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Differential expression of FMR1, FXR1 and FXR2 proteins in human brain and testis

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Lack of expression of the fragile X mental retardation protein (FMRP) results in mental retardation and macroorchidism, seen as the major pathological symptoms in fragile X patients. FMRP is a cytoplasmic RNA-binding protein which cosediments with the 60S ribosomal subunit. Recently, two proteins homologous to FMRP were discovered: FXR1 and FXR2. These novel proteins interact with FMRP and with each other and they are also associated with the 60S ribosomal subunit. Here, we studied the expression pattern of the three proteins in brain and testis by immunohistochemistry. In adult brain, FMR1, FXR1 and FXR2 proteins are coexpressed in the cytoplasm of specific differentiated neurons only. However, we observed a different expression pattern in fetal brain as well as in adult and fetal testis, suggesting independent functions for the three proteins in those tissues during embryonic development and adult life.

INTRODUCTION

Fragile X syndrome is a common form of inherited mental retardation with an incidence of ~1:4000 in males and 1:6000 in females (1). The syndrome is characterized by mental retardation, macroorchidism, typical facial appearance and various degrees of autistic behaviour (2). The fragile X syndrome is caused by the expansion of a highly polymorphic CGG repeat present in the 5′ untranslated region of the *FMR1* gene (3,4). When the repeat expands to more than ~230 CGG units, the promotor region and the repeat itself become hyper-methylated and as a consequence no FMR1 transcription (5) and thus no translation occurs (6,7). The absence of the FMR1 protein (FMRP) leads to mental retardation.

The FMR1 transcript is alternatively spliced and generates splice variants coding for different FMRP isoforms with a molecular weight varying from 70 to 80 kDa (7). FMRP is widely expressed in most adult and fetal tissues and high levels are found particularly in brain and testis (6). The protein is predominantly localized in the cytoplasm, although occasionally nuclear staining has been reported (6,7).

One clue as to the function of FMRP is derived from the identification in the FMR1 sequence of motifs observed in several RNA-binding proteins (8,9). Two heterogeneous nuclear ribonucleoprotein K homology (KH) domains and one RGG box are located in the middle and in the C-terminus of FMRP respectively. Data have been presented that show that FMRP can bind RNA *in vitro* with some degree of sequence specificity (8,9), but the functional evidence for the importance of the RNA-binding capacity was illustrated by a severe fragile X patient with a point mutation in the second KH domain of FMRP resulting in reduced RNA binding (10–12). Interestingly, in addition to its RNA-binding capacity, an association of FMRP with the ribosomal 60S subunit has recently been described (13,14) and it has been suggested that this binding occurs via RNA (15,16).

Recently we demonstrated at the electron microscopical level that FMRP is associated with free and membrane-bound ribosomes and, surprisingly, also in the granular component of the nucleolus (17). Our findings were supported by the identification of a nuclear location signal (NLS) as well as a nuclear export signal (NES) in the FMR1 protein (16,18,19). The NES of FMRP is functionally similar to the export signal identified in REV, the regulatory protein of human immunodeficiency virus type 1 (HIV-1), which mediates the export of the viral RNA from the nucleus to the cytoplasm. Taken together with the ribosomal association, these data led to the hypothesis that FMRP might play a role in the transport of RNA or ribosomal particles between nucleus and cytoplasm and possibly in the regulation of the translation and turnover of these RNAs.

Finally, two novel human proteins that interact with FMRP were discovered (20,21). These proteins, named FXR1P and FXR2P, are very similar in overall structure to FMRP (~60% amino acid identity) and they both have two KH domains and one RGG box that can bind artificial RNA homopolymers *in vitro*. Like FMRP, FXR1P and FXR2P have been found to be associated predominantly with the ribosomal 60S subunit (14). Furthermore, FMRP can form homo- and heteromultimers with FXR1P and FXR2P *in vitro* as well as *in vivo*, suggesting that this interaction could be a mechanism of functional autoregulation of this new family of RNA-binding proteins. So far, no common immunohistochemical studies for the three proteins have been performed, despite the possible importance of FXR1P and FXR2P in the functioning of FMRP, or vice versa. Only an RNA *in situ* hybridization analysis of the murine FXR1 has been reported,

