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Rescue of behavioral phenotype and neuronal protrusion morphology in *Fmr1* KO mice

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Abstract

Lack of fragile X mental retardation protein (FMRP) causes Fragile X Syndrome, the most common form of inherited mental retardation. FMRP is an RNA-binding protein and is a component of messenger ribonucleoprotein complexes, associated with brain polyribosomes, including dendritic polysomes. FMRP is therefore thought to be involved in translational control of specific mRNAs at synaptic sites. In mice lacking FMRP, protein synthesis-dependent synaptic plasticity is altered and structural malformations of dendritic protrusions occur. One hypothesized cause of the disease mechanism is based on exaggerated group I mGluR receptor activation. In this study, we examined the effect of the mGluR5 antagonist MPEP on Fragile X related behavior in *Fmr1* KO mice. Our results demonstrate a clear defect in prepulse inhibition of startle in *Fmr1* KO mice, that could be rescued by MPEP. Moreover, we show for the first time a structural rescue of Fragile X related protrusion morphology with two independent mGluR5 antagonists.

Keywords

Fragile X syndrome; spines; dendrite branching; MPEP; fenobam; prepulse inhibition of startle; metabotropic glutamate receptor; primary hippocampal neuron culture

Introduction

Fragile X syndrome (FXS) is the most common heritable form of mental retardation. The syndrome is caused by a lack of expression of FMRP (fragile X mental retardation protein), which is the protein product of the *FMR1* gene. In most cases, the lack of expression is caused by expansion of a CGG repeat (>200 units) in the 5′ UTR of the *FMR1* gene, leading to methylation of both the CGG repeat and the promoter region, accompanied by transcriptional silencing. FMRP is an RNA binding protein that associates with polyribosomes and is localized in neurons in the form of granules that move in a microtubule dependent manner with the speed of RNA transport (Antar et al., 2005; De Diego Otero et al., 2002; Wang et al., 2007). Moreover,

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FMRP has been shown to influence the translation efficacy of several of its target mRNAs (reviewed in (Bagni and Greenough, 2005; Bardoni et al., 2006; Zalfa et al., 2007), which also implicates local translation at synaptic sites (Greenough et al., 2001; Muddashetty et al., 2007; Weiler et al., 1997; Weiler et al., 2004). In most cases, FMRP acts as a translational repressor (Laggerbauer et al., 2001; Lu et al., 2004). Therefore, FMRP is thought to be involved in the transport and/or the regulation of local mRNA translation at synaptic sites (Bagni and Greenough, 2005; Miyashiro et al., 2003; Weiler et al., 1997; Weiler et al., 2004). The presumed loss of translational regulation at synaptic sites might underlie the cognitive impairment in FXS (Huber et al., 2000).

Over the last few years, the metabotropic glutamate receptor (mGluR) theory of FXS has gained much support (Bear et al., 2004). The mGluR theory states that AMPA receptor internalization triggered by mGluR5 stimulation (Snyder et al., 2001), is exaggerated in *Fmr1* KO mice, accounting for the enhanced hippocampal LTD found in knockout mice (Bear et al., 2004; Huber et al., 2002). Recently it was shown that FMRP deficient dendrites indeed show aberrant AMPA receptor trafficking resulting in a significantly reduced number of AMPA receptors at the plasma membrane (Nakamoto et al., 2007). Moreover, *Fmr1* KO mice that are crossbred with mice that have a 50% reduction in mGluR5 expression were shown to be rescued in several phenotypic aspects (Dolen et al., 2007). It is hypothesized that FMRP normally is involved in the inhibition of the translation of several local mRNAs that are important for the mediation of AMPA receptor internalization. Since the amount of AMPA receptors in the postsynaptic density is correlated with protrusion shape, this might also explain the immature protrusion morphology that has been found in different brain areas of both fragile X patients and *Fmr1* KO mice (Comery et al., 1997; Galvez and Greenough, 2005; Grossman et al., 2006; Hinton et al., 1991; Koekkoek et al., 2005; Nimchinsky et al., 2001). The mGluR theory has also boosted the search for therapeutic targets for FXS. An antagonist of mGluR5 receptors would theoretically counteract the increased amount of AMPA receptor internalization in *Fmr1* KO neurons. Behavioral studies have shown that *Fmr1* KO mice treated with the mGluR5 antagonist MPEP (2-methyl-6-(phenylethynyl)-pyridine hydrochloride) clearly display less sensitivity to audiogenic seizures and more wild type-like behavior in an open field test compared with untreated mice (Yan et al., 2005). Also in a *Drosophila* model based on loss of function of *dfmr1*, the single homolog of the *FXR* family of genes in the *Drosophila* genome, MPEP was able to rescue courtship behavior and mushroom body defects (McBride et al., 2005). However, the molecular mechanisms behind the effects of MPEP have not been elucidated.

