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Ultrastructural analysis of the functional domains in FMRP using primary hippocampal mouse neurons

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Abstract

Fragile X syndrome is caused by lack of the protein FMRP. FMRP mediates mRNA binding, dendritic mRNA transport and translational control at spines. We examined the role of functional domains of FMRP in neuronal RNA-granule formation and dendritic transport using different FMRP variants, including the mutant FMRP_I304N and the splice-variant FMRP_Iso12. Both variants are absent from dendritic RNA-granules in *Fmr1* knockout neurons. Co-transfection experiments showed that wild-type FMRP recruits both FMRP variants into dendritic RNA-granules. Co-transfection of *FXR2*, an FMRP homologue, also resulted in redistribution of both variants into dendritic RNA-granules. Furthermore, the capacity of the variants to transport their mRNAs and the mRNA localization of an *FMR1* construct containing silent point-mutations affecting only the G-quartet-structure was investigated. In conclusion, we show that wild-type FMRP and FXR2P are able to recruit FMRP variants into RNA-granules and that the G-quartet-structure in *FMR1* mRNA is not essential for its incorporation in RNA-granules.

Keywords

Fragile X syndrome; FMRP; *Fmr1*; mRNA transport; FXR2P; RNA-granules

Introduction

Fragile X syndrome (FXS) is the most prevalent form of inherited mental retardation (Imbert et al., 1998; Turner et al., 1996). The syndrome is characterized by moderate to severe mental retardation, macroorchidism, mild facial abnormalities and behavioral manifestations (Hagerman et al., 1996). The main cause of the syndrome is an expansion of the CGG repeat in the 5'-untranslated region of the *FMR1* gene. If the expansion exceeds 200 CGG repeats, the adjacent CpG island and promoter region of the *FMR1* gene are methylated, resulting in

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transcriptional silencing of the gene. The lack of *FMR1* protein (FMRP) is responsible for the fragile X syndrome phenotype (de Vries et al., 1998).

FMRP is expressed abundantly in the brain and testes. It has several conserved functional domains, containing three RNA-binding motifs -two KH-domains and a RGG-box-, a nuclear localization sequence (NLS) and a nuclear export sequence (NES). The importance of the second KH-domain was illustrated by the study of a patient with a missense mutation in the second KH-domain (Ile304Asn) who has been diagnosed with a severe phenotype of fragile X syndrome (De Boulle et al., 1993). This mutation results in the expression of mutant FMRP that no longer associates with translating polyribosomes, and loses its function as a translational repressor (Laggerbauer et al., 2001; Siomi et al., 1994). The RGG-coding region in FMRP can bind intramolecular G-quartet structures in target mRNAs (Schaeffer et al., 2001).

FMRP has two autosomal homologues, FXR1P and FXR2P (Fragile X-related proteins). These proteins are very similar to FMRP and contain the same conserved functional domains in addition to two Nucleolar Targeting Signals (NoS). The precise function of FXR2P is still unknown, although the *Fxr2* KO mice show some behavioral abnormalities similar to *Fmr1* KO mice (Bontekoe et al., 2002). FXR1P is mainly expressed in striated muscle, testis and brain and the *Fxr1* KO mice displays neonatal lethality (Mientjes et al., 2004).

FMRP appears to mediate transport and local translation of several mRNA targets at postsynaptic sites in neurons (Bakker et al., 2000; De Diego Otero et al., 2002; Devys et al., 1993; Feng et al., 1997b; Wang et al., 2008). Moreover, FXS patients and *Fmr1* KO mice both show structural malformations of dendritic protrusions, (Comery et al., 1997; De Vrij et al., 2008; Hinton et al., 1991; Irwin et al., 2001; McKinney et al., 2005) and aberrant synaptic plasticity (Huber et al., 2002; Koekkoek et al., 2005; Nosyreva and Huber, 2006). Clearly, dendritic mRNA transport and local protein synthesis are critical for synaptic plasticity and are widely studied in FXS. However, the exact mechanism of mRNA binding, transport kinetics and regulation of translation by FMRP is still largely unknown. FMRP has been suggested to transport target mRNAs from the nucleus, using its NES and NLS, to the cytoplasm. Although the presence of a NLS and NES suggests a role for FMRP in the nucleus, it has never been shown that it is necessary for FMRP to associate with target-mRNAs in the nucleus before it can be incorporated in dendritic RNA-granules.

To learn more about FMRP and its incorporation in RNA-granules, we studied a naturally occurring isoform of FMRP (FMRP_Iso12) and FMRP with the pathogenic mutation Ile304Asn (FMRP_I304N). The localization of FMRP-positive RNA-granules containing either normal or the FMRP variants was studied in cultured primary *Fmr1*-knockout mouse neurons with and without co-expression of wild-type FMRP. In addition, we also studied *FMR1* mRNA localization in transfected *Fmr1*-knockout neurons expressing different variants of FMRP, including FMRP_Iso12, FMRP_I304N and an *FMR1* construct that has silent point mutations that affect the G-quartet-structure in the mRNA.

