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The fragile X mental retardation protein–RNP granules show an mGluR-dependent localization in the post-synaptic spines

Francesca Ferrari,[a,1] Valentina Mercaldo,[a,b,1] Giovanni Piccoli,[c] Carlo Sala,[c] Stefano Cannata,[b] Tilmann Achsel,[a] and Claudia Bagni[a,b,*]

[a]*Fondazione Santa Lucia, Rome, Italy*
[b]*Dipartimento di Biologia, Università "Tor Vergata", Rome, Italy*
[c]*CNR, Istituto di Neuroscienze e Farmacologia, Milan, Italy*

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The localization of RNA/mRNA in dendrites plays a role in both local and temporal regulation of protein synthesis, which is required for certain forms of synaptic plasticity. A key molecule in these processes is the fragile X mental retardation protein (FMRP). Using in situ hybridization coupled to immunofluorescence confocal microscopy, we find that the FMRP–RNP particle contains *αCaMKII* and *BC1* RNAs as well as Staufen and CPEB proteins. Furthermore, following mGluR activation, the FMRP–mRNP complex moves into spines as shown by co-localization with the PSD-95 and Shank proteins. This study shows, for the first time, that the translationally inactive FMRP–mRNP complex relocates into neuronal spines after stimulation and that de novo protein synthesis mainly contributes to the pool of FMRP at synapses.

Keywords: Fragile X mental retardation protein; Spines; Dendritic mRNA; CPEB; Staufen; *BC1* RNA; *FMR1* mRNA; *αCaMKII* mRNA

Introduction

The fragile X Syndrome (FXS) is the most common form of inherited mental retardation. Almost all FXS cases are caused by expansion of a triplet repeat in the *FMR1* gene, which leads to hypermethylation of the promoter region, transcriptional silencing and loss of the fragile X mental retardation protein, FMRP (Sutcliffe et al., 1992). FMRP is an RNA-binding protein that plays an important role in mRNA localization and in the synaptic plasticity, which depends on local protein synthesis and in spine maturation. The syndrome is characterized by mental retardation, macroorchidism and behavioral abnormalities (recently reviewed in Bagni and Greenough, 2005). At the cellular level, the FXS phenotype includes defects in synaptic spine structure and function. FXS patients and the mouse model for the syndrome, *FMR1*-KO mice, display defects in dendritic spines which appear to be longer, thinner and more numerous compared to the control mice (Irwin et al., 2000; Restivo et al., 2005; Grossman et al., 2006). Fragile X features suggest that impaired regulation of mRNA localization and translation in FXS may affect spine pruning and/or maturation and stabilization.

At the molecular level, it is therefore of considerable interest to understand how the mRNAs that are required at synapses are selected by FMRP from the pool of mRNAs produced in the nucleus, how they are transported along the neuronal processes and how they are finally translated (apparently largely post-synaptically in spines and dendrites) in a regulated manner in response to pre-synaptic and other inputs.

FMRP target mRNAs encode proteins important for synaptic plasticity (Brown et al., 2001; Miyashiro et al., 2003; Zalfa et al., 2003; Lu et al., 2004) and FMRP-associated mRNAs display altered translational profiles in human FX lymphoblastoid cells and *FMR1*-KO mouse brain (Brown et al., 2001; Zalfa et al., 2003). The regulation of FMRP expression and localization is likely to be relevant for the response and strength of the synapse. Indeed, FMRP translation has been reported to increase in synaptoneurosomes after the activation of type-I metabotropic glutamate receptors (Weiler et al., 1997). Moreover, mGluR5 activation also increases the levels of dendritic FMRP and *FMR1* mRNA trafficking in cultured mature hippocampal neurons (Antar et al., 2004). It has also been reported that in the absence of FMRP, mGluR activation does not trigger incremental protein synthesis in synaptoneurosomes (Weiler et al., 2004).

GFP-FMRP travels along the neurites of PC12 cells in granules (De Diego Otero et al., 2002) that resemble mRNP transport granules. More recently, Antar and colleagues (2004) showed that endogenous FMRP has a similar dendritic pattern in hippocampal neurons and that microtubules are required for FMRP localization

* Corresponding author. Università "Tor Vergata", Via della Ricerca Scientifica 1, Rome, Italy. Fax: +39 06 2023500.
E-mail address: claudia.bagni@uniroma2.it (C. Bagni).
[1] These two authors equally contributed to the work.
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and mGluR-dependent transport (Antar et al., 2005). They have also reported that FMRP and *FMR1* mRNA are localized in the synaptic compartment (colocalization with synapsin) and that mGluR activation diminishes the localization of FMRP, but not of *FMR1* mRNA, at these sites (Antar et al., 2004). Using a biochemical approach, Kanai and collaborators (2004) isolated mRNP complexes (transport granules) that contain more than forty proteins including FMRP and some of its target mRNAs (*Arc* and *αCaMKII* mRNAs).

Using a highly specific antibody for FMRP, we present here a characterization of the FMRP–mRNP in primary neurons, during in vitro development, showing that FMRP is highly present at the branch points together with ribosomal components as well as with molecules involved in the regulated transport and translation of messenger RNAs. Finally, we show for the first time a co-localization of FMRP with mRNA/RNA (*αCaMKII* mRNA and the non-coding RNA *BC1*) that are part of the transport granules. Interestingly, after DHPG treatment, the FMRP–mRNP complex moves into the post-synaptic compartment, as shown by a co-localization with PSD-95 and Shank proteins supporting the activity-dependent function of the fragile X mental retardation protein in mediating mRNA translation at the spines. Finally, this effect is partially dependent on de novo protein synthesis.

Results

FMRP is present in specific subcellular neuronal compartment

In previous reports, it has been shown that neuronal FMRP is localized in proximal dendrites and in dendritic spines of pyramidal cells in the frontal cortex and in the CA1 area of the hippocampus as well as in synaptoneurosomes from occipital and parietal cortex (Feng et al., 1997; Weiler et al., 1997). Lately, a more detailed study was also performed in cultured hippocampal cells, showing a punctuate localization of FMRP (Antar et al., 2004). These experiments were performed using the monoclonal antibody 1C3 (Devys et al., 1993). This antibody robustly recognizes FMRP, however, it also reacts slightly with the two related proteins, FXR1P and FXR2P, as previously reported (Khandjian et al., 1998) because it was raised against the N-terminal region of the protein, which is highly homologous between the three FMRP/FXR proteins. Although the function of FXR1P and FXR2P have not been clarified yet, there are similarities among all three FMRP/FXR proteins in the gene structure, amino acid sequence, expression pattern in brain and in developing hippocampal neurons (Tamanini et al., 1997; Agulhon et al., 1999; Bakker et al., 2000). Interestingly, *FMR1/FXR2* double-KO mice have an exaggerated behavioral phenotype in open-field activity, prepulse inhibition of acoustic startle response and contextual fear conditioning when compared with *FMR1*-KO mice, *FXR2*-KO mice or wild-type littermates (Spencer et al., 2006). These findings suggest that *FMR1* and *FXR2* genes contribute in a cooperative manner to pathways controlling mice behavior.