In the present study, we show a defect in prepulse inhibition of acoustic startle (PPI) in *Fmr1* KO mice compared to wild type littermates and a rescue of this behavioral phenotype by the mGluR5 antagonist MPEP. In addition, we demonstrate an altered protrusion morphology in *Fmr1* KO primary hippocampal neurons that could be rescued using two different mGluR5 antagonists, MPEP and fenobam, rendering protrusion densities indistinguishable from wild type neurons.

Materials and methods

Mouse models

Fmr1 KO mice were generated in our lab as described previously (Bakker et al., 1994; Mientjes et al., 2006). Both lines were used and were backcrossed to C57Bl/6J mice at least seven times. No differences were observed between both *Fmr1* KO lines.

Prepulse inhibition of startle

Prepulse inhibition of startle (PPI) was measured by analysis of eye blink reactions of mice to acoustic stimuli, based on the magnetic distance measurement technique (MDMT) used for eye blink conditioning (Koekkoek et al., 2002; Koekkoek et al., 2005). Adult *Fmr1* KO mice (n=8) and wild type littermates (n=9) were anesthetized with an oxygenated mixture of nitrous oxide and isoflurane. A dental acrylic pedestal was placed on the skull and animals were allowed to recover for three days. Prior to the experiment the mice were very briefly sedated using the isoflurane/nitrous oxide mixture. A sensorholder with an airchannel and a magnetsensor was attached to the pedestal. A small neobdimium iron borium magnet (0.8*1.6*0.2 mm) was glued to the lower eyelid with a minute drop of cyanoacryllate and a silicon body harness was put on to protect the mice from strain on the pedestal. Mice were placed inside their own cages within soundproof training chambers and allowed to recover until normal behavior (grooming, eating) returned, usually this was within 15 minutes. To test and calibrate the MDMT system air puffs were given as a measure of full eyelid closure.

A background noise level of 60 dB white noise was present. Subsequently, the mice were presented with a white noise startle stimulus of 90 dB, which in the prepulse inhibition condition was preceded by a 70 dB white noise prepulse, 50 ms before the startle stimulus.

Each mouse was subjected to seven blocks of trials consisting of one air puff and three repeated measures of a startle stimulus followed fifty seconds later by a prepulse/startle stimulus with a fifty seconds intertrial interval. The next day the same mice were analyzed again in the same way after MPEP treatment. MPEP treatment was administered by i.p. injection of 20 mg/kg MPEP dissolved in PBS, 30 minutes before the experiment. Percentages of PPI of startle were compared by non-parametric Mann Whitney U test.

Primary hippocampal neuron culture

In short, E18 wild type and *Fmr1* KO mice litters were planned on the same day. Embryos were decapitated after which hippocampi were removed and dissociated by trypsin and mechanical treatment. Neurons were plated on poly-L-lysine (100 μg/ml, Sigma) and laminin (50 μg/ml, Sigma) coated 30mm glass coverslips. The neurons were attached to the substrate in a drop of Neurobasal medium (Gibco), containing penicillin/streptomycin (Gibco), Glutamax (Gibco) and B-27 (Gibco) supplements. After 2 hours, medium volume was adjusted to 2ml per coverslip in 6-well plates. After 20 days *in vitro* cells were transfected, using Lipofectamine 2000 (Invitrogen), with an mCherry construct under control of a chicken *β*-actin promoter to ensure neuron-specific expression. One day after transfection, cells were treated for four hours with 200 μm MPEP (Sigma), 300 μm fenobam (Sigma), 100 μm D-AP-5 (Sigma) or left untreated in supplemented Neurobasal medium. After treatment, neurons were fixed in 4% formaldehyde in PBS, washed in PBS and mounted in Mowiol mounting solution (Mowiol 4-88, Hoechst).