Materials and Methods

Primary hippocampal neuron culture

Primary hippocampal neurons were cultured as described by De Vrij et al (De Vrij et al., 2008). Hippocampi of *Fmr1* knockout mice (Mientjes et al., 2006) were dissected from E18 mouse brain and placed in Dulbecco's modified Eagle's medium (DMEM, Gibco, BRL). After dissection, the hippocampi were dissociated using trypsin and mechanical treatment. The neurons were plated on coverslips coated with poly-D-lysine (100 µg/ml, Sigma) and laminin (50 µg/ml, Sigma). In a drop of Neurobasal medium (Gibco) containing penicillin/streptomycin (Gibco), Glutamax (Gibco) and B-27 (Gibco) supplements, 100,000 cells were allowed to

attach to the substrate. After two hours the medium volume was adjusted to 2 ml per coverslip in a six-well plate. After 20 days *in vitro*, the hippocampal neurons were transfected with several variants and wild type human *FMRI* constructs under control of a chicken β -actin promoter.

Expression vectors and transfection

MCherry or *EGFP* coupled fusion constructs were constructed by cloning the EcoRI fragment containing *FMRI* from pCMV-*EGFP-FMRI* or pCMV-*EGFP-FMRI_I304N* (Castren et al., 2001) into the EcoRI site of the β actin-*Cherry* or β actin-*EGFP* vector. To clone the natural splice variant of *FMRI* missing exon 12 and exon 14 (encoding for FMRP_Iso12) (Sittler et al., 1996) into β actin-*EGFP*, we digested CMV-*EGFP-FMRI_Iso12* (Tamanini et al., 1999b) with EcoRI and ligated *FMRI_Iso12* fragment into the β actin-*EGFP* that had also been digested with the same restriction enzyme. The G-quartet mutated construct (*FMRI_ΔG1+2*) has been described before (Didiot et al., 2008). This construct contains point-mutations in the G-quartet structure of *FMRI* mRNA without changing the amino acid sequence of the protein. The G-quartet mutant was also cloned behind *EGFP* to create a fusion protein. Finally, β actin-*FXR2* fusion constructs were created using human cDNA and the following FXR2 primers: forward –cggactcagatctgagctcaagcttgaat– and reverse –gagaagtactagtcgactggatcctgaatt–. The PCR product encoding for FXR2P was digested by *BglII* and *Sall* and the fragment was cloned in frame behind β actin-*EGFP* or β actin-*mCherry*, also digested with *BglII* and *Sall*. All fusion constructs were tested by sequencing (Fig. 1). The expression of all fusion proteins was verified with Western Blot on transfected HEK 293 cells (Supplementary data, S1).

After ~14 days *in vitro* (DIV), cells were transfected (1 μ g DNA) or co-transfected (0.5 μ g of each construct) with different *FMRI*-fusion constructs using Lipofectamine 2000 (Invitrogen). One day after transfection, cells were fixed with 4% paraformaldehyde in PBS, washed in PBS and mounted in Mowiol mounting solution (Mowiol 4-88, Hoechst).

Immunocytochemistry and antibodies

Neurons cultured for 14 days *in vitro* (DIV) were fixed with 4% paraformaldehyde in PBS and washed in PBS. For blocking and permeabilization we used “staining buffer” containing 0.05M Tris, 0.9% NaCl, 0.25% gelatin, and 0.5% Triton-X-100, pH 7.4. The following antibodies were used: human anti-ribosomal P antigen (RLP0, 1:2000; Immunovision) and polyclonal anti-FXR2P (Ab1937, 1:200) (Tamanini et al., 1999a) and mouse anti-FMRP (T1A, 1:200). Primary antibodies were diluted in staining buffer and were incubated overnight at 4 °C. The next day, the cells were washed in PBS and incubated with donkey anti-human-Cy3 antibody (1:200; Jackson ImmunoResearch) and donkey anti-rabbit Cy2 antibody (1:200; Jackson ImmunoResearch) or donkey anti-mouse Cy3 antibody (1:200; Jackson ImmunoResearch) and diluted in staining buffer for 1 hour at room temperature. Finally, the coverslips were washed in PBS and mounted in Mowiol mounting solution (Mowiol 4-88, Hoechst).

Riboprobes

The *FMRI* cDNA used to generate riboprobes for *in situ* hybridisation is an 800 bp fragment complementary to the 3' UTR of *FMRI*. To clone the 3' UTR fragment, we used cDNA of total human RNA and the following primers: forward –GTGAATGGAGTACCCTAACTGCA– and reverse –CCTTCCTATCTCTCCAAAATAAGCATT–. The cDNA was then cloned into the TOPOII vector with a dual promoter (Invitrogen) and linearized. *FMRI* sense and antisense probes were synthesized by *in vitro* transcription in the presence of the appropriate RNA polymerase, and digoxigenin (DIG)-conjugated UTP according to the manufacturer's protocol (Roche).