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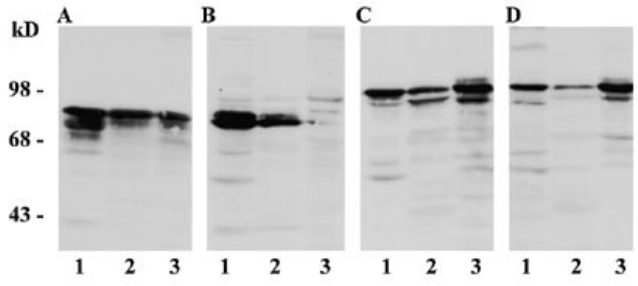


Figure 1. Western blot analysis of total protein incubated with the four different antibodies. Protein homogenates from human lymphoblastoid cell line, human brain and mouse brain (lanes 1, 2 and 3 respectively) were tested for the presence of FMRP with Ab1C3 (**A**), FXR1P with Ab1934 (**B**) and FXR2P with AbA42 and Ab1937 (**C** and **D** respectively).

which pointed to a different gene expression between FMR1 and FXR1 in tissues as testis, muscle and heart (22).

To characterize the *in vivo* distribution of FMRP, FXR1P and FXR2P, we studied the expression of the three proteins in human brain and testis by immunohistochemistry. Here we report a common expression pattern for FMRP, FXR1P and FXR2P in the cytoplasm of specific differentiated neurons, while fetal brain as well as adult and fetal testis showed a different distribution, which might suggest a possible independent function for the three proteins in those tissues. Finally, we show biochemically that FMRP and FXR2P were present in synaptosomes purified from mouse brains.

RESULTS

Characterization of the antibodies

For the detection of FMRP we used the mouse monoclonal antibody 1C3 (6) (Fig. 1A). To study the FXR1 protein we produced an anti-FXR1 rabbit polyclonal antibody (Ab1934). Because of the high rate of amino acid homology among the three proteins, the antibody Ab1934 was raised against an epitope in the C-terminus of FXR1P (amino acids 588–613) which is physically absent in the short form of FXR1P (amino acids 1–539) (20). The band seen with the antibody Ab1934 in lymphoblastoid cells and human brain (Fig. 1B, lanes 1 and 2) corresponds to the long isoform of FXR1P as described by Siomi *et al.* (20). The low expression of FXR1P in mouse brain (Fig. 1B, lane 3) can be the result of a poor recognition of the mouse sequence by our anti-FXR1P antibodies or can result from a low expression of the long isoform of FXR1P in mouse. The expression of the human FXR2 protein was studied by using the mouse monoclonal antibody (A42) which has been described recently (21) and a new anti-FXR2 rabbit polyclonal antibody (Ab1937). The results obtained on Western blot with both antibodies were similar (Fig. 1C and D).

Expression of FMRP, FXR1P and FXR2P in adult and fetal brain

FXR1 and FXR2 proteins were described to be localized in the cytoplasm of HeLa cells as the only cell lines tested so far (20,21). The developmental expression of the murine FXR1 gene was shown by RNA *in situ* hybridization (22), whereas the expression of FMRP has been characterized both at the RNA and protein level (6,23,24). Here, we show a comprehensive view of the expression patterns of FMR1, FXR1 and FXR2 proteins in different human tissues.

In human adult cerebellum, the strongest expression of FMRP was observed in neurons and especially in the Purkinje cells at the interface between the granular layer and the molecular layer (Fig. 2A). Similarly, a high expression for FXR1P and FXR2P in these Purkinje neurons is found (Fig. 2B and C respectively). All three proteins showed a clear cytoplasmic localization. In human adult cortex and brain stem the three proteins were not only localized to the cytoplasm of neurons but, in addition, compared to the Purkinje cells, a stronger labelling was seen in the proximal dendrites (data not shown). In brain tissues of a fragile X patient, as expected, no FMRP signal was detected as is demonstrated in cerebellum (Fig. 2D), while FXR1P and FXR2P expression was unchanged compared to the normal control (data not shown).