Quantification of protrusion density and dendrite branching

Images of *β*-actin-mCherry transfected neurons were acquired using a Zeiss LSM510 confocal microscope. Twenty to forty neurons from three independent experiments were imaged for each condition. For each neuron, a z-stack of 10 × 0,3μm was made. The projected images were analyzed for protrusions with Metamorph software (Molecular Devices, Sunnyvale, CA). Two distal dendritic segments of 70-100 μm were chosen per neuron for protrusion morphometric analysis. For each protrusion, the length and the width were measured. The length was defined as the distance from the base to the tip of the protrusion; width was defined as the maximum distance perpendicular to the long axis of the protrusion. In order to make an objective distinction between spines and filopodia, we calculated the ratio of the width and the length for each protrusion. Protrusions with a ratio above or equal to 0,5 were considered as

spines and conversely, protrusions with a ratio below 0.5 were considered as filopodia (Okamura et al., 2004). Averages of protrusion densities of three independent experiments were compared with unpaired two-tailed Student's T-tests.

Dendrite branching of *β*-actin-mCherry transfected neurons was quantified by performing Sholl analyses of stacked Zeiss confocal generated images (40× objective, stack of 20 × 0,2μm). With Metamorph software, concentric equally spaced circles (every 20μm) were drawn around the cell soma of each neuron and subsequently, the amount of dendrite crossings were counted per circle. Averages of counts of three independent experiments were compared with unpaired two-tailed Student's T-tests.

Results

MPEP rescues prepulse inhibition of startle defect in Fmr1 KO mice

One of the most common clinical features of FXS is heightened sensitivity to sensory stimulation (Frankland et al., 2004; Miller et al., 1999). PPI is a widely used model to study basic sensorimotor processing and has shown to be related to mGluR signaling (Grauer and Marquis, 1999). In our mouse model, we examined PPI in wild type and *Fmr1* KO mice. Mice were presented with a startling acoustic stimulus of 90dB, which in the prepulse condition was preceded by a 70dB pulse, 50 ms before the startle stimulus. In wild type mice, the startle response after a prepulse was inhibited by 73% compared to the response after a startle stimulus alone (Fig.1). This indicates good PPI in the wild type mice. In the *Fmr1* KO mice however, the startle response was inhibited by only 30% when a prepulse preceded the startle stimulus, illustrating that PPI is defective in *Fmr1* KO mice. To study if MPEP can rescue a behavioral FXS phenotype in our mouse model, we examined PPI in wild type and *Fmr1* KO mice with or without MPEP treatment. Treatment of *Fmr1* KO mice with 20 mg/kg MPEP thirty minutes prior to the experiment, rescued the PPI to a level of 70%, indistinguishable from the wild type PPI response (Fig.1). Interestingly, wild type mice also responded to MPEP treatment with higher PPI levels. This effect was not further examined in this study.

Rescue of protrusion phenotype of Fmr1 KO primary hippocampal neurons

Although MPEP has been shown to rescue several behavioral FXS phenotypes in mice and *Drosophila* (McBride et al., 2005; Yan et al., 2005), the molecular mechanism behind these rescue effects remains elusive. Therefore we decided to study if mGluR5 antagonists are also able to rescue FXS related altered protrusion morphology in an established *in vitro* model of primary hippocampal neurons.

In order to characterize our *in vitro* model, we first examined the basic neuronal properties of dendrite branching and protrusion morphology of our primary hippocampal cultures. Primary hippocampal neurons of wt and *Fmr1* KO mice were cultured in parallel. After twenty days *in vitro*, neurons were transfected with a βactin-mCherry construct in order to visualize the neurons, including dendritic protrusions. The next day, neurons were fixed, after which transfected neurons were imaged by confocal microscopy (Fig.2). In order to quantify dendrite branching, we used Sholl analysis, which measures the number of dendrite crossings with equally spaced concentric circles around the cell soma. Quantification of three independent experiments comparing dendrite branching of *Fmr1* KO neurons and wild type neurons did not reveal any significant difference (Fig.3).