We decided to examine the endogenous distribution of FMRP in single neuronal cells, using specific, polyclonal antibodies (called rAM2). The C-terminal region of the human FMRP (a peptide spanning amino acid 516 to amino acid 632 of the human FMRP) was used for the production of the rabbit antibodies employed in these studies. As reported in Fig. 1A, out of 117 amino acids only 21 are conserved among the three proteins, making a cross reactivity with FXR1P and FXR2P very unlikely. The antibodies recognize three FMRP isoforms in mouse brain extracts while they do not recognize any protein in the *FMR1*-KO mice (Fig. 1B, right panel). Instead, the monoclonal antibody 1C3 very weakly recognizes some proteins also in the *FMR1*-KO mice, possibly the FMRP-related protein(s) (Fig. 1B, left panel). Specificity of rAM2 antibodies was also documented by immunostaining of wild-type and *FMR1*-KO mouse brain sections (Fig. 1C); as expected, the *FMR1*-KO mouse exhibited no staining (right panel) while a clear labeling is observed in the wild-type mouse (left panel).

To further characterize the rAM2 antibodies, we transfected COS cells with a full-length *FMR1* cDNA (ISO7) and a deleted *FMR1* cDNA (ISO4), which does not contain the C-terminus of FMRP (Fig. 1D). rAM2 antibodies show a high specificity for the C-terminal part of the protein detecting only the full-length protein (Fig. 1D, lane 1) and the C-terminus produced in *E. coli* (Fig. 1D, lane 3). The monoclonal antibody 1C3, recognizing the N-terminus of the protein, can detect both ISO7 and ISO4 (Fig. 1D, lanes 4 and 5).

To gain more insights on FMRP function during brain development, we investigated the level of expression during in vitro development of hippocampal and cortical neurons (Fig. 1E). Performing Western blot analysis from cortical and hippocampal neurons, we observed that FMRP expression level decreases during in vitro development (from 7 to 15 days in vitro, DIV) and that the basal level of expression is higher in cortical compared to hippocampal neurons. A two-way ANOVA with brain area (between factor) and time in culture (within factor) as main factors show a significant "brain area" ($F_{1,4}=3.35$) × "time in culture" ($F_{1,4}=20.77$; $p<0.05$) interaction ($F_{1,4}=9.70$; $p<0.05$). Subsequent pair-wise comparison revealed a significant difference in FMRP expression level (post hoc Tukey, $p<0.05$) between cortex and hippocampus at 7 DIV and within cortex during time in culture (Fig. 1E). The results are quantified in Fig. 1E, right panel.

Immunofluorescence for FMRP in cultured hippocampal neurons using 1C3 and rAM2 antibodies show no major differences, although a granular pattern is more evident using rAM2 antibodies (Fig. 1F).

The subcellular distribution of FMRP in neurons was analyzed in hippocampal neurons isolated from embryonic stage 19 (E19) and cortical neurons derived from embryonic stage 15 (E15) during in vitro development. In double immunolabeling with antibodies directed against MAP2 (Fig. 2A, b, e, h), a specific dendritic marker (Blichenberg et al., 1999; Aronov et al., 2002), Tau (Fig. 2A, k–n) an axonal marker (Binder et al., 1985) and FMRP–rAM2 (Fig. 2A, panels a, d, g, j, m) showed that FMRP immunoreactivity is mainly cytoplasmatic but is also localized along the dendrites in a punctuate pattern and in the axon (Fig. 2A, panel j) of hippocampal cells as recently reported by Antar and colleagues (2006). The expression of FMRP in axonal processes is also showed in hippocampal brain sections of the CA1 region (Fig. 2A, panels m–o).

As indicated by the arrows, in both cell cultures and brain sections, FMRP shows a very unique and interesting pattern (Fig. 2A, a, g, m). The protein is present in high concentrations at the branching points of the dendrites where it forms bigger granular structures reminiscent of ribonucleoparticles, a macromolecular structure that functions as motile unit for the translocation of

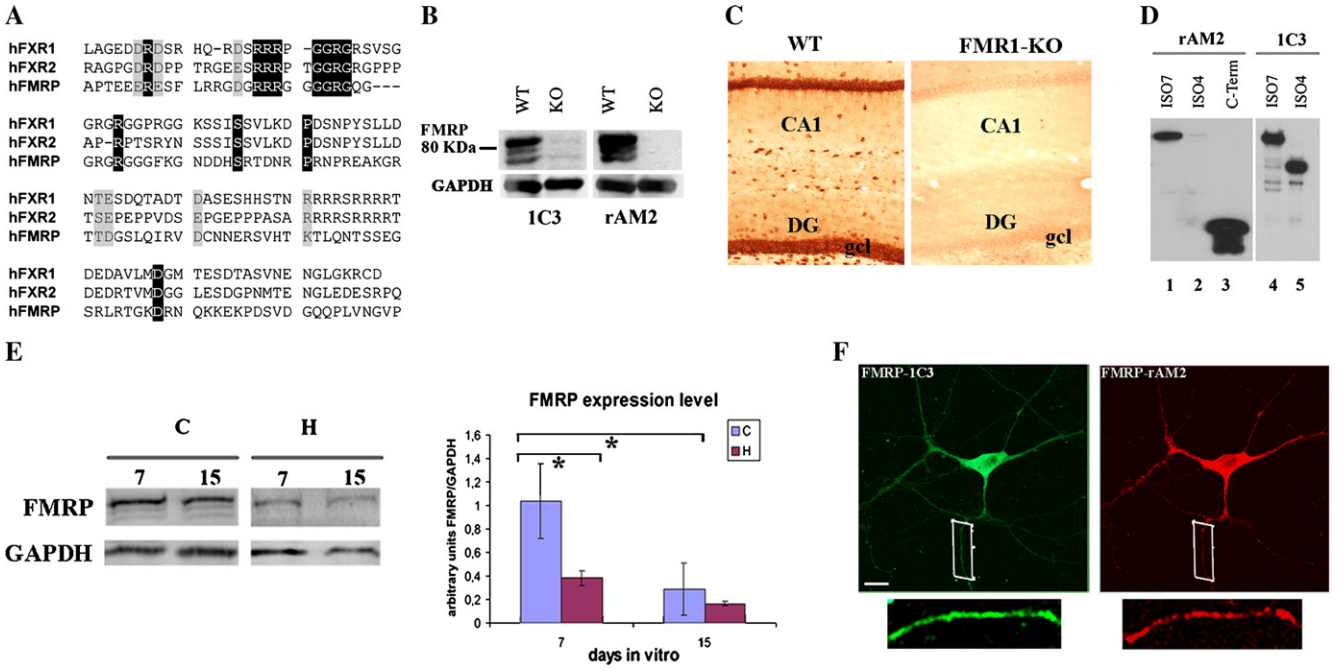


Fig. 1. FMRP expression in neuronal cells during in vitro development. (A) Comparison of the C-terminus of hFMRP (aa 516–632) with the respective regions of hFXR1 and hFXR2. The sequences were aligned by the ClustalW algorithm; identical residues are boxed in black, conserved residues in grey. Note that the C-terminus of hFXR2 contains another 90 aa that do not align: these residues are not shown. The overall identity in the domain shown here is 18%. (B) Proteins from WT and *FMR1*-KO total brain extracts were analyzed with 1C3 and rAM2 antibodies. (C) Immunohistochemistry performed with rAM2 antibodies on WT and *FMR1*-KO hippocampal brain sections. Abbreviations: CA1 = area of Ammon's horn; DG = dentate gyrus; gcl = granule cell layer. (D) COS cells were transfected with full-length *FMR1* (ISO7, lane 1 and 4) or *FMR1* lacking the C-terminus (ISO4, lane 2 and 5) and extracts analyzed with rAM2 and 1C3. Lane 3 shows the recombinant FMRP fragment used for the rAM2 antibody production. (E) Left panel, FMRP and GAPDH proteins were detected in cortical (C) and hippocampal (H) cells cultured for 7 and 15 days. GAPDH has been used as loading control. Right panel, a graph representing a quantification of FMRP expression levels in cortical and hippocampal neurons at 7–15 DIV after normalization for GAPDH and expressed as arbitrary units. Histograms indicate mean± SEM of experiments performed on three independent cortical and hippocampal cultures. $*p<0.05$. (F) Immunofluorescence of an hippocampal neuron labeled with 1C3 (green) and rAM2 (red) antibodies. The scale bar indicates 10 μm.

