutamate receptors to spine dynamics and spine stability that could be mediated by different signaling pathways. Notably the activity-dependent spine stabilization mechanisms probably require to maintain some functionality of metabotropic glutamate receptor signaling, consistent with the notion that MCPG interferes with induction of LTP (Anwyl, 2009). This is also in line with the notion that activity-dependent spine stabilization may depend upon specific signaling systems and regulations of the cytoskeleton that are defective in Fmr1-KO mice (Chen et al., 2010; Seese et al., 2012). However, although MCPG did not fully reverse the spine dynamics deficit reported here, it is interesting that chronic blockade of mGluR5 was recently reported to restore many cortical deficits in Fmr1-KO mice, including altered dendritic spine phenotypes, excessive LTD, mammalian target of rapamycin signaling and avoidance learning (Michalon et al., 2012).

In addition to MCPG, our study suggests that another interesting molecule to reverse phenotypes in Fmr1-KO mice is BpV, a PTEN
inhibitor. Application of BpV enhances PI3K activity, a signaling pathway activated by many surface receptors including BDNF that also inhibits LTD (Jurado et al., 2010) and contributes to LTP signaling (Tang et al., 2002). Consistent with this, our experiments show that BpV could restore activity-dependent spine stabilization. These results thus suggest that in addition to an exaggerated LTD type of signaling, another defect present in *Fmr1*-KO mice could involve defective LTP mechanisms. Our LTP experiments support this interpretation and are in line with those of several other studies (Lauterborn et al., 2007; Meredith et al., 2007; Hu et al., 2008; but see also Krueger & Bear, 2011). A question, however, remains regarding the specific role of PI3K signaling in FXS. Two recent studies showed that PI3K activity and AKT phosphorylation are already upregulated in *Fmr1*-KO mice (Gross et al., 2010; Sharma et al., 2010), a result at variance with another study (Hu et al., 2008). It should be noted, however, that the PI3K-AKT signaling pathway is downstream of many cell surface receptors and implicated in many interactions with other systems, possibly through differential regulations of AKT phosphorylation sites (Hemmings & Restuccia, 2012). Although a precise understanding of the complex signaling mechanisms implicated in PI3K signaling is beyond the scope of this study, our data clearly show that BpV treatment enhanced AKT phosphorylation on the S473 site both under in vitro and in vivo conditions, and was able not only to reverse the deficits in spine dynamics observed in *Fmr1*-KO mice, but also to increase LTP and improve *Fmr1*-KO mice behavior in a Morris water maze learning task. Consistent with these results, PI3K signaling has been shown to contribute to LTP mechanisms (Tang et al., 2002), to be involved in BDNF signaling, which also rescued LTP in *Fmr1*-KO mice (Lauterborn et al., 2007), to regulate synaptogenesis and spinogenesis in hippocampal neurons (Jaworski et al., 2005; Cuesto et al., 2011), and more importantly to be also deficient in a mouse model of Angelman syndrome, another neurodevelopmental disorder with severe cognitive deficits and autistic traits (Cao et al., 2013). It might therefore represent an interesting target for reversing cognitive deficits.

Conflict of interest
The authors declare no competing financial interests.

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Abbreviations

| AKT | protein kinase B | BDNF | brain-derived neurotrophic factor | BpV | dipo-tassium bispero(5-hydroxy-2-carboxyloxy)oxovanadate | Fpx | Fragile X syndrome | KO | knockout | LTD | long-term depression | LTP | long-term potentiation | MCPG | α-Methyl-4-carboxyphenylglycine | PI | proximity index | PI3K | phosphoinositol-3 kinase | PTK | phosphatase and tensin homolog | TBS | theta-burst stimulation | WT | wild-type |

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