Protrusions were quantified and measured for their length and width with Metamorph software. Based on these measurements, they were classified objectively as spines or filopodia (immature spines). Mature spines have a mushroom shaped appearance with a large spine head, while immature spines or filopodia have a long and thin appearance. Therefore, protrusions whose

width was equal to or more than half the size of its length, were judged as standard mature mushroom spines. If this ratio was less than half the size of the length, protrusions were considered to be filopodia. Using the Metamorph software we compared the protrusions of wt and *Fmr1* KO neurons. Based on the above described criteria, *Fmr1* KO neurons had an excess of filopodia when compared to wt neurons (Fig.4).

After characterization of our *in vitro* model and establishing an *Fmr1* KO phenotype in protrusion morphology, we continued to study the effect of MPEP on protrusion morphology. In addition, we also studied the effects of the more specific mGluR5 antagonist fenobam. Fenobam was originally discovered as an anxiolytic agent with unknown molecular target, that later was discovered to be a potent mGluR5 antagonist with an allosteric modularity site shared by MPEP, but different in structure (Porter et al., 2005). In parallel, wild type and *Fmr1* KO neurons were subjected to treatment with the mGluR5 antagonists. Treatment of *Fmr1* KO neurons with 200 μM MPEP or 300 μM fenobam for four hours, rescued the protrusion phenotype (Fig.5). The number of filopodia in treated *Fmr1* KO neurons was significantly lower than that in untreated *Fmr1* KO neurons, and indistinguishable from wild type neurons (Fig.5C). Protrusion numbers of wild type neurons were not significantly altered by MPEP or fenobam treatment.

The total protrusion density did not differ significantly between wt and *Fmr1* KO neurons with or without treatments (Fig.5A). Although the average number of protrusions classified as spines in *Fmr1* KO neurons were not statistically different from wt neurons (Fig.5B), the average percentage of spines compared to filopodia per neuron was significantly lower in *Fmr1* KO neurons and was also rescued by either MPEP or fenobam treatment (Fig.6). In other words, mGluR5 antagonist treatment restored the spine/filopodia ratio of *Fmr1* KO neurons to wild type levels.

Discussion

In this study we have shown a clear defect in PPI in *Fmr1* KO mice measured by eye blink in response to loud sound. In support of the mGluR theory of FXS, this defect was rescued to wild type levels after treatment of the mice with 20 mg/kg of the mGluR5 antagonist MPEP. The impaired PPI response in *Fmr1* KO mice is in line with sensorimotor gating deficits in FXS patients (Frankland et al., 2004). However, in the Frankland study, PPI was found to be increased rather than decreased in *Fmr1* KO mice. One explanation could be that the measurement of startle eyelid responses with the magnetic distance measurement technique (MDMT) as performed in our study is more sensitive than standard wholebody startle measurements such as used in the Frankland study. Eyelid measurements of startle include the very first components of the startle response, whereas wholebody startle measurements require induction of very strong startle responses. Therefore, eyelid startle measurement allows for a better dissection of the more subtle differences in startle behavior. In addition, our method allows us to reduce the sound pressure levels necessary for startle induction which is relevant since *Fmr1* KO mice react strongly to loud acoustic stimuli and are highly susceptible to audiogenic seizures (Musumeci et al., 2000). In another study, PPI in *Fmr1* KO mice was not significantly altered (Spencer et al., 2006). This could also be attributed to differences in the sensitivity of the methods used to measure PPI. Interestingly, we found that wild type mice showed increased PPI after MPEP treatment which is in contrast with earlier studies in rats (Henry et al., 2002; Zou et al., 2007). The underlying molecular mechanisms of the increased PPI in MPEP-treated wild type mice are unknown and beyond the scope of this study. Nevertheless, the rescue of PPI levels in the *Fmr1* KO mice underscores the therapeutic potential of MPEP (and/or other mGluR5 antagonists) for treatment of Fragile X related behavior. The PPI as measured in this study has therefore proven to be a valid behavioral test to study mGluR5 targeted therapeutic intervention in FXS patients. In this study, an acute effect

of MPEP was measured (thirty minutes after i.p. injection). However, in consideration of potential future therapeutic interventions in patients, it would be interesting to study these effects in a chronic model for MPEP treatment after long-term exposure (e.g. 2 months) of mice to MPEP. In addition, other mGluR5 antagonists that are more specific for the mGluR5 receptor and show less side effects are due to be tested in clinical trials in the future.