mRNAs to particular subcellular domains. This peculiar distribution is present in both hippocampal (Fig. 2A, a–f) and cortical (Fig. 2A, g–i) neurons as pointed by the arrows (Fig. 2A, panels a, c, g, i). Finally FMRP co-localizes also with the ribosomal protein S6 (Fig. 2B, a–c), in agreement with their partial co-sedimentation on a sucrose gradient (Zalfa et al., 2003) and co-localization with the ribosomal RNA (Antar et al., 2005).

FMRP biochemically co-fractionates with Staufen

To investigate the level of association of FMRP with other RNA-binding proteins involved in mRNA transport and translation such as Staufen, cultured hippocampal neurons were extracted at 18–20 DIV while still attached to the Petri dishes in several steps with detergents of increasing strength, namely Digitonin, Triton X-100 and Tween-40 plus Deoxycholate (Allison et al., 1998). As shown in Fig. 3A, most of MAPK and the free part of β-tubulin became soluble when neurons were extracted with Digitonin. Instead, both FMRP and Staufen proteins are mostly concentrated in the same fraction that is solubilized by Triton X-100 and are therefore less soluble than cytosolic MAPK or free β-tubulin. The results obtained by Western blot analysis from several independent experiments are quantified in Fig. 3A, right panel. Moreover, in neurons, Staufen and FMRP (Fig. 3B) show the same subcellular distribution and partially co-localize in the cell body and dendrites of cortical neurons, forming dendritic granular structures as highlighted by arrows in panels c and d. The FMRP–Staufen co-localization and solubility to Triton X-100 is consistent with their mild association to cytoskeleton and their movement in granules along microtubules (Antar et al., 2005; Johnson et al., 2006).

FMR1 mRNA and FMRP are assembled into transport ribonucleoprotein complexes

In neurons, most of the mRNAs present in the cell are localized in the soma, but a subset of mRNAs is transported to dendrites as granules composed of mRNAs/RNA and proteins that move along microtubules (Steward and Schuman, 2003).

Here we analyzed the nature of the FMRP–RNP complex using in situ hybridization coupled to confocal microscopy in neuronal cells. We observed that *FMR1* mRNA co-localizes with Staufen (Fig. 4, panels a–d), CPEB, cytoplasmic polyadenylation element-binding protein (Fig. 4, panels e–h) and FMRP (Fig. 4, panels i–l) in cell bodies and dendrites of hippocampal neurons (the co-localization in the dendrites is highlighted by arrows in the details, panels d, h, l). Interestingly, we have found a putative CPE element in the 3′UTR of the mouse and human *FMR1* mRNA (Carosi and Bagni, unpublished). As it has been previously shown that FMRP regulates *αCaMKII* mRNA translation in synaptoneurosomes (Zalfa

et al., 2003), we next investigated the localization of the *αCaMKII* mRNA-FMRP complex in neurons. As shown in Fig. 4 (panels m–t), *αCaMKII* mRNA is present in both Staufen (Fig. 4, panels m–p, see arrows in panel p) and CPEB (Fig. 4, panels q–t, see arrows in panel t) containing granules in cell bodies and proximal/distal dendrites, confirming the co-localization of CPEB and *αCaMKII* mRNA (Huang et al., 2003) as well as the presence of *αCaMKII* mRNA and Staufen in the same granules (Kanai et al., 2004). Finally, to investigate if some of the FMRP-containing granules may be translationally silent, we examined its co-localization with the small non-coding RNA *BC1* (Fig. 4, panels u–x) previously shown to be a translational repressor in vitro (Wang et al., 2002) and acting together with FMRP to repress translation in vivo (Zalfa et al., 2003; Johnson et al., 2006). As shown in Fig. 4 (panels u–x), there is a high degree of co-localization between FMRP and *BC1* RNA in granular structures indicating that FMRP and *BC1* RNA are present in the same particles.

The specificity of the dendritic localization of *FMR1*, *αCaMKII* mRNAs and *BC1* RNA is demonstrated by the detection of an mRNA restricted to the cell body such as *α-tubulin* (Fig. 4, panels y, z).

mGluR-driven synaptic stimulation leads to a localization of FMRP–mRNP into the post-synaptic spines

Spine abnormalities (Bagni and Greenough, 2005 and references therein) and alterations of synaptic plasticity (Huber et al., 2002; Li et al., 2002; Chuang et al., 2005; Giuffrida et al., 2005; Larson et al., 2005; Zhao et al., 2005), which are both features present in fragile X patients as well as in *FMR1*-KO mice, indicate a possible role for FMRP in the modulation of synaptic activity. An activity-dependent re-localization of FMRP at synaptic boutons has been previously reported (Antar et al., 2004). To investigate the localization of FMRP in the post-synaptic compartment after stimulation, we performed double immunostaining for FMRP/PSD-95 (Fig. 5A, panels a–h) and FMRP/Shank (Fig. 5A, panels i–p) in control and DHPG-treated neuronal cells to visualize the response of FMRP to DHPG, a specific agonist of group I mGluR (Huber et al., 2002; Antar et al., 2004; Hou et al., 2006). As shown in Fig. 5, in basal conditions, the majority of FMRP is present along the dendrites and only partially co-localizes with PSD-95 and Shank (Fig. 5A, panels c, d; k, l) because it is mainly absent from post-synaptic sites (as pointed by arrows in panels d and l). After DHPG treatment, FMRP moves into the post-synaptic compartment, near the post-synaptic density and therefore partially co-localizes with PSD-95 and Shank as evident from the enlargement of the merged image (Fig. 5A, panels h and p, see arrows). Quantification of the DHPG effect on FMRP post-synaptic localization is reported as a graph in Fig. 5B (left panel): there is a significant ($p<0.001$) increase of FMRP at the synapses, while the post-synaptic localization of Shank essentially does not change. Evaluation of the FMRP/PSD-95-labeled micrographs gives the same result (data not shown). Finally, to address if the increase in FMRP immunoreactivity in spines following DHPG treatment was due to translation of *FMR1* mRNA, which has been detected in both dendrites (Antar et al., 2004) and synaptoneurosomes (Zalfa et al., 2003), neuronal cells were stimulated in presence and absence of cycloheximide (60 μM; 30 min) before DHPG treatment. Interestingly, as shown in Fig. 5B (left panel), the majority of FMRP that moves into the spine after synaptic stimulation comes from contribution of de novo protein synthesis (one-way ANOVA, $F_{2,57}=58.89$; $p<0.001$) although a minor but significant contribution is due to the pool of pre-existing FMRP ($p<0.05$). Furthermore, as shown in Fig. 5B (right panel), the total amount of FMRP is not affected by DHPG and cycloheximide treatments in agreement with the slow turnover of FMRP (estimated to be around 30 h: Ceman et al., 2003).