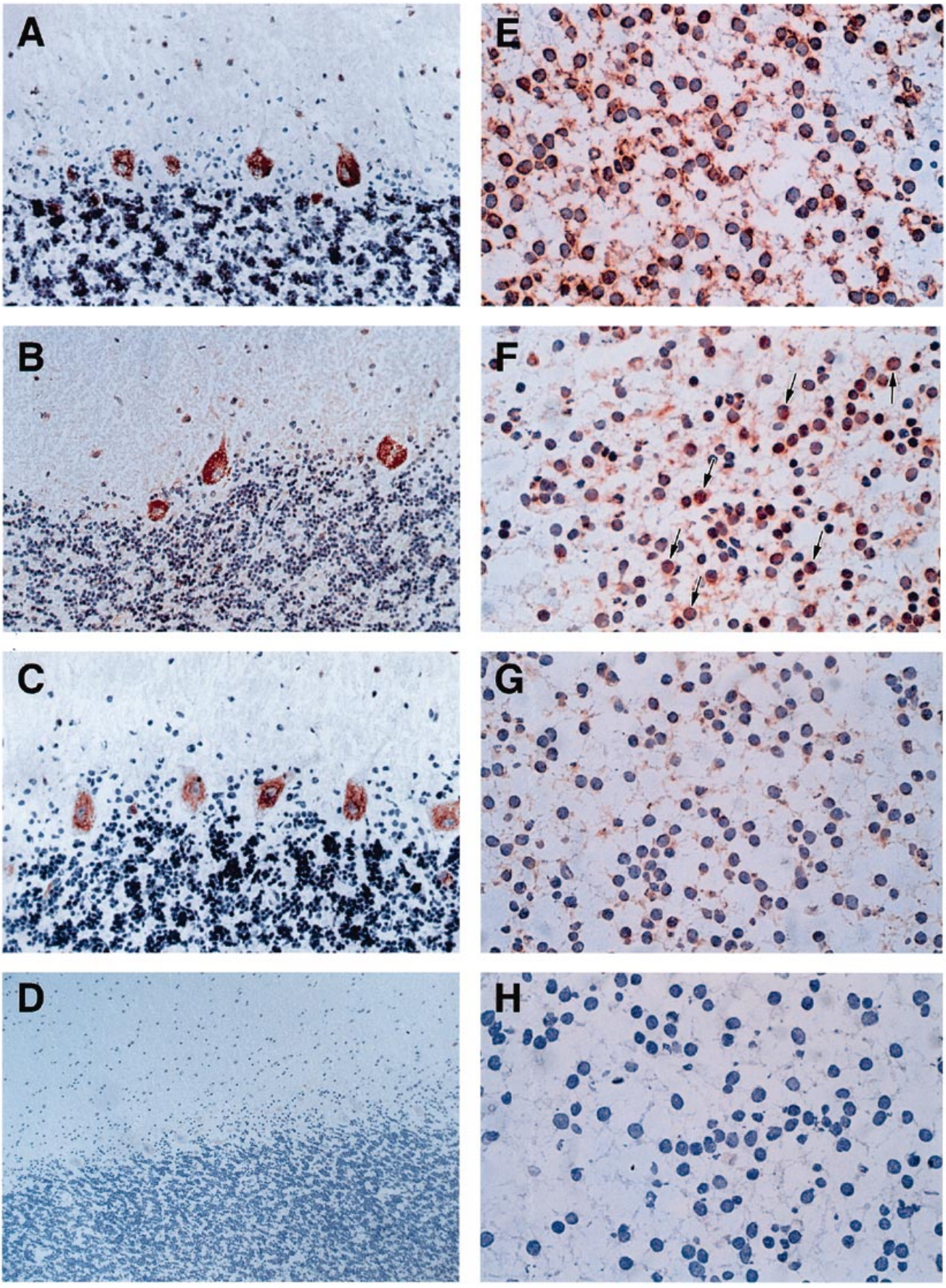
We performed a similar analysis in brain of a control fetus (18 weeks) and a fragile X fetus (18 weeks). The pattern of labelling of FMRP was comparable to the pattern seen in adult brain with a clear cytoplasmic signal in all the neurons (Fig. 2E). Since fetal brain contains mostly undifferentiated neurons, evident brain structures are difficult to determine. FXR1P showed an expression pattern in the cytoplasm of all the neurons but a strong nuclear signal was also present in a substantial number of neurons (Fig. 2F). FXR2P expression was also cytoplasmic, but weaker (Fig. 2G). In brain of a fragile X fetus no FMRP could be detected (Fig. 2H). In contrast, FXR1P and FXR2P labelling was identical to the pattern observed in brain of a normal control fetus (data not shown).