In situ hybridization

Fmr1 KO neurons were transfected with the different *FMRI* fusion constructs (described above). The following day, neurons were fixed with 4% PFA for 20 minutes. The coverslips were thoroughly washed with PBS and permeabilized with PBS-Triton X (0.1%), rinsed in 2× SSC for 5 min, dehydrated in an ethanol series, and air-dried. The coverslips were hybridized overnight at 55 °C in hybridization mix (50% formamide, 5× SSC, 5× Denhardt's, 250 µg/ml baker's yeast RNA, 500 µg/ml salmon sperm DNA) with the antisense or sense riboprobe concentration of 500 ng/ml. The *FMRI* sense probe was used as a negative control. After hybridization, the coverslips were washed in 4× SSC and treated with RNase (10 µg/ml) for 30 min at 37 °C. The coverslips were then subjected to subsequent washing steps with 2× SSC/50% formamide, 1× SSC/50% formamide at 55 °C and 0.1× SSC.

Immunodetection of the DIG-labeled riboprobe was preceded by preincubating the sections for 30 min in buffer 1 (0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl) containing 2% blocking reagent (Roche). Subsequently, the coverslips were incubated for 2 hours at room temperature with Cy3-conjugated mouse anti-DIG (Jackson ImmunoResearch 1:500) and polyclonal anti-EGFP (Abcam 1:1000) in buffer 1 containing 2% blocking reagent. After thorough washing in buffer 1 containing 0.5% Triton X, the coverslips were incubated with the secondary antibodies donkey anti-mouse Cy3 and donkey anti-rabbit Cy2 in buffer 1 for 1 hour at room temperature. Finally the coverslips were washed in buffer 1 and mounted in Mowiol mounting solution (Mowiol 4-88, Hoechst).

Quantification of dendritic granules

Images of neurons transfected with different constructs were acquired using a Zeiss LSM510 confocal microscope. To quantify the number of macromolecular granules in dendrites after transfection of different FMRP constructs, detailed images of two dendrites per neuron of at least 5 different neurons per experiment were quantified for each construct. Granules (defined as larger or equal to 0.1 µm²) were counted in equally sized regions of each dendrite using ImageJ software (developed by the National Institutes of Health). The average number of granules of three independent experiments was statistically compared using the unpaired tailed Student's *T*-test.

Results

To study the function of the conserved functional domains of FMRP in mRNA binding and dendritic transport, we used hippocampal primary neurons of *Fmr1* KO mice. Cultures were grown for 10 to 21 days which allowed us to study granule formation and transport into the dendrites and spines. The use of *Fmr1* KO neurons provided an Fmrp background-free cell system, preventing misinterpretation of effects caused by interactions between mutant and endogenous Fmrp.

FMRP is known to be present in RNA-granules trafficking into the dendrites. These granules consist of mRNAs, ribosomal subunits, motor-proteins and other RNA binding proteins (RBPs). In *Fmr1* KO neurons, Fmrp expression is totally absent and therefore the number of RNA-granules or the structure of these granules could be affected (Aschrafi et al., 2005). To study this possibility, *Fmr1* KO neurons and wild-type neurons were immunostained for two proteins that are known to be present in RNA-granules: ribosomal subunit RPLP0 and a homologue of Fmrp, Fxr2p. Both wild-type and *Fmr1* KO hippocampal neurons showed P0 positive and Fxr2p positive granules (Supplementary data, S2). The dendritic RNA-granule population is known to be heterogeneous (reviewed in (Kiebler and Bassell, 2006)), which is reflected in the staining pattern of P0 and Fxr2p in these neurons. Some dendritic RNA-granules contained P0 ribosomal subunit, but did not contain Fxr2p and vice versa. No obvious

differences in RNA-granule size or number was observed between wild-type and *Fmr1* KO neurons with both markers.

Consistent with the endogenous localization of Fmrp in wild-type primary neurons, mCherry-FMRP, transiently transfected into *Fmr1*-knockout primary hippocampal neurons was present in the cell soma and in a granular pattern within the dendrites (Fig.2a). Several mutant *FMR1* constructs were developed to study the conserved functional domains of FMRP. The expression pattern of the construct carrying the pathogenic missense mutation I304N (EGFP-FMR1_I304N) showed EGFP-FMRP_I304N localization in the cell soma and in the dendrites. However, the localization pattern of EGFP-FMRP_I304N in the dendrites was more diffuse than the granular pattern of wild-type mCherry-FMRP in dendrites (Fig.2b). We quantified this difference between EGFP-FMRP_I304N expression and wild-type mCherry-FMRP expression in *Fmr1* KO neurons by counting the number of FMRP-positive RNA-granules in distal dendrites. We defined dendritic RNA granules as having a size of 0.1 μm^2 or more. As figure 3 shows, EGFP-FMRP_I304N was less abundant in RNA-granules than wild-type mCherry-FMRP.