In an attempt to study the effect at the cellular level, we have shown altered protrusion morphology of *Fmr1* KO neurons in an established *in vitro* model. Primary hippocampal neurons of E18 wild type and *Fmr1* KO mice were cultured for 21 days, a time at which dendritic spines have matured and form synaptic contacts characteristic of those seen *in vivo* (Papa 1995). Protrusion morphology in *Fmr1* KO neurons was significantly different from wild type neurons. *Fmr1* KO had more filopodia than wild type neurons, corresponding to a more immature phenotype (Fig.4). This is in accordance with literature for both FXS patients and *Fmr1* KO mice (Comery et al., 1997;Galvez and Greenough, 2005;Grossman et al., 2006;Hinton et al., 1991;Koekkoek et al., 2005;Nimchinsky et al., 2001). In primary hippocampal neurons, reported quantities of protrusions tend to differ in literature. One study has even described fewer protrusions in hippocampal cultures of *Fmr1* KO mice (Braun 2000). Another more recent study showed increased density of filopodia-like spines in cultured *Fmr1* KO hippocampal neurons, but with many more protrusions per distance (3-5 filopodia/ 10 μm) than in our study (Antar et al., 2006). However, these cultures were not fully matured and different culture methods (such as use of glial cell feeder layers) might influence the protrusion number. In contrast, it was reported that specifically in hippocampal area CA1, *Fmr1* KO neurons have more stubby spines as opposed to filopodia (Grossman et al., 2006). In light of all these seemingly different findings, we analyzed our own culture system extensively and used an objective measurement technique to distinguish mature mushroom-like protrusions from immature filopodia-like protrusions. With these criteria, *Fmr1* KO neurons in our culture system showed a decreased spine to filopodia ratio. Furthermore, we have shown rescue of this altered protrusion morphology in *Fmr1* KO primary hippocampal neurons by two independent mGluR5 antagonists, MPEP and fenobam. As total protrusion density is not different between wild type and *Fmr1* KO neurons, we conclude that the excess of filopodia in *Fmr1* KO neurons can successfully be changed into or replaced by spines.

Since spine shape is correlated with the number of AMPA receptors in the postsynaptic density (Matsuzaki et al., 2001), these data correlate with the rescue effect of MPEP on AMPA receptor trafficking as shown by Nakamoto et al. (Nakamoto et al., 2007). In the latter study, the concentrations of MPEP used on primary neurons (10-50 μM) differed from our experiments due to different time courses of the experiments. In our study, a higher MPEP concentration was needed to visualize fast effects (within 4 hours) on protrusion morphology, whereas Nakamoto et al. studied MPEP effects after 16 hours and up to three days. In wild type cerebellar Purkinje cells, daily treatment with 30 μM MPEP for ten days changes normal protrusion morphology into a more immature phenotype with more filopodia like protrusions (Catania et al., 2001). In the present study, acute MPEP treatment had no significant effect on the protrusion morphology of wild type hippocampal neurons (Fig. 4).

Others have shown that MPEP can target NMDA receptors at high concentrations (Lea et al., 2005; Popoli et al., 2004). However, it is unlikely that the rescue effect in this study is mediated by NMDA receptors, as we also see the rescue effect with the structurally different, more specific mGluR5 antagonist fenobam. Moreover, we also tested the effect of the NMDA specific antagonist D-AP-5 (100 μM) on protrusion morphology, which did not show rescue of the *Fmr1* KO protrusion phenotype (data not shown).

In conclusion, our *in vitro* model of primary hippocampal neurons and the *in vivo* measurement of PPI form excellent tools to further study the molecular mechanisms that underlie therapeutic

intervention with mGluR5 antagonists in FXS patients and have great potential for testing newly developed drugs.