In summary, our results indicated that the expression pattern of the three proteins was clearly overlapping in normal human adult brain showing the same cell type specificity as well as a cytoplasmic localization. On the other hand, in fetal brain, FXR1 protein was also strongly expressed in the nuclei of some neurons.

Characterization of FMRP and FXR2P in mouse brain synaptosomes

To study the cytoplasmic expression of the neurons in more detail, we extended the analysis on cultured mouse hippocampal neurons. FMRP labelling in these neurons was strong in the perikaryon and the proximal dendrites but, in contrast, the distal dendrites and the axon contained little or no FMRP (Fig. 4A). Interestingly, we detected a similar labelling signal for FXR2P in the perikaryon and in the dendrites (Fig. 4B). It has been demonstrated that FMR1 and FXR1 and FXR2 are bound to ribosomes (14). Since ribosomes are localized within the neurons to the perikaryon, the proximal part of the axon and the dendrite (25), the reported protein distribution in the neurons could be due

Figure 2. FMRP, FXR1P and FXR2P expression in adult and fetal brain. Light-microscopic micrographs of cryostat sections from adult cerebellum (**A**–**C**) and fetal brain (**E**–**G**) of control individuals and adult cerebellum **(D)** and fetal brain (**H**) of a fragile X patient. Sections were immunostained for FMRP (A, D, E and H), FXR1P (B and F) and FXR2P (C and G) with the antibodies 1C3, 1934 and A42, respectively. A positive signal with the indirect immunoperoxidase technique results in a brown precipitate. Arrows indicate the nuclei of neurons which are positive for FXR1P (F).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to the association of FMRP and FXR2P with ribosomes. Next we tested whether FMRP and FXR2P were also present at the synapse. Therefore, a synaptosome preparation was purified from brains of wild type and *Fmr1* knockout mice, separated on SDS–PAGE gel and probed with the antibodies 1C3 and Ab1937. Using Western blotting we confirmed the presence of FMRP and FXR2P in our synaptosome preparation (Fig. 5). Synaptophysin (p38), a transmembrane glycoprotein of synaptic vesicles, was demonstrated to be present in the isolated synaptosomes that we used (Fig. 5, lane 5). The antibody Ab1934 does not recognize the murine FXR1 protein (Fig. 1B, lane 3).

Expression of FMRP, FXR1P and FXR2P in adult and fetal testis

Sections of human adult testis were examined for expression of the three proteins. As previously reported (6), we found a prominent labelling for FMRP in the cytoplasm of cells adjacent to the basal membrane of the seminiferous tubules, corresponding to spermatogonia (Fig. 3A). The labelling was often in a punctuated pattern, probably due to a large amount of FMRP concentrates in the cytoplasm. FXR1P was also detectable in spermatogonia. However, in addition, a predominant expression of FXR1 protein was found in the cytoplasm of cells which were more inside the tubuli seminiferi, corresponding to maturing spermatogenic cells (Fig. 3B). Finally, FXR2P gave a third, slightly different, pattern of expression in this tissue. The labelling of FXR2P is also punctuated, however, of lower intensity and present in all the cells of the seminiferous tubules (Fig. 3C). Figure 3D illustrates the localization of FMRP in adult testis from a fragile X patient. The normal expression of FMRP in spermatogonia is in agreement with previous studies, where it is reported that adult fragile X males with the fully expanded repeat in the somatic cells show only premutation length repeats in their sperm (26,27). The same material was tested for FXR1P and FXR2P expression and showed similar results as seen in a control testis (data not shown).