In addition, we studied the localization of EGFP-FMRP_Iso12, a natural splice variant of FMRP that lacks exon 12 and 14, the latter of which contains the NES. Importantly, the C-terminal part of the protein is frame shifted due to alternative splicing. *EGFP-FMR1_Iso12* transfected neurons showed a predominantly nuclear localization of the protein, with slightly diffuse cytoplasmic expression (Fig.2c). Also protein seems not to be incorporated into dendritic RNA-granules. In contrast to wild-type FMRP expression, this natural splice variant does not show EGFP-FMRP_Iso12-positive dendritic RNA-granules, as quantified in figure 3.

Effect of wild type FMRP on localization of FMRP variants

In vitro studies have demonstrated that FMRP_I304N has lost the ability to form homo-oligomers (Laggerbauer et al., 2001) and shows an abolished binding to elongating polyribosomes (Feng et al., 1997a). Laggerbauer *et al* showed *in vitro* that FMRP_I304N was still able to form dimers with wild-type FMRP, but this was never shown *in vivo*. Therefore, additional transfection experiments were carried out with the presence of wild-type mCherry-FMRP to test whether wild-type FMRP can recruit FMRP_I304N in RNA-granules. First, we studied the expression pattern of EGFP-FMRP_I304N in *Fmr1* knockout primary hippocampal neurons when co-transfected with wild-type mCherry-FMRP. These co-transfected neurons showed dendritic RNA-granules that were positive for wild-type mCherry-FMRP and most of these granules showed co-localization with EGFP-FMRP_I304N (Fig. 4a). In addition, we quantified the number of RNA-granules positive for EGFP-FMRP_I304N in distal dendritic regions. In these co-transfection studies the number of EGFP-FMRP_I304N-positive dendritic RNA-granules was similar as in wild-type mCherry-FMRP transfected neurons (quantified in figure 3), demonstrating that EGFP-FMRP_I304N can be incorporated in RNA-granules and co-localizes with wild-type FMRP. These results suggest that the two proteins can heterodimerize.

In addition, we also tested whether the presence of wild-type mCherry-FMRP affects the predominantly nuclear and diffuse dendritic distribution of EGFP-FMRP_Iso12. Co-transfection of *EGFP-FMR1_Iso12* and wild-type *Cherry-FMR1* in *Fmr1*-knockout primary hippocampal neurons resulted in mCherry-FMRP positive RNA-granules in the dendrite that were also positive for EGFP-FMRP_Iso12 (Fig. 4b). Quantification of the number of EGFP-FMRP_Iso12 RNA-granules revealed a number of granules similar to wild-type mCherry-FMRP-positive granules in a single transfection (Fig 3), showing that also FMRP_Iso12 can be incorporated in RNA-granules together with wild-type FMRP. Finally, when *EGFP-FMR1_Iso12* and *Cherry-FMR1_I304N* were co-transfected, a small proportion of both

proteins was also incorporated together in a significant number of dendritic RNA-granules (Fig. 3). Colocalization of both FMRP variants in dendritic RNA-granules is shown in figure 4c.

Role of FMRP-homologue FXR2P in dendritic granule formation

FXR2P is a homologue of FMRP that contains the same functional domains as FMRP. FMRP in addition to two Nucleolar Targeting Signals (NoS) in the 3' terminal of the protein. We were interested to see whether FXR2P is capable of recruiting FMRP_I304N or FMRP_Iso12 in dendritic RNA-granules. First we studied EGFP-FXR2P expression in *Fmr1* KO hippocampal neurons. Figure 5 illustrates the presence of EGFP-FXR2P in both the cell soma of neurons and dendritic RNA-granules (Fig. 5a). To visualize spines of EGFP-FXR2P transfected neurons, mCherry protein was co-expressed, which clearly showed that EGFP-FXR2P is also present in dendritic spines (Fig. 5a, merge). *EGFP-FXR2* and *Cherry-FMR1* co-transfection showed that all dendritic RNA-granules contained FXR2P and FMRP (Fig. 5b).