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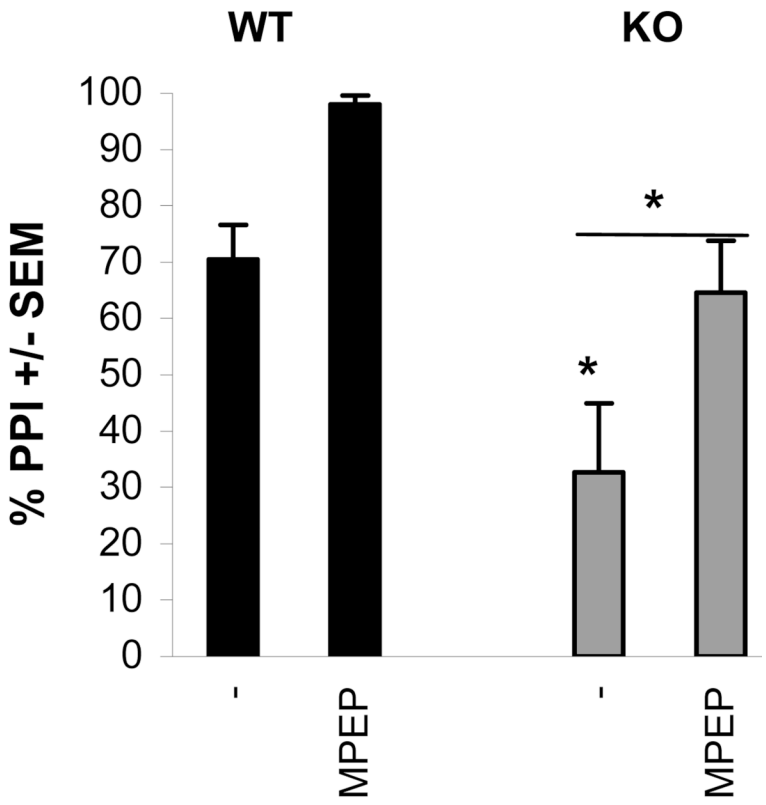

Fig.1. Rescue of prepulse inhibition of startle in *Fmr1* KO mice

Both wild type and *Fmr1* KO mice were subjected to prepulse inhibition of startle procedures. Fmr1 KO mice displayed a dramatic impairment of PPI on day 1 (baseline levels). This reduction was rescued to wild type levels on day 2 by injection of 20 mg/kg MPEP 30 minutes prior to training. Interestingly, the wild types showed an equal improvement of PPI performance after injection of MPEP.

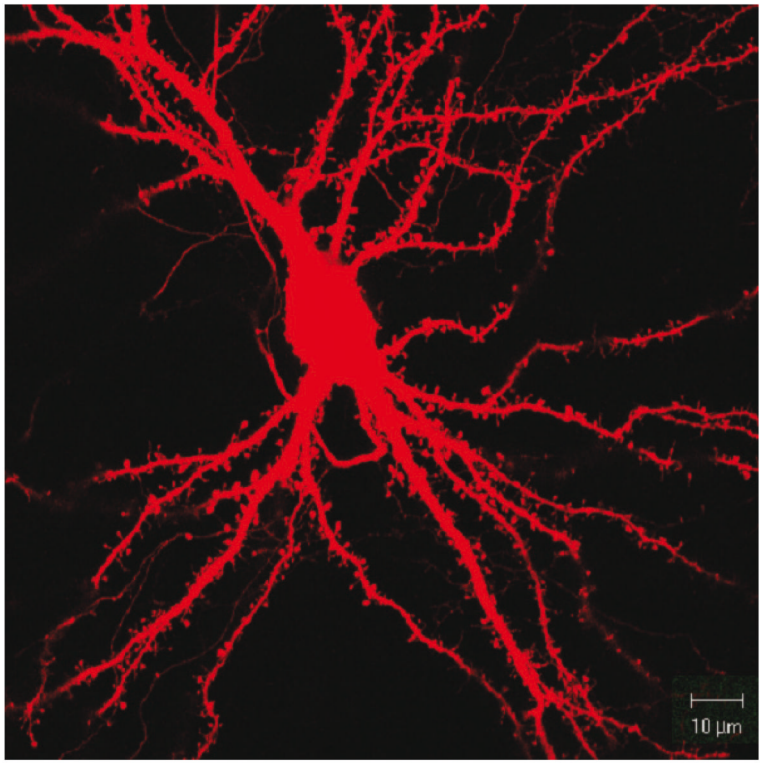

Fig.2.
Representative image of a wild type E18 hippocampal mouse neuron (DIV21), transfected with a β-actin-mCherry construct.