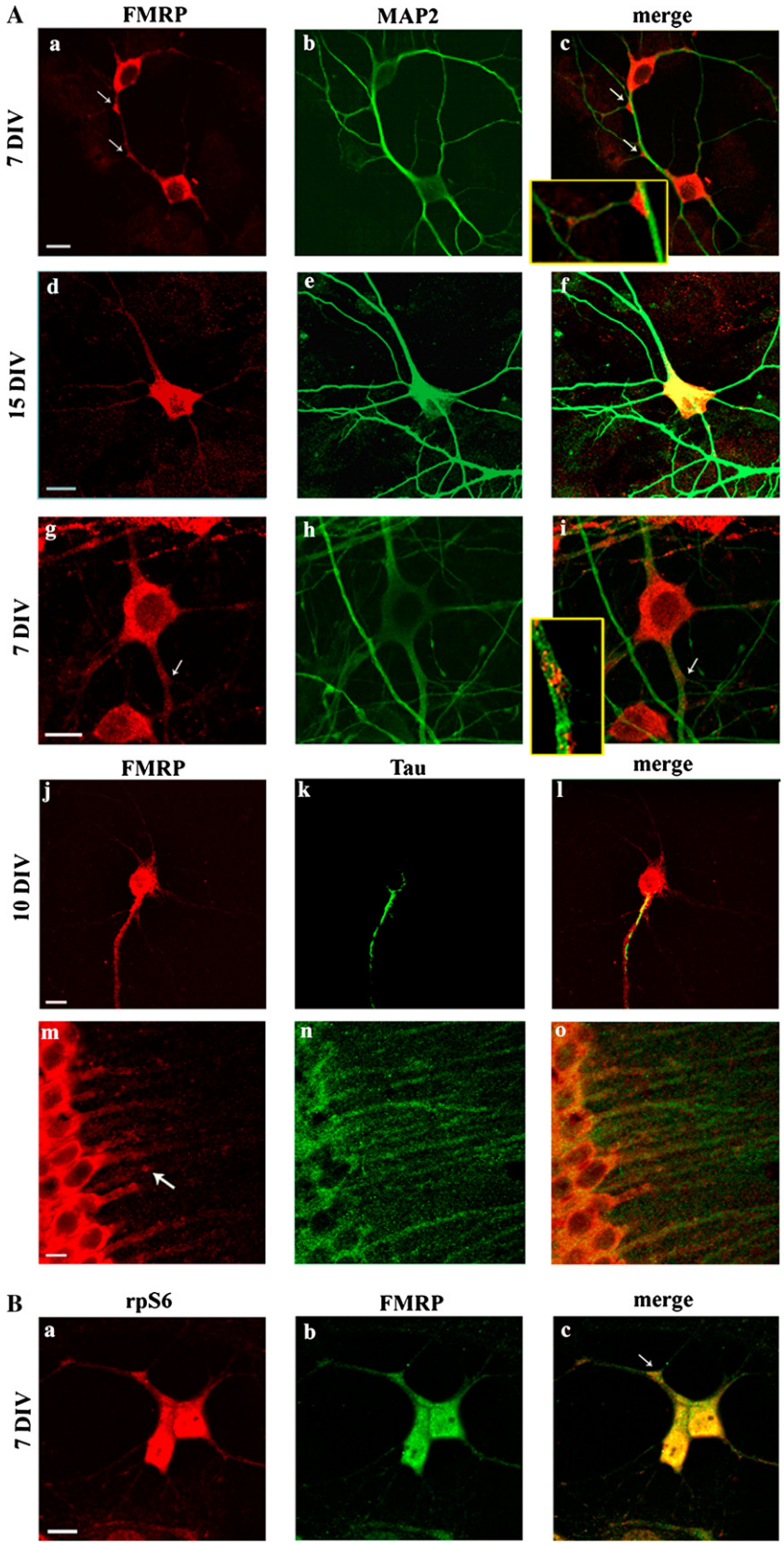
To further investigate if the distribution of FMRP–mRNP complexes in neurons would be affected by synaptic stimulation, hippocampal neurons were stimulated with DHPG and *αCaMKII* mRNA, one of the mRNAs regulated by FMRP in dendrites (Zalfa et al., 2003; Hou et al., 2006), was detected together with FMRP and Shank. In control conditions, *αCaMKII* mRNA co-localizes with FMRP in the cell body and in dendrites (Fig. 6, panel d). DHPG treatment causes a re-localization of the FMRP–*αCaMKII* mRNA complex into the post-synaptic compartment (as pointed by arrows in Fig. 6, panel h) suggesting that synaptic activity recruits FMRP–mRNPs complexes into the spines.

Discussion

In the present study, we have better characterized the subcellular distribution of the fragile X mental retardation protein FMRP and showed a re-localization of the FMRP–*αCaMKII* mRNA after synaptic stimulation in neuronal cell cultures. Previous studies were performed with the monoclonal antibody 1C3 directed against the N-terminus of the protein (Devys et al., 1993) and demonstrated a dendritic localization of FMRP in mouse brain sections (Feng et al., 1997; Weiler et al., 1997) and in primary hippocampal neurons (Ohashi et al., 2002; Antar et al., 2004). Here, we used polyclonal antibodies produced in our laboratory that are directed against the C-terminus of FMRP. In contrast to the N-terminus, this domain diverges considerably between the proteins of the FXR family (Fig. 1A), and the resulting antibodies are thus monospecific (Fig. 1B). Using this antibody provided by our laboratory, Villace and colleagues (2004) showed that FMRP is present in the neurites of differentiated neuroblastoma cells. We now confirmed and fine-mapped the dendritic localization of FMRP in primary cell culture.

FMRP expression levels decrease during in vitro development of the neurons (between 7 and 15 DIV, Fig. 1E), and the basal expression level is significantly higher in cortical compared to hippocampal neurons, reflecting basal differences in the physiol-