To study possible recruitment of mutant FMRP by FXR2P in dendritic RNA-granules, *EGFP-FXR2* and *Cherry-FMR1_I304N* were co-transfected in *Fmr1* KO neurons. The results indeed showed dendritic RNA-granules that contained EGFP-FXR2P as well as mCherry-FMRP_I304N (Fig. 5c). This suggests that EGFP-FXR2P is able to recruit mCherry-FMRP_I304N in dendritic RNA-granules. Also the natural splice variant EGFP-FMRP_Iso12 was recruited into dendritic RNA-granules by mCherry-FXR2P (Fig. 5d).

mRNA localization of mutant FMR1 mRNA

One of the mRNA targets of FMRP is *FMR1* mRNA itself (Dolzhanskaya et al., 2003; Schaeffer et al., 2001; Sung et al., 2000). Therefore, to study the transport of *FMR1* mRNA by different FMRP variants, we examined the mRNA localization of both wild-type and *FMR1* mRNA variants in *Fmr1* KO neurons using *in situ* hybridization.

FMR1 mRNA in *Fmr1* KO neurons transfected with *EGFP-FMR1* is transported into the dendrite and co-localizes with FMRP in dendritic granules (Fig. 6a). Most FMRP-positive RNA-granules also contained *FMR1* mRNA. The pathogenic mutation in the second KH-domain of EGFP-FMRP_I304N may affect the mRNA binding capacity of the mutant protein. *EGFP-FMR1_I304N* transfected in *Fmr1* knockout neurons showed a co-localization of *FMR1_I304N* mRNA with EGFP-FMRP_I304N and correspondingly showed a diffuse dendritic mRNA distribution compared to wild-type *FMR1* mRNA positive RNA-granules (Fig. 6b). *Fmr1* KO neurons transfected with *FMR1_Iso12* showed that *FMR1_Iso12* mRNA was mainly localized in the cytoplasm and only detected marginally in proximal dendritic RNA-granules (Fig. 6c). When co-transfected with FMRP_I304N the mRNAs of both variants were also incorporated in dendritic RNA-granules similar to the proteins (Fig 6d), although we were unable to distinguish between the two mRNAs since both are recognized simultaneously by the same probe.

As shown above, FXR2P was able to recruit both FMRP variants in dendritic RNA-granules. Therefore, we were also interested to study whether the mRNAs of both variants were also incorporated in the dendritic RNA-granules when the neurons were co-transfected with FXR2P combined with one of the FMRP variants. Therefore, *Fmr1* KO neurons were co-transfected either with *EGFP-FXR2* and *EGFP-FMR1_I304N* or with *EGFP-FXR2* and *EGFP-FMR1_Iso12* followed by an *in situ* hybridization for *FMR1* mRNA. *FMR1_I304N* and *FMR1_Iso12* mRNAs were indeed incorporated in dendritic RNA-granules and co-localized with the EGFP fusion-proteins. (Fig. 7a and 7b).

In addition to wild-type *FMRI*, *FMRI_I304N* and *FMRI_Iso12* constructs, we also examined a construct with a mutation that prohibits the formation of the G-quartet structure in *FMRI* mRNA without affecting the amino acid sequence of the protein (*EGFP-FMRI_ΔG1+2*) (Didiot et al., 2008). Since the amino acid sequence is not changed but only the G-quartet structure in the mRNA is affected, the protein shows the same granular pattern as wild-type Cherry-FMRP. Neurons transfected with *EGFP-FMRI_ΔG1+2* showed the same mRNA distribution as wild-type *FMRI* mRNA in *EGFP-FMRI* transfected cells (Fig. 6e). The *FMRI_ΔG1+2* mRNA was incorporated in dendritic RNA-granules and co-localized with EGFP-FMRP.

Discussion

It has been proposed that FMRP binds target mRNAs and mediates mRNA transport into the dendrite. During this dendritic mRNA transport, FMRP might play a role in silencing its target mRNAs. Upon arrival of the target mRNAs in spines, FMRP seems to play a role in local translation of these mRNAs (Brown et al., 2001; Weiler et al., 2004). Moreover, spine morphology in both fragile X patients and in *Fmr1* KO mice is also affected, showing an immature morphology (Comery et al., 1997; Hinton et al., 1991; Irwin et al., 2001; McKinney et al., 2005). In 2004, the mGluR theory was proposed to explain the spine and electrophysiological characteristics seen in FXS (Bear et al., 2004). The spine phenotype in FXS is explained by exaggerated AMPA-receptor internalization after mGluR5 stimulation and increased protein synthesis resulting in immature spines and enhanced LTD. Despite these findings, not much is known about the basic properties of FMRP-mRNA binding, target mRNAs and transport kinetics.

Our hippocampal neuron cultures of the *Fmr1* KO mice are the perfect tool to study RNA-granule formation and dendritic mRNA transport. Hippocampal neurons of the *Fmr1* KO mice allow us to solely study the effects of mutant forms of FMRP on FMRP-mRNA binding, target mRNAs and transport kinetics without endogenous Fmrp that might interfere with results.