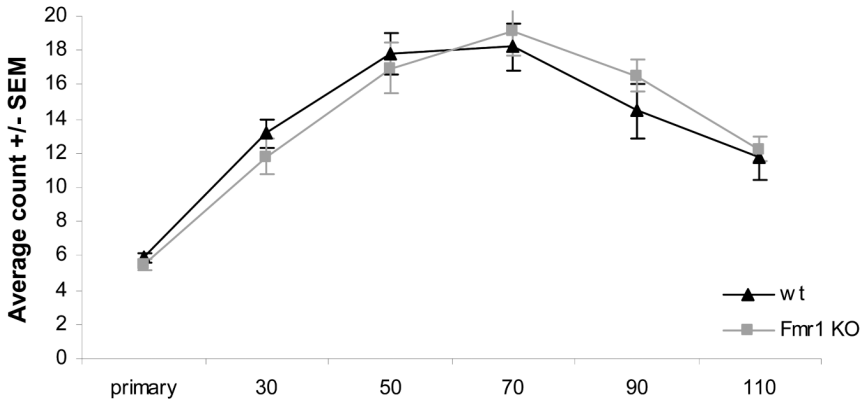

Fig.3. Dendrite branching is normal in *Fmr1* KO primary hippocampal neurons

Sholl analysis of wild type and *Fmr1* KO primary hippocampal neurons cultured in parallel was performed with Metamorph software. Average of three independent experiments.

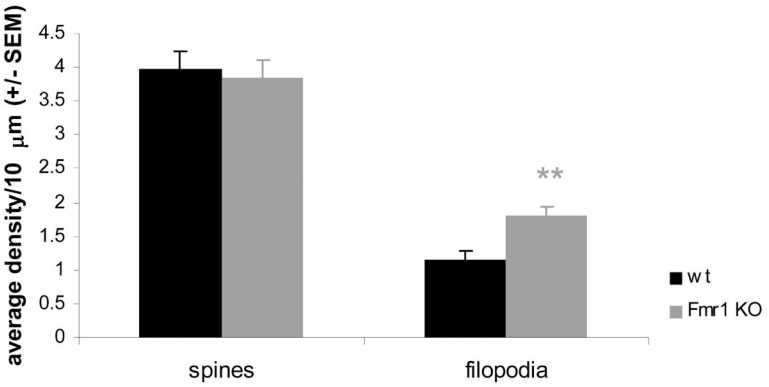

Fig.4. *Fmr1* KO primary hippocampal neurons have an immature protrusion phenotype

Protrusion densities of wild type and *Fmr1* KO primary hippocampal neurons cultured in parallel were counted with Metamorph software. *Fmr1* KO neurons had significantly more filopodia than wild type neurons ($p<0,001$), corresponding to an immature phenotype. Averages of 3 independent experiments, compared with Student's T tests. The distinction between spines and filopodia was made objectively by using a threshold ratio of 0,5 for the width/length ratio of protrusions.