Testis from a normal fetus (20 weeks) and a fragile X fetus (18 weeks) were analysed for the three proteins. In normal fetal testis, FMRP was predominantly present in all primordial germ cells (PGCs) (Fig. 3E). Also, FXR1P could be localized predominantly in primordial germ cells; however, the non-spermatogenic cells were also labelled, although to a lesser extent (Fig. 3F). In contrast, FXR2P was only strongly expressed in interstitial cells (Fig. 3G). Testis from a fragile X fetus showed FMRP labelling in some primordial germ cells (Fig. 3H) whereas the majority of the testis was FMRP-negative. The few FMRP-positive PGCs might correspond to cells which most likely have a premutation allele due to full mutation contraction (28). FXR1P and FXR2P distribution in the testis from a fragile X fetus was similar to the normal fetus (data not shown).

From our results we concluded that, in adult and fetal testis, FMRP, FXR1P and FXR2P are expressed at various levels in different cell types, and they may, therefore, have independent functions. Conversely, in spermatogonia, the only cell type positive for FMRP, we detected an evident coexpression of the three proteins.

DISCUSSION

Recently, two human homologs of FMR1 were identified, named FXR1 and FXR2, and the corresponding proteins were indicated to interact with FMRP, the protein product of the FMR1 gene (20,21). Like FMRP, FXR1P and FXR2P have domains characteristic of RNA-binding proteins and all three are associated with ribosomes, predominantly with the 60S ribosomal subunit (14). *In vitro* and *in vivo* binding studies showed that FMRP, FXR1P and FXR2P form homo- and heterodimers, suggesting that this oligomerization may have a biological function (21). However, knowledge about the repertoire of FMRP/FXRPs combinations which exist in human tissues is still limited, particularly in brain and testis. Here we report that FMRP, FXR1P and FXR2P are differentially expressed in fetal brain as well as in adult and fetal testis, while the expression pattern in adult brain was identical.

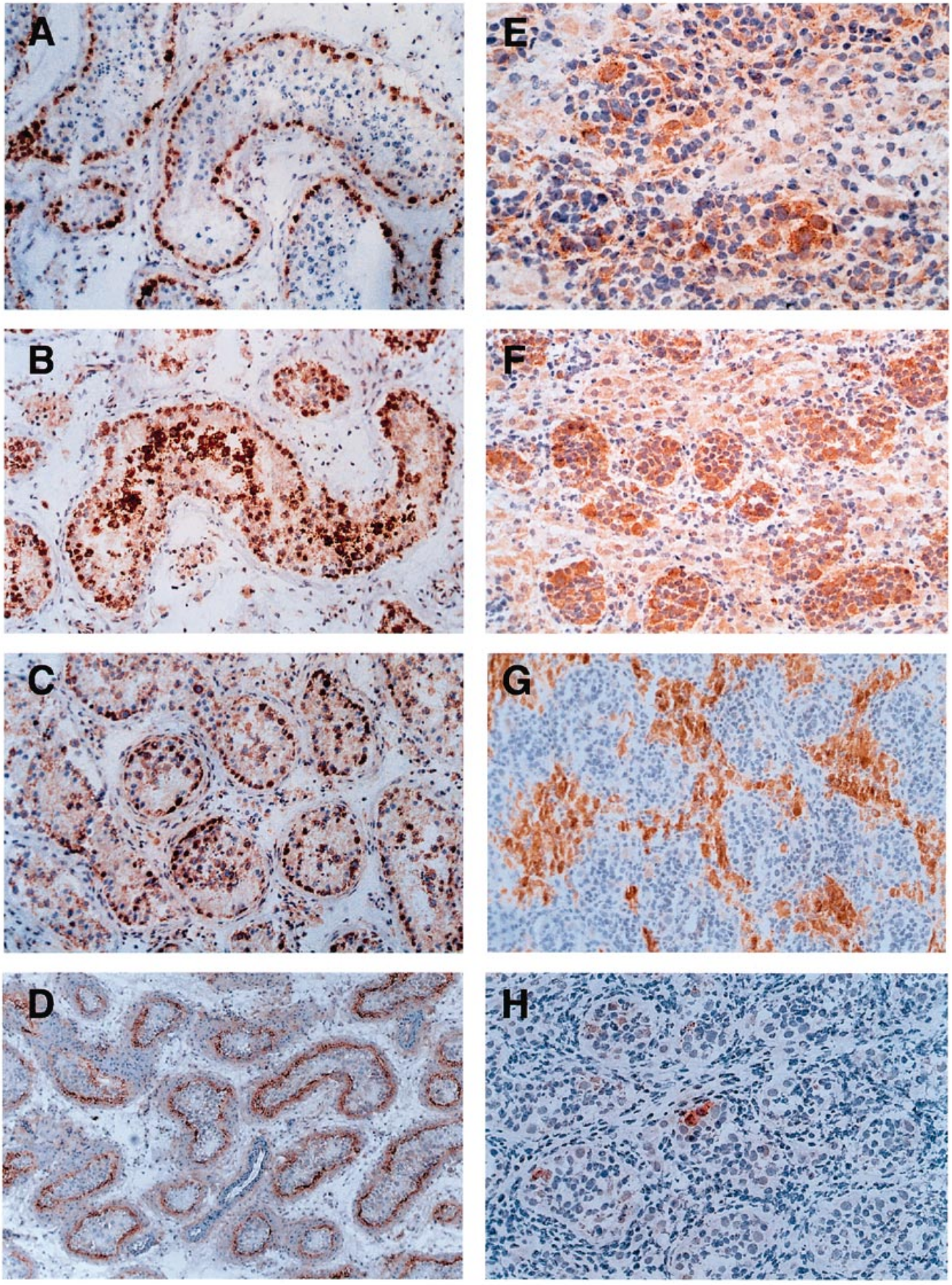
In adult brain, examination of several neuronal populations revealed a common cytoplasmic localization and a high expression of the three proteins in Purkinje, cortical and brain stem neurons. FMRP is abundantly expressed in neurons and not detectable in non-neuronal cells such as glia, astrocytes and oligodendrocytes (6). Importantly, we showed that, like FMRP, FXR1 and FXR2 proteins were also only expressed in differentiated neurons.