Fig. 2. Subcellular distribution of FMRP in neurons. (A) Double immunostaining for FMRP (a, d, g) and MAP2 (b, e, h) in hippocampal (a–f) and cortical (g–i) neurons, during in vitro development, at 7 DIV (a–c; g–i) and 15 DIV (d–f). Panels c, f and i represent merged images and (c, i) contain enlargements of the regions pointed by arrows (upper arrow for panel c). In panels a, c, g and i, arrows point to a granular localization of FMRP at the branch points. Panels j, k and l represent double immunostaining of hippocampal cells at 10 DIV for FMRP (j) and Tau (k), an axonal marker. Panel l shows the merged image. Panels m–o show double immunostaining of FMRP (m) and Tau (n) in hippocampal brain sections. Panel o is merged image. (B) Double immunostaining for the ribosomal protein S6 (rp-S6, a) and FMRP (b) in cortical cells cultured for 1 week. Panel c is merged image. The arrow points the co-localization between FMRP and rp-S6. The scale bars indicate 10 μm.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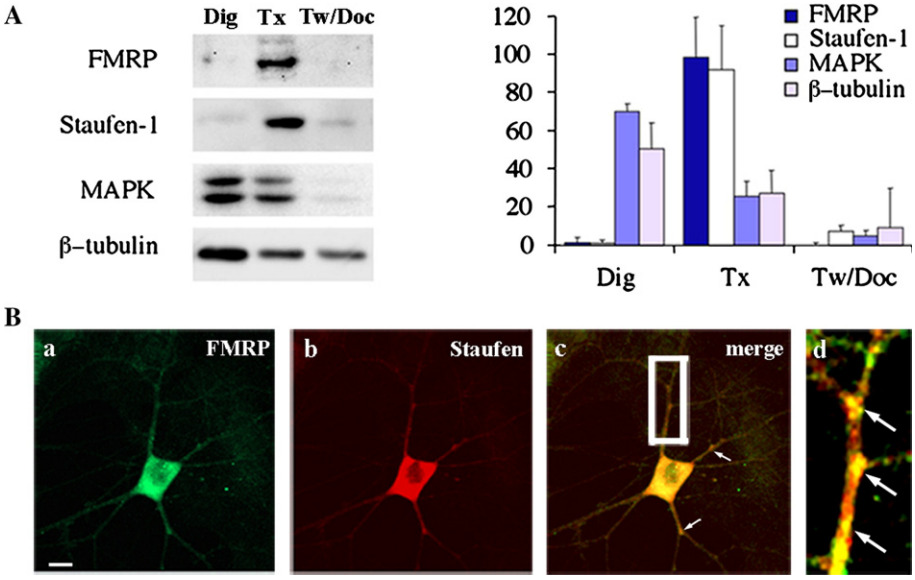

Fig. 3. Biochemical association of FMRP with components of the cytoskeleton. (A) Left panel, Western blot of protein fractions obtained after incubation with buffer containing different detergents as indicated on top of each column; antibodies used for the Western blot are indicated on the left side of each panel. Dig, Digitonin; Tx, Triton X-100; Tw/Doc, Tween-40 plus Deoxycholate. Right panel, histograms indicate mean±SD of percent of total distribution of the indicated proteins among each fraction. Quantification refers to four independent fractionation experiments. (B) Immunofluorescence of Staufen (b) and FMRP (a, monoclonal 1C3). Panel d is an enlargement of the merged panel c. Arrows point to regions of co-localization. The scale bar indicates 10 μm.

ogy of cells that belong to specialized areas of the brain. Indeed, in vivo alterations of long-term potentiation have been reported in the cortex but not in the hippocampus of *FMR1*-KO mice (Li et al., 2002; Larson et al., 2005) confirming a different role in these two brain areas.

The developmental change in culture (decreasing level of FMRP) is best explained by different requirements for FMRP activity once the dendrites have branched out and the main synaptic network has formed.

In high-resolution images, we observed a concentration of granular FMRP structures at the branch points (Fig. 2). It is tempting to hypothesize that FMRP could control actin remodeling by regulating synthesis of proteins related to actin at specific locations such as the branch points. Indeed, two of the mRNA targets of mammalian FMRP, *ArgBP2* (Brown et al., 2001) and *MAP1b* (Zalfa et al., 2003; Lu et al., 2004; Hou et al., 2006), encode proteins that are responsible for actin cytoskeleton organization (Kioka et al., 2002; Cestra et al., 2005) and microtubule/actin microfilament polymerization and dynamics (Gonzalez-Billault et al., 2004), respectively. Furthermore, it has been shown that FMRP negatively regulates profilin protein expression in *Drosophila* (Reeve et al., 2005) and interferes with Rac1 pathway in FMRP-deficient murine fibroblasts, where the level of phospho-ADF/Cofilin, a major mediator of Rac1-dependent actin remodeling, is reduced (Castets et al., 2005).

Overall, these data support the interaction between FMRP and actin cytoskeleton dynamics and are in agreement with the abnormal spine morphology observed in *FMR1*-KO mice.

The presence of FMRP in granules of different sizes prompted us to investigate their possible role in mRNA trafficking. First we looked at its association with the cytoskeleton. Because one of the best-characterized proteins involved in neuronal transport is Staufen, we fractionated the cells and analyzed the distribution of FMRP and Staufen. FMRP and Staufen are both concentrated in the same fraction that is solubilized by Triton X-100 and contains protein complexes mildly associated with the cytoskeleton (Fig. 3A). This co-fractionation agrees with the detection of Staufen and FMRP in the same macromolecular complex that also contains other proteins involved in translational control, such as EF-1α (elongation factor 1α), FXR1P, FXR2P and Purα (Kanai et al., 2004). Staufen is directly responsible for the delivery of RNA to the dendrites (Tang et al., 2001). The presence of Staufen and FMRP in the same particle therefore indicates that the same granules regulate both RNA localization and activity-dependent translation within dendrites (Krichevsky and Kosik, 2001; Weiler et al., 2004).

Once localized, the translationally silent RNA granules (Kohrmann et al., 1999; Kiebler and DesGroseillers, 2000; Krichevsky and Kosik, 2001; Kindler and Monshausen, 2002; Kanai et al., 2004) are local storage compartments that can rapidly shift, in response to stimulation, from the silent granule fraction to the actively translated polysomes (Krichevsky and Kosik, 2001). FMRP co-localization with the ribosomal protein S6 (Fig. 2B), particularly pronounced at dendritic branch points (Fig. 2B), could assure a rapid shift of mRNAs between mRNPs and polysomes in response to stimuli, in line with the role of FMRP as translational regulator (Laggerbauer et al., 2001; Li et al., 2001; Schaeffer et al., 2001; Mazroui et al., 2002; Zalfa et al., 2003). Several papers have demonstrated that FMRP is involved in synaptic activity. It has been reported to be necessary for neurotransmitter-activated protein translation at synapses (Weiler et al., 2004) and for type-I metabotropic glutamate receptor-dependent increase of PSD-95 (Todd et al., 2003). Moreover, deletion of the *FMR1* gene leads to a reduced association of mGluR5 receptors with Homer proteins in the synaptic plasma membrane, together with reduced Homer

