Developmentally-programmed FMRP expression in oligodendrocytes: a potential role of FMRP in regulating translation in oligodendroglia progenitors

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The fragile X mental retardation protein (FMRP) is a selective RNA-binding protein whose function is implicated in regulating protein synthesis of its mRNA targets. The lack of FMRP leads to abnormal synapse development in the brain and impaired learning/memory. Although FMRP is predominantly expressed in neurons of the adult brain, whether FMRP also functions in glia during early development remains elusive, since expression of FMRP in glia has not been rigorously examined. This is an important question because recent studies revealed important roles of glia in synaptic development. Here we report that in addition to the observed neuronal expression, FMRP expression is detected in oligodendroglia progenitor cells (OPCs), immature oligodendrocytes and oligodendroglia cell lines, where it interacts with a subgroup of oligodendrocyte-specific mRNAs, including the myelin basic protein (MBP) mRNA. FMRP expression gradually declines as oligodendrocytes differentiate in vitro and in the developing brain. The decline of FMRP expression during oligodendrocyte differentiation is associated with a vigorous up-regulation of the MBP protein. In addition, we show that the MBP 3′ untranslated region (3′UTR) is necessary and sufficient for binding FMRP, and mediates translation inhibition of a reporter gene by FMRP specifically in oligodendrocytes. These results support the hypothesis that FMRP may participate in regulating translation of its bound mRNAs in oligodendroglia during early brain development.

INTRODUCTION

The absence of FMR1 expression leads to fragile X syndrome, a frequent cause of familial mental retardation affecting 1 in 4000 males and 1 in 8000 females (1,2). The protein encoded by FMR1, the fragile X mental retardation protein (FMRP), is a messenger-ribonucleoprotein (mRNP) that selectively binds a subclass of brain mRNAs (3–6). FMRP harbors RNA-binding motifs (7,8) as well as domains for shuttling between the nuclear-cytoplasmic compartments (9–12). At steady state, FMRP is predominantly localized in the cytoplasm (13), associated with translating polyribosomes in many types of cells (3,9,10,14,15). Therefore, the function of FMRP has been postulated to regulate translation of its bound mRNAs (1,16,17). Consistent with this idea, the absence of FMRP leads to abnormal polyribosome-association of mRNAs that normally bind to FMRP (3), suggesting misregulation of translation. Indeed, FMRP has been shown to act as a translation suppressor in vitro and in transfected fragile X fibroblasts (18–20). Under appropriate molar concentrations, FMRP selectively suppresses translation of its bound mRNAs. For example, FMRP binds the 3′untranslated region (3′UTR) of the myelin basic protein (MBP) mRNA, and suppresses MBP translation in a MBP 3′UTR dependent manner in vitro (18). However, how FMRP may influence translation during brain development remains largely unknown.

In the adult brain, FMRP is detected in the somatodendritic compartments of neurons including dendritic spines, with negligible expression in glia (10,13,21). The lack of FMRP in fragile X patients as well as in Fmr1 knockout mice results...
in abnormal dendritic spine maturation (22–24). The most severe dendritic spine abnormality in Fmr1 knockout mice was observed during the developmental window for active synaptogenesis and gliogenesis (24). However, whether the delayed synaptic maturation in fragile X brain is solely due to neuronal abnormality remains elusive, because recent studies revealed critical roles of glia in synapse maturation (25). In fact, whether FMRP is expressed during early glia development remains unclear, since expression of FMRP at early stages of brain development has not been carefully examined.

Here we report that FMRP was detected in the soma and the developing processes of oligodendroglial progenitor cells (OPCs) and immature oligodendrocytes in the neonatal brain, in primary cultures of oligodendrocytes, as well as in oligodendrocyte cell lines. The level of FMRP declined as oligodendrocytes differentiated, suggesting that FMRP may function transiently in early oligodendroglia development. FMRP was found to associate with polyribosomes in oligodendrocytes, and preferentially interacted with a subclass of oligodendrocyte-specific mRNAs including those that encode the myelin basic protein (MBP). When cells express high levels of FMRP, MBP protein was undetectable regardless of the expression of the MBP mRNA. The decline of FMRP during oligodendrocyte differentiation was accompanied by a robust expression of the MBP protein, supporting the hypothesis that FMRP may be involved in the translation inhibition of MBP. Indeed, the 3’untranslated region (3’UTR) of the MBP mRNA was necessary and sufficient for binding FMRP, which mediated translation inhibition by FMRP specifically in cells derived from the oligodendroglial origin. These observations suggest that FMRP may play a role in regulating translation of its bound mRNAs in oligodendroglia, including the MBP mRNA, during neonatal brain development.