In fetal brain, FXR2P, like FMRP, is expressed in the cytoplasm of the neurons. However, the expression of FXR2P in fetal brain is much lower than in adult brain. Also, for FXR1P, a different localization pattern is seen in fetal brain compared to adult brain. In adult brain FXR1P is only found in the cytoplasm of the neurons, while in fetal brain a substantial number of neurons also showed a nuclear localization.

The three proteins are very homologous; they have RNA-binding properties and a ribosomal association, which indicates a role in the ribosomal and RNA metabolism of neurons. Together with the common subcellular distribution it is suggestive to accept a complementary effect of the proteins. However, the absence of FMRP in the fragile X syndrome leads to mental retardation despite the observed normal expression of FXR1P and FXR2P in the neurons of fragile X patients. Therefore our result confirms that FXR1P and FXR2P cannot complement the absence of FMRP in fragile X patients, indicating that FMR1, FXR1 and FXR2 proteins may have independent, although similar, cellular functions. Another explanation is that the clinical phenotype seen in the fragile X syndrome (mental retardation) occurs during embryonic development where we demonstrated that the complementary effect of FXR2P or FXR1P is less likely than in adult brain due to a reduced expression and a different localization respectively in fetal neurons.

Furthermore, the three proteins were expressed in different cell types in adult as well as in fetal testis. In adult testis, FMRP was strongly expressed in spermatogonia. Despite the fact that our