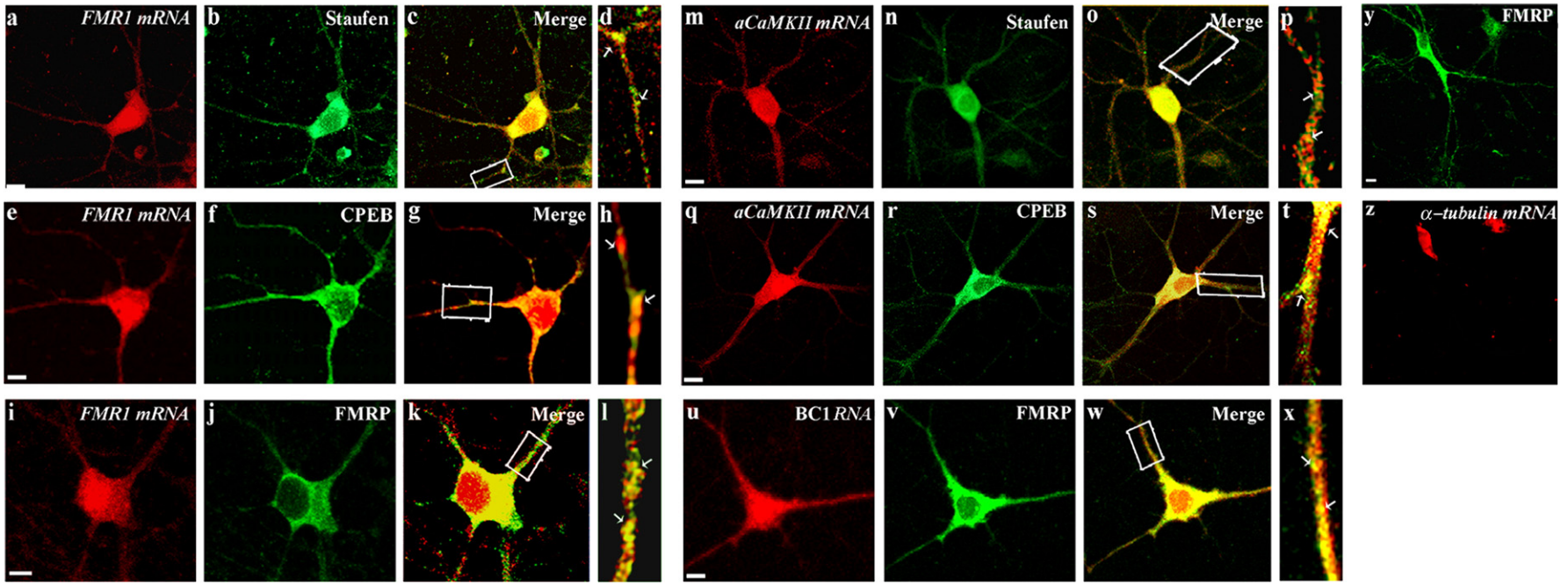

Fig. 4. Co-localization of RNA and proteins in putative translationally silent granules. In situ hybridization for *FMR1* mRNA (a, e, i) and immunofluorescence for Staufen (b), CPE binding protein (CPEB, f) and FMRP (j) in hippocampal neurons. Panels c, g and k are merged images. Panels d, h and l are enlargements of white rectangles in panels c, g and k, respectively. In situ hybridization for α*CaMKII* mRNA (m, q) and immunofluorescence for Staufen (n) and CPEB (r). Panels o, s are merged images. Panels p and t are enlargements of white rectangles in panels o and s, respectively. In situ hybridization for *BC1* RNA, (u) and immunostaining for FMRP (v) in hippocampal cells (10 DIV). Panel w is a merged image. Panel x is an enlargement of the white rectangle in panel w. Panels y and z show the immunostaining for FMRP and the in situ hybridization for α-*tubulin*, a non dendritically localized mRNA. Scale bars indicate 10 μm.

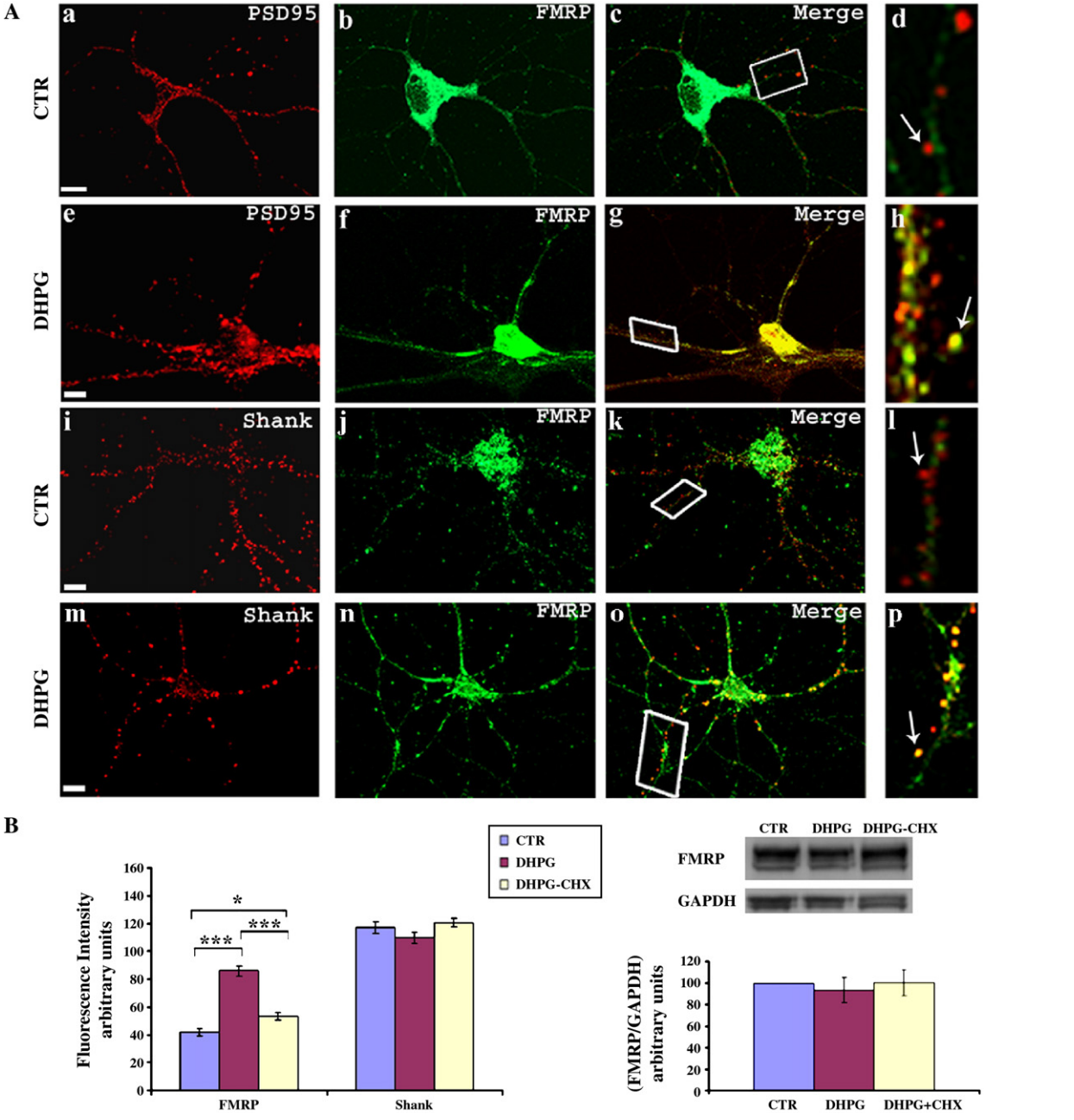

Fig. 5. Localization of FMRP–mRNP in activated spines. (A) Double immunostaining for PSD-95 (a, e)/FMRP (b, f) and Shank (i, m)/FMRP (j, n) in hippocampal neurons (10 DIV) in control conditions (a–d; i–l) and after 5 min of treatment with 100 μM DHPG (e–g; m–o). Panels c, g, k and o represent merged images. Panels d, h, l and p are enlargements of white rectangles in panels c, g, k and o, respectively. Co-localization of PSD-95/FMRP and Shank/FMRP inside the spines is pointed by arrows. The scale bars indicate 10 μm. (B) Left panel, the graph represents the quantification of the fluorescence intensity in Shank-positive domains in control (CTR), DHPG, DHPG + cycloheximide (CHX) conditions. Each histogram results from the average ± SEM of twenty cells (200 spines). *$p<0.05$; ***$p<0.001$. Right panel, Western blot for FMRP and GAPDH from untreated (CTR) and treated cell cultures (DHPG and DHPG + CHX). The graph represents a quantification of two independent treatments.

tyrosine phosphorylation (Giuffrida et al., 2005), indicating a strong link between FMRP and PSD proteins. These data point to an activity-dependent function of FMRP at the level of spines. Probably, stimulation of specific pathways recruits FMRP-containing granules to assure the local synthesis of proteins that are responsible for the activity-mediated reorganization of synapses. In agreement with this hypothesis, FMRP moves into the post-synaptic compartment after DHPG treatment and partially co-localizes with post-synaptic proteins (PSD-95 and Shank, Fig. 5) and with *αCaMKII* mRNA (Fig. 6). Further, we show that the FMRP that localizes in the spines comes mainly from de novo protein synthesis (Fig. 5B).