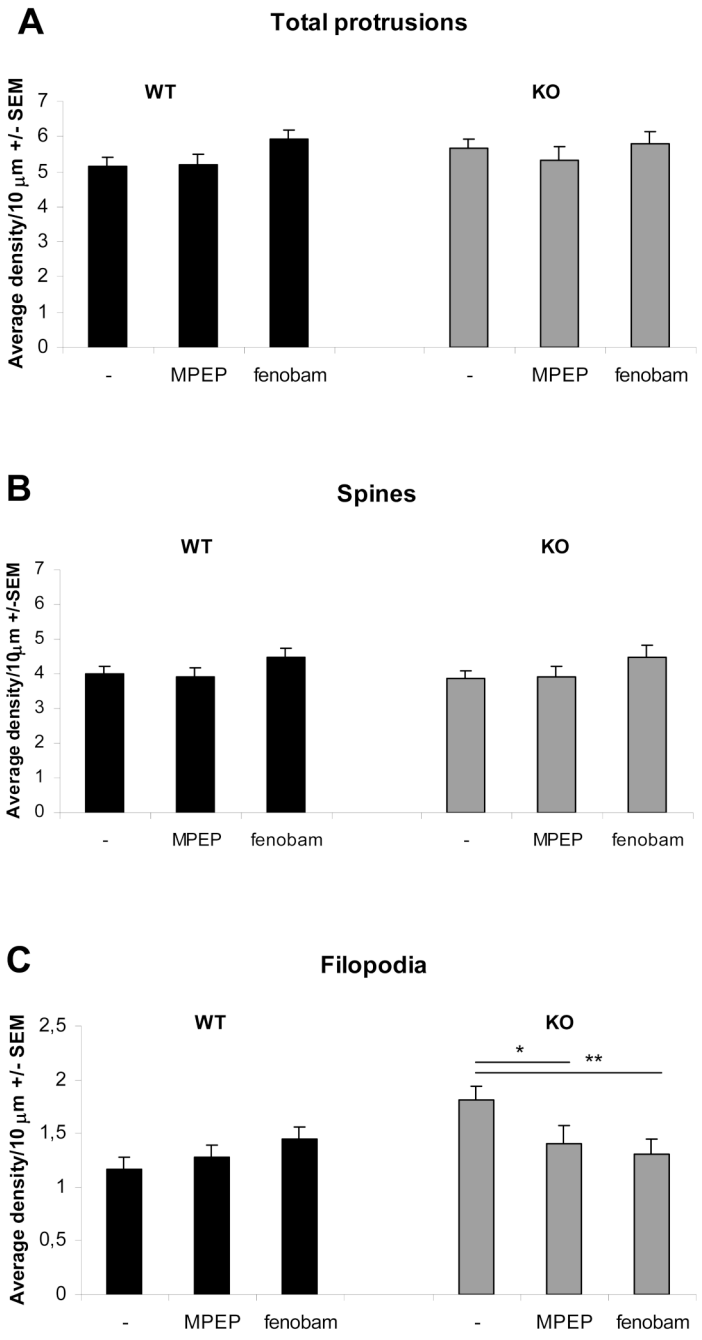

Fig.5. Rescue of protrusion morphology in *Fmr1* KO primary hippocampal neurons

Fmr1 KO and wild type neurons were treated for four hours with 200 µm MPEP or 300 µm fenobam. The total amount of protrusions (A) and the amount of mature spines (B) were unaffected by mGluR5 antagonist treatment. The *Fmr1* KO phenotype showing an increased number of filopodia was completely rescued by both mGluR5 antagonists (C). Averages of 3 independent experiments, compared with Student's T tests (*=p<0,05, **=p<0,01).

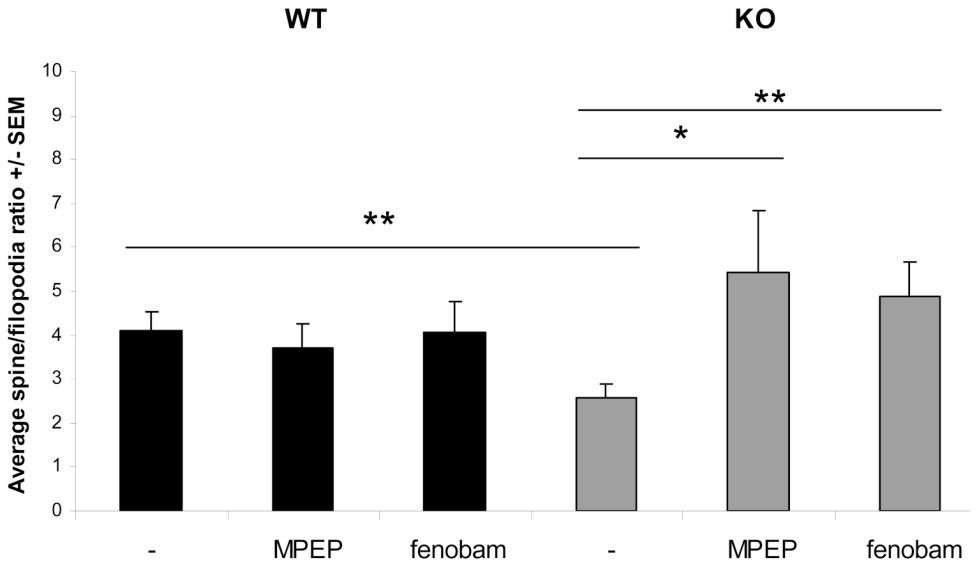

Fig.6. mGluR5 antagonist treatment changes the distribution of spines and filopodia in *Fmr1* KO neurons

The average spine/filopodia ratio changes significantly in *Fmr1* KO primary hippocampal neurons after treatment with two independent mGluR5 antagonists. As total protrusion density is not different between wild type and *Fmr1* KO neurons, we can conclude that the excess of filopodia in *Fmr1* KO neurons can successfully be changed into or replaced by spines.