Figure 3. FMRP, FXR1P and FXR2P expression in adult and fetal testis. Light-microscopic micrographs of cryostat sections from adult (**A**–**C**) and fetal testis (**E**–**G**) of control individuals and adult (**D**) and fetal testis (**H**) of a fragile X patient. Sections were immunostained for FMRP (A, D, E and H), FXR1P (B and F) and FXR2P (C and G) with the antibodies 1C3, 1934 and A42 respectively. Again, a brown precipitate illustrates a positive signal.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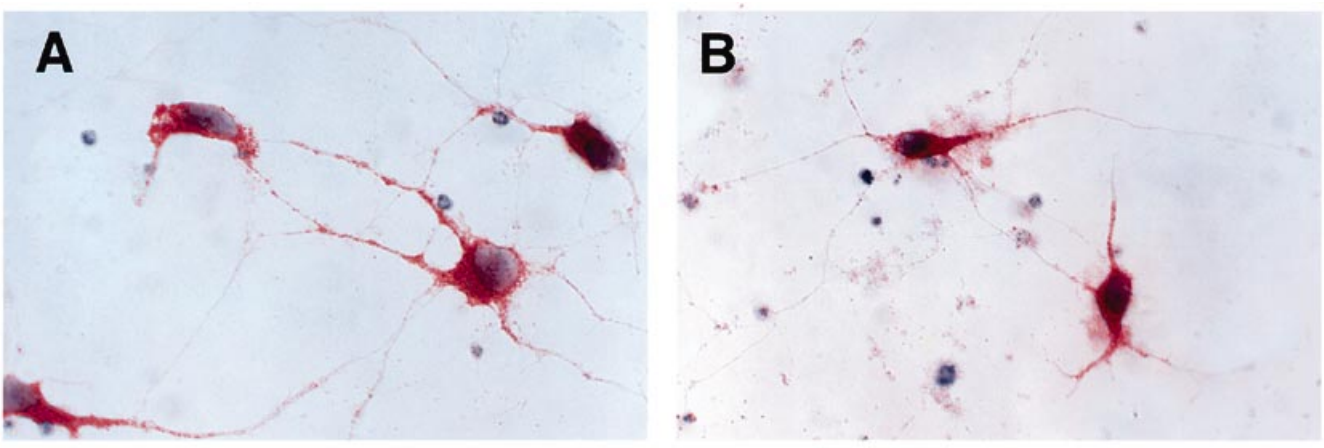

Figure 4. Localization of FMRP and FXR2P in cultured neurons. Cultured mouse hippocampal neurons (9 days in culture) were immunostained for FMRP (**A**) and FXR2P (**B**) with the antibodies 1C3 and A42 respectively, using an indirect alkaline phosphatase technique. With this method a positive reaction is illustrated by a red precipitate.

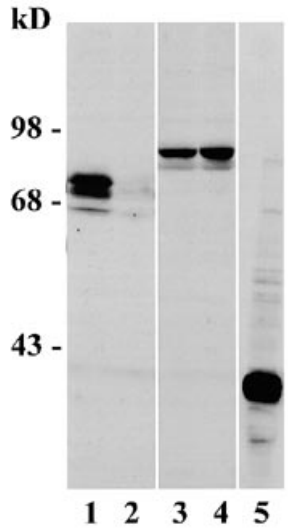

Figure 5. Equal amount of synaptosomes suspension derived from wild type mouse brain (lanes 1 and 3) and from *Fmr1* knock out brain (lanes 2 and 4) were tested for the presence of FMRP with Ab1C3 (lanes 1 and 2) and FXR2P with Ab1937 (lanes 3 and 4). Lane 5 shows the presence of Synaptophysin in the isolated synaptosomes.

cryostat sections did not permit recognition of the stages of spermatogenic differentiation, it is clear that FXR1P is predominantly expressed in maturing spermatogenic cells. This is supported by RNA *in situ* hybridization studies on mouse which showed that the strongest signal for FXR1 was restricted to spermatids (22). Finally, FXR2P showed a labelling signal in all the cells throughout the tubuli seminiferi.

In fetal testis, FMRP was only expressed in primordial germ cells (PGCs); FXR1P was also present in PGCs, but not exclusively. However, FXR2P was not detectable in PGCs but strongly present in the interstitial cells (Fig. 3G), resulting in a third different pattern of expression. The expression of FMRP in some PGCs of a fragile X fetus of 18 weeks (Fig. 3H) is in agreement with our recent model, suggesting that a reduction of full mutation alleles results in a premutation in sperm in adult patients (28).

Our results showed that FMR1, FXR1 and FXR2 proteins are colocalized in differentiated neurons, like Purkinje cells, while in fetal brain and testis each of the proteins has an independent cellular distribution. Neurons in particular need accurate protein synthesis and transport of mRNA from the nucleus to specific compartments as dendrites and synapses. With this in mind, it is possible that differentiated neurons may coexpress FMRP, FXR1P and FXR2P to enhance a particular cellular task which is not yet known. The presence of FMRP and FXR2P in dendrites and in synaptosomes could be in line with this hypothesis (29). Whether or not FMRP, FXR1P and FXR2P have complementary functions in neurons might be resolved by the generation of knockout mice for FXR1 and FXR2 and crossing with the *Fmr1* knockout (30) successively to generate double knockout mice.