Fmr1 KO neurons transfected with *FMRI_I304N* showed a diffuse pattern in the dendrite and a significantly lower number of EGFP-positive RNA-granules. This is in line with previous work showing that FMRP_I304N display a more diffuse pattern in cultured PC12 cells and hippocampal neurons (Schrier et al., 2004; Wang et al., 2008). However, the disadvantage of cells used in these studies is that there is endogenous Fmrp present which interfere with the distribution of the FMRP mutants (Fig. 4). The significantly reduced number of FMRP_I304N-positive granules can be due to the inability of the mutant protein to bind to polyribosomes, leaving only single FMRP_I304N present in RNP particles bound to microtubules to be transported into the dendrite. Indeed, Wang *et al* showed that GFP-FMRP_I304N could still be found in granules although these granules were much smaller than RNA-granules containing wild-type FMRP (Wang et al., 2008). Strikingly, if neurons were co-transfected with wild-type *Cherry-FMRI* and *EGFP-FMRI_I304N* constructs, both proteins co-localized in dendritic RNA-granules. Previously it was shown that *in vitro* that FMRP_I304N lost the function to dimerize with itself, but that FMRP_I304N can still dimerize with wild-type FMRP, FXR1P and FXR2P (Laggerbauer et al., 2001), although Feng *et al* using I304N-patient lymphoblasts found no co-sedimentation in sucrose-gradient of FXR2P and FMRP_I304N (Feng et al., 1997a). In agreement with these results, we now show in primary hippocampal neurons that wild-type mCherry-FMRP can mediate, probably by dimerisation, the presence of EGFP-FMRP_I304N in RNA-granules.

EGFP-FMRP_Iso12 is an alternatively spliced variant of FMRP (Sittler et al., 1996). Several experiments have shown that FMRP without a NES (FMRP-NES) accumulates in the nucleus in different cell lines (Fridell et al., 1996; Hu et al., 2005; Tamanini et al., 1999a; Willemsen

et al., 1996), although some studies also have shown the presence of small quantities of FMRP-NES in the cytoplasm (Kim et al., 2009). *Fmr1* KO neurons transfected with *EGFP-FMR1_Iso12* showed a predominantly nuclear localization, while the dendrites did not show dendritic EGFP-FMRP_Iso12-positive RNA-granules. Since the FMRP_Iso12 construct lacks a NES signal, the fusion protein cannot be exported out of the nucleus and accumulates in the nucleus. Moreover, the lack of EGFP-FMRP_Iso12-positive RNA-granules indicates that FMRP_Iso12 cannot be incorporated directly after synthesis in the cytoplasm in RNA-granules before it is transported into the nucleus. In theory, the absence of EGFP-FMRP_Iso12-positive RNA granules could also result from the lack of the RNA-binding RGG-box in this construct. However, earlier experiments by Sittler *et al* using a FMRP-NES construct that lacks only exon 14 without a frameshift, showed a similar localization pattern as the natural FMRP-Iso12 in COS cells (Sittler et al., 1996). Furthermore, it has been shown that the C-terminal part of FMRP is important for microtubule-dependent transport into the dendrites via kinesin (Dictenberg et al., 2008). Thus, FMRP_Iso12 might show a diffuse distribution pattern because it cannot bind to kinesin and consequently is not transported along microtubules. Moreover, the small amount of EGFP-FMRP_Iso12 found in the dendrite and cell soma might arise from a different nuclear export mechanism (Kim et al., 2009) or might reflect newly synthesized fusion proteins waiting for nuclear shuttling. Although there is some cytoplasmic FMRP_Iso12, our results suggest that FMRP requires to be first transported into the nucleus to pick up target mRNAs or interact with other RNP proteins, before it can be incorporated in RNA-granules and subsequently transported into the dendrite.

Neurons co-expressing EGFP-FMRP_Iso12 and wild-type Cherry-FMRP show positive labeling of RNA-granules for both proteins. This indicates that EGFP-FMRP_Iso12 can be incorporated into dendritic RNA-granules in the presence of wild-type FMRP. It is already known that FMRP can dimerize with itself and FXR1P and FXR2P (Laggerbauer et al., 2001; Tamanini et al., 1999b). The observation that EGFP-FMRP_Iso12 could be incorporated into RNA-granules together with wild-type FMRP suggests that the variant EGFP-FMRP_Iso12 most likely dimerize with wild-type FMRP in the nucleus and in this way can escape accumulation in the nucleus. Surprisingly, when neurons were co-transfected with *Cherry-FMR1_I304N* and *EGFP-FMR1_Iso12*, RNA-granules were labeled positive for both proteins. This result suggests that EGFP-FMRP_Iso12 and mCherry-FMRP_I304N may dimerize using their coiled-coiled domains and ultimately be incorporated in RNA-granules using each others conserved domains, i.e. the NES of FMRP_I304N to exit the nucleus and the normal KH2 domain of FMRP_Iso12.