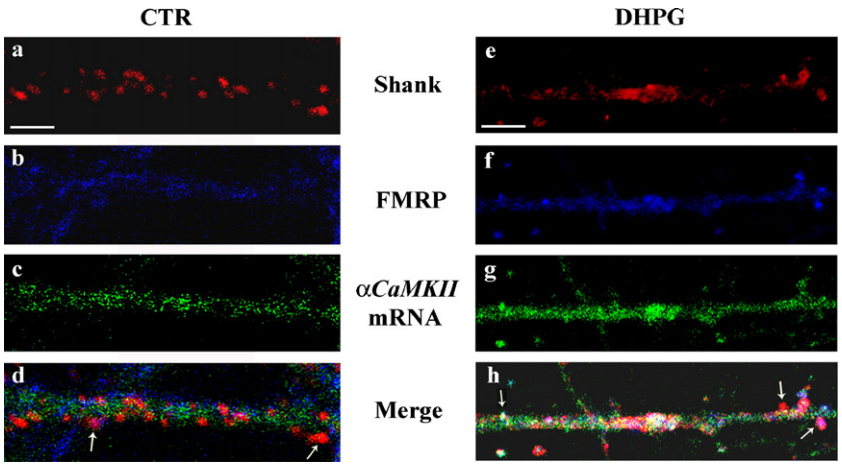

Fig. 6. Localization of the FMRP–*αCaMKII* mRNA complex in activated post-synaptic compartments. In situ hybridization for *αCaMKII* mRNA (c, g) and immunofluorescence for FMRP (b, f) and Shank (a, e) in hippocampal neurons (10 DIV) in control and after DHPG stimulation. Panels d and h are merged images. Arrows indicate the following: red = Shank puncta, pink = Shank + FMRP puncta, white = Shank + FMRP + *αCaMKII* mRNA puncta. The scale bars indicate 10 μm.

This phenomena indicates that synaptic activity triggers an increase of the FMRP-mRNP complexes in the spines. We believe that FMRP modifications such as phosphorylation (Ceman et al., 2003) or methylation (Dolzhanskaya et al., 2006; Stetler et al., 2006) will then release the translational inhibition, leading to the activity-dependent synaptic response.

Recently, Antar and collaborators (2004) showed a decrease in the content of FMRP granules in spines after DHPG treatment. The apparent discrepancy between our findings and those of Antar et al. can be due to several differences in these two studies. Most likely, the usage of different synaptic markers (Antar and colleagues used synapsin while we used Shank and PSD-95) changes the outcome. In the study published by Antar et al., the authors considered the percentage of synapsin puncta that had a detectable FMRP signal in them. In our study, we have used two post-synaptic markers, PSD-95 and Shank, since it has been shown that the co-localization of post-synaptic markers with the pre-synaptic ones is not 100% (Gerrow et al., 2006; Morita et al., 2006), there is a possibility that part of the FMRP present in the post-synaptic compartment has not been considered in the study from Antar and colleagues. Furthermore, since FMRP is present in both the pre and post-synaptic compartment (Feng et al., 1997; Antar et al., 2006), part of the FMRP leaving the spine after stimulation could be the one present in the pre-synaptic compartment.

Using in situ hybridization coupled with immunofluorescence, we showed that *FMR1* mRNA co-localizes with Staufen, CPEB and FMRP in hippocampal neurons (Fig. 4), indicating the presence of *FMR1* mRNA and CPEB protein in Staufen–FMRP-containing granules. CPEB recognizes cytoplasmic polyadenylation elements (CPEs). In the case of the *αCaMKII* mRNA, the CPE promotes cytoplasmic polyadenylation-induced translation in response to synaptic stimulation and also facilitates mRNA transport to dendrites (Huang et al., 2002, 2003). The co-localization of *αCaMKII* mRNA with Staufen and CPEB (Fig. 4) suggests the presence of *αCaMKII* mRNA/CPEB in Staufen-containing granules, which supports the notion that transport and translational control take place in the same particles (see above). *FMR1* mRNA also contains putative CPE elements in the 3′ untranslated region (Carosi and Bagni, unpublished observations), suggesting a possible interaction between the CPEB and *FMR1* mRNA. The notion that such a complex exists is supported by the ability of *FMR1* mRNA to respond to activity-dependent translation (Gabel et al., 2004; Weiler et al., 2004), similar to *αCaMKII* (Huang et al., 2002; Shin et al., 2004). Indeed, activation of NMDA and group I mGluR receptors causes phosphorylation of CPEB by Aurora kinase and therefore polyadenylation-induced translation of bound mRNAs (Huang et al., 2002; Shin et al., 2004) such as *αCaMKII* mRNA (Wu et al., 1998) and the tissue plasminogen activator (*tPA*), a secreted protease required for some forms of long-term synaptic plasticity (Shin et al., 2004).

To further characterize the nature of the FMRP–mRNPs, we performed experiments to visualize *BC1* RNA/FMRP (Fig. 4). The presence of *BC1* RNA in FMRP ribonucleoparticles has been reported in a previous study (Zalfa et al., 2003) and recently by Johnson and colleagues (Johnson et al., 2006). Using double-RNA immunoprecipitation from brain extracts, Johnson and colleagues found Purα, a protein that controls *BC1* RNA expression and distribution within dendrites (Kobayashi et al., 2000; Ohashi et al., 2000), together with Staufen or FMRP on *BC1* RNA together with specific neuronal mRNAs. We therefore decided to investigate the co-localization of FMRP with *BC1* RNA in neuronal cells. As shown in Fig. 4, the two molecules co-localize in the cell body as well as in dendrites, in agreement with previous papers reporting the interaction between FMRP and *BC1* RNA (Zalfa et al., 2003, 2005; Gabus et al., 2004; Johnson et al., 2006).

Experimental methods

Animal treatment

Animal care was conducted conforming to the institutional guidelines that are in compliance with national (DL N116, GU, suppl. 40, 18-2-1992) and international laws and policies (European Community Council Directive 86/609, Oja L 358, 1, December 12, 1987; National Institute of Health Guide for the Care and Use of Laboratory Animals, US National Research Council, 1996).

Antibody production

Rabbits were immunized against the human C-Terminus of FMRP. The DNA plasmid containing the human FMRP C-terminus fragment (nt 1545–1899) was a generous gift of Salvatore Adinolfi (MRC, London). The construct was expressed in *E. coli* strain BL21 (DE3). Serum was affinity purified using a HiTrap NHS-activated HP column (Amersham Pharmacia Biotech) conjugated to the C-Terminus.