MATERIALS AND METHODS

Antibodies and Western blotting

Polyclonal antibodies were raised in rabbits against synthetic peptides covering amino acid positions 588–613 of FXR1 (Ab1934) and amino acid positions 625–641 of FXR2 (Ab1937). Protein samples separated on 10% SDS–polyacrylamide gels were electroblotted onto nitrocellulose membrane (Schleicher & Schuell). Immunodetection was carried out using the mouse monoclonal antibody Ab1C3 (1:2500), the rabbit polyclonal antibody Ab1934 (1:2500), the mouse monoclonal antibody AbA42 (1:5000) and the rabbit polyclonal antibody Ab1937 (1:500). The secondary antibody (1:4000) was coupled to peroxidase allowing detection with chemiluminescence method (ECL KIT, Amersham). A monoclonal anti-Synaptophysin was purchased from Sigma and for Western blotting used in a 1:1000 dilution.

Immunohistochemistry

Tissues were embedded in Tissue-Tek (Miles, Inc.) and snap frozen in liquid nitrogen. Cryostat sections (7 μm) were fixed with 3% paraformaldehyde (10 min) followed by a methanol step (20 min). Endogenous peroxidase was inhibited by 30 min incubation in PBS–hydrogen peroxide–sodium azide solution (100 ml 0.1 M PBS + 2 ml 30% H_2O_2 + 1 ml 12.5% sodiumazide). Subsequently, sections were incubated either with Ab1C3 (produced in

Tecno/mouse system; 1:200) or Ab1934 (1:200) or AbA42 (1:200) for 1 h at room temperature. Subsequently, a 1 h incubation with a peroxidase conjugated secondary antibody was performed and the signal was detected using 3,3′-diamino-benzidine.HCl (Serva) as a substrate. The sections were counterstained with hematoxylin. The immunohistochemistry of cultured neurons was performed using an alkaline phosphatase conjugated secondary antibody and New Fuchsin Red as substrate (DAKO).

Synaptosome purification and cultured neurons

Synaptosomes were prepared from the forebrain of 20 day old mouse pups following the protocol of Rao *et al*. (31). A 20% (w/v) homogenate was prepared from chopped forebrain in 0.35 M Sucrose, 10 mM Tris pH 7.4, 0.5 mM EGTA solution. The homogenate was centrifuged at 2000 *g* for 1 min using a JA-20 fixed angle rotor to remove a nuclear pellet (P1). P1 was washed once by resuspension in the homogenizing buffer and repelleting. The supernatant was combined with the supernatant from the first centrifugation, and the pellet was discarded. The pulled supernatant (S1) was centrifuged at 23 000 *g* for 4 min to yield a crude mitochondrial pellet (P2), which was washed once and repelleted. P2 was then made up to 6 ml total volume with the same buffer. This suspension was layered onto a discontinuous gradient of 5 and 13% Ficoll in 0.35 M Sucrose that had been allowed to equilibrate at 4°C for 1 h prior to loading the sample. The gradient was centrifuged at 45 000 *g* for 45 min using a SW50.1 swinging bucket rotor. A synaptosomal fraction was collected from the 5–13% interface and diluted in 0.35 M Sucrose and centrifuged at 23 000 *g* for 20 min. The synaptosomal pellet was resuspended and equal amount of protein in Laemmli buffer were loaded on a 10% SDS–polyacrylamide gel.

Hippocampi were dissected out from 2–5 day old mouse brains and placed in Hank's balanced salt solution (HBSS) without Ca^{2+} and Mg^{2+}. Quickly the Hippocampi were subdivided into small pieces, incubated for 10 min with trypsine + DNase I and mechanically dissociated in HBSS w/o Ca^{2+} and Mg^{2+} supplemented with DNase I. Divalent cations were restored by dilution with 2 vol of HBSS. After allowing non dispersed tissues to settle for 3 min, the supernatant was centrifugated for 1 min at 200 *g*. Subsequently, the cells were plated onto D-polylysine coated glass coverslip and cultured for 1–2 weeks in Neurobasal Medium (Life Technologies) supplemented with B27 and 25 µM glutamate.

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