Finally, we were interested to see whether the FMRP-homologue FXR2P was also capable of binding the FMRP variants resulting in FMRP-positive dendritic mRNA-granules. FXR2P shares all the conserved domains of FMRP and therefore it is suggested that the homologues might be able to compensate to some extent for the lack of FMRP in *Fmr1* KO mice. Like FMRP, FXR2P is incorporated in dendritic mRNA-granules and is localized in a subpopulation of spines (Fig. 5a). Although endogenous Fxr2p levels in the neurons are most likely not sufficient to recruit both variants into RNA-granules, co-transfection of *EGFP-Fxr2* together with one FMRP variants resulted RNA-granules containing both mutant FMRP and FXR2P. In addition, similar to FMRP, FXR2P was able to recruit mutant forms of FMRP in dendritic RNA-granules. Our results suggest that FXR2P and FMRP can functionally interact in neurons.

mRNA localization

There are several studies showing that FMRP can bind its own mRNA (Dolzhanskaya et al., 2003; Schaeffer et al., 2001; Sung et al., 2000). However, we were interested whether mutant proteins still possess this function. Therefore, *Fmr1* KO neurons were transfected with several *FMR1* variants, followed by an *in situ* hybridization to study the subcellular localization of

FMR1 mRNAs. As expected, wild-type FMRP co-localized with its mRNA in RNA-granules as previously described (Antar et al., 2004). However, some granules were only positive for *FMR1* mRNA and not for FMRP. This suggests that *FMR1* mRNA can also bind to other mRNA-binding proteins to be incorporated in RNA-granules and transported into the dendrite.

Furthermore, we studied *FMR1* mRNA localization of the mutant *FMR1_I304N*. The mutation is located in the second KH-domain, which is involved in binding of mRNAs with a sequence-specific element in a complex tertiary structure termed the FMRP kissing complex (Darnell et al., 2005). *FMR1-I304N* mRNA was also diffusely localized in the dendrites, like FMRP_I304N. Wang *et al* showed with propidium iodide, which stains all mRNAs, that mutant FMRP-I304N co-localizes with mRNAs, suggesting that it still has the property to bind mRNAs (Wang et al., 2008). In contrast, Siomi *et al* showed that FMRP_I304N has impaired mRNA binding properties (Siomi et al., 1994). Our results suggest that FMRP_I304N can still bind its own mRNA but that the protein/RNA complex has lost the property to be incorporated in RNA-granules.

We also studied the *FMR1_Iso12* mRNA localization. The protein lacks the NES in exon 14 and due to a frameshift mutation the protein also does not contain exon 15 to 17. However, this frameshift does not hamper the G-quartet structure in the mRNA. *FMR1_Iso12* mRNA is found in the cell soma of neurons, but was hardly detected in dendritic RNA-granules. This suggests that *FMR1_Iso12* mRNA incorporation in dendritic RNA-granules is very inefficient or delayed, probably due to accumulation of EGFP-FMRP_Iso12 in the nucleus that would otherwise transport the mRNA. Interestingly, when both variants were co-transfected in neurons, dendritic RNA-granules that are positive for both EGFP-FMRP_Iso12 and EGFP-FMRP_I304N also showed the incorporation of *FMR1* mRNA.

In a similar fashion, FXR2P was able to recruit not only the FMRP variants in dendritic RNA-granules, but also their corresponding mRNAs. Unfortunately, we could not discriminate whether FXR2P or the FMRP variant is able to bind to the *FMR1* mRNA. However, our results suggest that FXR2P interacts with *FMR1* mRNA in neurons.

Finally, the *FMR1_ΔG1+2 in situ* hybridization experiments showed that *FMR1-ΔG1+2* mRNA co-localize with FMRP-positive RNA-granules and was still transported into the dendrite. Recently it has been reported that this mutant *FMR1_ΔG1+2* mRNA has a disrupted binding to FMRP, but that the mRNA is normally localized in HeLa cells (Didiot et al., 2008). Our results suggest that *FMR1-ΔG1+2* mRNA can still be transported into the dendrite and therefore the G-quartet structure is not essential for incorporation of *FMR1-ΔG1+2* mRNA in dendritic RNA-granules.

In conclusion, we show that the KH2-domain and the C-terminal part of FMRP are important for *FMR1* mRNA transport from the nucleus into dendritic RNA granules in primary hippocampal neurons. Moreover, mutations in these domains can be functionally overcome by co-expression of wild type FMRP or FXR2P. This suggests that hetero-dimers of mutant and wild type FMRP or FXR2P are functionally formed in these neurons. Finally, the G-quartet RNA structure was found not to be necessary for the dendritic transport of *FMR1* mRNA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Schematic overview of constructs used for transfection experiments. All constructs, except *FMR1_G-quartet*, are driven by the chicken β-actin-promoter to promote neuronal expression. The *EGFP-FMR1_I304N* has a pathogenic point mutation in the second KH domain. The *EGFP-FMR1_Iso12* is a naturally occurring splice variant lacking exon 12 and 14 and the C-terminal part of the protein is frame shifted due to alternate splicing. The *FMR1_G-quartet* construct harbors silent point mutations in the mRNA resulting in disruption of the G-quartet structure of *FMR1* mRNA without affecting the amino acid sequence of FMRP. Finally, a construct encoding FXR2p fused to EGFP was used. For several constructs, both *mCherry* and *EGFP* fusion constructs were developed for co-transfection experiments. No differences between EGFP or mCherry coupled fusion proteins were observed.