Hippocampal/cortical neuron cultures and drug treatments

Hippocampal and cortical neuron cultures were prepared from embryonic day (E) E19 and E15 mouse, respectively. The brains were removed, neocortices or hippocampi were freed of meninges, treated with 0.025% trypsin, minced and plated on poly-L-lysine (Sigma-Aldrich) wells in MEM supplemented with 5% FBS, 5% HS, 25 mM glucose and 2 mM glutamine. The medium was replaced 24 h later with neurobasal supplemented with 2% B27, 1 mM glutamine and 100 μg/ml gentamicin (GIBCO BRL) and cells were maintained at 37 °C and 5% CO2. Cells were treated at 10 DIV before fixation with 100 μM (*S*)-3,5-dihydroxyphenylglycine for 5 min (DHPG; Sigma-Aldrich), a specific agonist of group 1 mGluR (Huber et al., 2002; Antar et al., 2004; Hou et al., 2006). Cycloheximide (60 μM) was added to the cultures for 30 min before DHPG treatment.

Preparation of cRNA probes

RNA in situ hybridization experiments were performed using four different mouse probes (*FMR1*-3′UTR, α*CaMKII*-coding, α-*tubulin*-coding and entire *BC1*) in both sense and antisense orientations. The primers used to clone the partial cDNAs from total brain RNA are:

FMR1-3′UTR up: 5′-GGT AAA GAT CGT AAC CAG AAG-3′
FMR1-3′UTR down: 5′-CAA GTA CAT CAG AGG CAG AAC-3′
α*CaMKII* up: 5′-GGG AGC CAT CCT CAC CAC TAT GCT GG-3′
α*CaMKII* down: 5′-AGA GAG CAG GGA CCC TGG CCT GGT CC-3′
α-*Tubulin* up: 5′-TTT TCC ACA GCT TTG GTG GGG G-3′
α-*Tubulin* down: 5′-TCT TGA TGG TGG CAA TGG CAG-3′
BC1 up: 5′-GTT GGG GAT TTA GCT CAG TGG-3′
BC1 down: 5′-AGG TTG TGT GTG CCA GTT ACC-3′

The cDNAs, except for the α*CaMKII* fragment, were cloned into the pGemTeasy vector and linearized with *Sac*II or with *Sac*I and transcribed with SP6 or T7 polymerases for antisense or sense Cy5-labeled cRNA probes, respectively. The cDNA for α*CaMKII* was cloned into the pBlueScript vector.

FISH and immunofluorescence

For FISH, hippocampal and cortical neurons were fixed at room temperature for 15 min with 4% paraformaldehyde, 2 mM $MgCl_2$, 5 mM EGTA in PBS 1× or 100% methanol. Coverslips were UV irradiated and cells permeabilized in PBS–Triton X-100. Neurons were prehybridized in 50% formamide, 2× SSC, 10 mM NaH_2P0_4 and hybridized overnight at 42 °C in 30% formamide, 10 mM NaH_2P0_4, 10% dextran sulphate, 2× SSC, 0.2% BSA, yeast tRNA 500 μg/ml, salmon sperm DNA, in vitro synthesized Cy5-labeled riboprobes (sense or antisense). After FISH, neurons were incubated with primary antibodies at the following concentrations: anti-Staufen (1:200, rabbit polyclonal, kindly provided by Juan Ortin); anti-CPEB1 (1:50, rabbit polyclonal, kindly provided by David Wells); anti-FMRP-rAM2 (1:500, rabbit polyclonal); anti-FMRP-1C3 (1:50, mouse monoclonal, Chemicon); anti-Tau (1:100, mouse monoclonal, Sigma); anti-rpS6 (1:50, rabbit polyclonal, Cell Signaling); anti-MAP2 (1:100, mouse monoclonal, Sigma-Aldrich); anti-Shank (1:400, rabbit polyclonal, kindly provided by Morgan Sheng); and anti-PSD-95 (1:400, rabbit polyclonal, kindly provided by Eunjoon Kim). Secondary antibodies were used as follow: TRITC-conjugated anti-rabbit secondary antibody (1:100, Jackson Research), FITC-conjugated anti-mouse secondary antibody (1:100, Jackson Research). The images were acquired using a confocal laser scanning microscope (LSM510, Zeiss). Quantitative analysis in double-labeled material was performed in different cell culture by counting 20 cells (total 200 spines) for each experimental condition using the image analysis tools present in the CLSM proprietary image analysis program (Zeiss, LSM 2.3).

Immunocytochemistry

Free-floating vibratome sections were heat-treated (95 °C for 5 min) to recover antigenicity. After antigen retrieval, sections were blocked for 2 h, at RT, in Mix solution (0.1 M Tris HCl, pH 7.4, 0.5% Triton X-100, 0.25% carrageenan lambda) plus 10% normal goat serum (NGS) and then incubated overnight with anti-FMRP-rAM2 antibodies (1:100) at 4 °C. Sections were washed with TBS and then incubated in biotin-conjugated secondary antibody (goat anti-rabbit IgG, 1:500). After washing, sections were reacted with DAB and H_2O_2 for the colorization reaction. Sections were mounted on poly-L-lysine slides, dehydrated through alcohols to xylene and coverslipped.

Cytoplasmic fractionation and Western blotting

Neurons at 18 DIV were treated, while attached to the Petri dish, at 4 °C in digitonin buffer (0.01% Digitonin, 10 mM Pipes, 300 mM sucrose, 100 mM NaCl pH 7.4 plus protease inhibitors cocktails from Sigma) for 10 min under gentle shaking. Proteins extracted with the Digitonin buffer were collected as Dig fraction. Neurons were then treated in Triton X-100 buffer (0.5% Triton X-100, 10 mM Pipes, 300 mM sucrose, 100 mM NaCl pH 7.4 plus protease inhibitors cocktails from Sigma) for 30 min at 4 °C. Triton X-100 buffer containing-proteins was collected as Tx fraction. After this extraction, neurons were incubated in Tween/Doc buffer (1% Tween-40, 0.5% Deoxycholate, 10 mM pipes, 300 mM sucrose, 100 mM NaCl pH 7.4 plus protease inhibitors cocktails from Sigma) for 10 min scraped and collected as Tw/Doc fraction. Each fraction was centrifuged at 2000×*g* for 15 min at 4 °C then stored at −20 °C. Fractions were precipitated twice in cold acetone 80%, 0.2 mM DTT, for 30 min at 16000×*g* at 4 °C. Protein pellets were resuspended in 200 μl of Laemmli buffer and 15 μl were loaded into 6–12% SDS–PAGE gels. Primary antibodies were applied overnight in blocking buffer (20 mM Tris pH 7.4, 150 mM NaCl, 0.1% Tween-20 and 3% dried non-fat milk); the secondary antibodies (HRP-conjugated anti-mouse, anti-rabbit or anti-goat from Amersham) were used 1:2000. The signal, detected using an ECL detection system (PerkinElmer Life Sciences), was captured by a Versadoc 1000 digital camera (Biorad) and quantified by means of ImageQuant software (Bio-Rad).

The quantification of signal enriched in specific fraction was expressed as the average percentage (±SD, obtained from at least four independent experiments) of the total which was calculated as the sum of the signal present in all the fractions.

Antibodies used for Western blotting

Rabbit anti-MAPK (1:1000; New England Biolabs), mouse anti-β-tubulin (1:1000; Sigma), mouse monoclonal 1C3 (1:5000; kindly provided by Ben Oostra), rabbit anti-FMRP-rAM2 (1:1000), rabbit anti-Staufen (1:1000; kindly provided by Stefan Kindler) and mouse monoclonal anti-GAPDH (1:10000; Chemicon).

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