Figure 2. Localization patterns of different FMRP constructs in *Fmr1* KO hippocampal neurons. Wild-type mCherry-FMRP (a) showed a granular pattern in the dendrite. In contrast, EGFP-FMRP_I304N (b) and EGFP-FMRP_iso12 (c) showed a more diffuse dendritic pattern. Moreover, EGFP-FMRP_iso12 showed accumulation of the protein in the nucleus.

Figure 3.

Quantification of FMRP-positive granules in dendrites of transfected *Fmr1* KO neurons. *Fmr1* KO neurons transfected with wild-type *FMRI* showed 5 granules per 20 μ m, while neurons transfected with *Cherry-FMRI_I304N* and *EGFP-FMRI_Iso12* show significantly less granules per 20 μ m ($P < 0.05$). *EGFP-FMRI_I304N* or *EGFP-FMRI_Iso12* co-transfection with wild-type *Cherry-FMRI* revealed more dendritic granules than with either one of the protein variant alone, similar to wild-type levels (counted number of granules containing the variant) ($P < 0.05$). Surprisingly, when mCherry-FMRP_I304N and EGFP-FMRP_Iso12 were co-expressed, both proteins were also incorporated in granules and the number of RNA-granules positive for both variants was not significantly different from the number of granules after wild-type *Cherry-FMRI* transfection.

Figure 4.

Localization patterns of FMRP variants and mCherry-FMRP after co-transfection in *Fmr1* KO hippocampal neurons. (a) *EGFP-FMRP_I304N* and *Cherry-FMRP* co-transfection resulted in co-localization of the two proteins in dendritic RNA-granules. (b) *EGFP-FMRP_Iso12* also co-localized with wild-type mCherry-FMRP and was incorporated in RNA-granules. Although *EGFP-FMRP_Iso12* was incorporated in dendritic granules, some *EGFP-FMRP_Iso12* remained in the nucleus. (c) Localization patterns of *EGFP-FMRP_Iso12* and mCherry-FMRP_I304N after co-transfection in *Fmr1* KO hippocampal neurons. *EGFP-FMRP_Iso12* co-localized with mCherry-FMRP_I304N and both proteins were incorporated in RNA-granules. Although *EGFP-FMRP_Iso12* was incorporated in dendritic granules, a small proportion was still present in the nucleus.

Figure 5.

(a) *Fmr1* KO neurons co-transfected with β actin-*Cherry* and *EGFP-FXR2* showed cytoplasmic EGFP-FXR2P and EGFP-FXR2P-positive dendritic RNA-granules. Note that a few spines also contained EGFP-FXR2P (examples indicated by arrow). (b) *Fmr1* KO neurons co-transfected with *EGFP-FXR2* and wild-type *Cherry-FMR1* showed dendritic RNA-granules containing both proteins. Like FMRP, FXR2P was able to incorporate mCherry-FMRP_I304N (c) and EGFP-FMRP_Iso12 (d) in dendritic RNA-granules.

Figure 6.

In situ hybridization for *FMR1* on transfected *Fmr1* KO neurons. (a) In neurons transfected with *EGFP-FMR1*, *FMR1* mRNA was localized in dendritic RNA-granules co-localizing with EGFP-FMRP. (b) *FMR1_I304N* mRNA was more diffusely localized in the dendrite compared to wild-type *FMR1* mRNA. (c) *FMR1_Iso12* mRNA was mainly localized in the cell soma with only some RNA in RNA-granules distributed in the proximal dendrite. (d) *FMR1_Iso12* and *FMR1_I304N* mRNAs (both in red) were incorporated in dendritic RNA-granules in neurons co-transfected with both variants (both in green). (e) *EGFP-FMR1_ΔG1+2* mRNA was localized in dendritic RNA-granules co-localizing with EGFP-FMRP.

Figure 7.

In situ hybridization for *FMR1* mRNA after co-transfection of FMRP variants and FXR2P in *Fmr1* KO neurons. (a) Neurons co-transfected with *EGFP-FMR1_I304N* and *EGFP-FXR2* or (b) *EGFP-FMR1_Iso12* and *EGFP-FXR2* showed that *FMR1* mRNAs were incorporated in dendritic RNA-granules.