RESULTS

The level of FMRP rapidly declines in the neonatal brain

We first examined FMRP expression in the brain at various postnatal ages to determine the developmental profile of FMRP. To evaluate the actual FMRP level, whole tissue lysate was prepared from the brain stem of wild type and Fmr1 ko littermates by sonication at postnatal ages indicated in Figure 1 followed by immunoblot analysis. In wild type mice younger than 7 days of age (P7), FMRP was expressed abundantly in the brain stem. After the first postnatal week, FMRP level rapidly declined and was maintained at low levels throughout the rest of life. The peak of FMRP expression was at least 1 week prior to the peak expression of PSD95, a marker protein for mature synapse, suggesting a functional requirement for high levels of FMRP in early brain development. As expected, FMRP was completely absent in the Fmr1 ko mice at all stages examined.

FMRP is expressed in oligodendroglia during early development and declines as oligodendrocyte differentiate

We questioned whether FMRP may be expressed in cells of the oligodendroglial lineage, because oligodendrogliogenesis is most vigorous in the neonatal brain (26) when FMRP is expressed at high levels (Fig. 1). We first examined FMRP expression in primary cultures of oligodendrocytes derived from neonatal rat brain (Fig. 2). Immunocytochemical studies revealed predominant cytoplasmic localization of FMRP in the soma as well as in the developing processes (Fig. 2). It is noticeable that the level of FMRP was highest in the oligodendrocyte progenitor cells (OPCs) that projected only two thin processes and expressed the earliest rat OPC marker A2B5. In more differentiated oligodendrocytes that extended numerous branched processes and expressed the later marker O1, much lower levels of FMRP were detected. We also performed immunostaining to neonatal mouse brain using antibodies against FMRP and NG2, a marker specific for mouse OPC in early development. As shown in Figure 3A, FMRP was clearly detected in NG2-positive cells in a wild type P2 mouse brain but undetectable in the Fmr1 ko age-matched control (data not shown). The majority of FMRP was detected in the soma, most likely in both the cytoplasm and in the nuclei. In addition, FMRP staining was diminished in later stages of oligodendroglial development when immature oligodendrocytes project a few processes and express low level of MBP. Furthermore, no FMRP was detected in oligodendrocytes that harbor fully developed processes and high levels of MBP (Fig. 3B). These findings suggest that FMRP may function in oligodendroglial progenitors and immature oligodendrocytes, but is down-regulated as oligodendrocytes differentiate.

We next examined whether FMRP is expressed in oligodendrocyte cell lines, which are more readily available and provide sufficient numbers of cells for biochemical analysis of FMRP. The immortalized oligodendrocyte cell line N20.1 expresses MBP mRNA but no MBP protein (Fig. 4A and B), characteristic of cerebral oligodendrocytes younger than P4 in vivo (27). Immunoblot analysis detected FMRP expression in N20.1 cells at a level at least comparable to that in the P4 brain stem (Fig. 4B). This is consistent with the above observations in vivo that FMRP is detected in immature oligodendrocytes before they express the MBP protein (Fig. 3). Furthermore, FMRP expression was also detected in two rat cell lines derived from

**Figure 1.** Developmental profile of FMRP expression in the postnatal brain stem. The level of FMRP in whole tissue lysates derived from brain stem of wild type (wt) and Fmr1 knockout (ko) littermates was evaluated by SDS-PAGE immunoblot analysis using the monoclonal antibody 1C3. The blots were re-probed with antibodies for house-keeping proteins including actin and eIF5α, as well as that for the neuron-specific protein PSD95. The postnatal age and genotype of each animal were indicated on top of the corresponding lanes. The signals for various proteins detected on the immunoblot were indicated on the left.
the oligodendroglia origin, CG4 and C6. Immunostaining revealed a pattern of subcellular localization of FMRP in all three oligodendrocyte cell lines (Fig. 4C), recapitulating what was observed in oligodendrocytes in primary cultures (Fig. 2).

To further delineate the decline of FMRP expression during oligodendrocyte differentiation, we performed immunoblot analysis to quantitatively measure the level of FMRP during induced differentiation of CG4 cells, a well-characterized cell line that can be induced to recapitulate differentiation of oligodendrocytes in primary cultures (28). As shown in Figure 5A, CG4 cells elaborated numerous processes when switched from the proliferating medium to the differentiation medium, morphologically mimicking primary cultured oligodendrocytes. Immunoblot analysis demonstrated a marked decline of FMRP upon induced differentiation (Fig. 5B). In contrast, translation initiation factor 5a (eIF5a) was maintained at a steady level during the entire differentiation profile. A similar reduction of FMRP was also observed in C6 cells upon induced differentiation (data not shown). When FMRP was expressed at high levels, MBP protein was undetectable. However, the down-regulation of FMRP expression was followed by a robust accumulation of the MBP protein. These observations in CG4 cells are consistent with the immunostaining result in oligodendrocytes in vivo (Figs 2 and 3), suggesting that FMRP may function transiently during the early stage of oligodendrocyte development. The MBP mRNA level was also elevated during the differentiation of CG4 cells, due to developmentally programmed transcription (Fig. 5C). However, the presence of MBP mRNA in CG4 cells without MBP protein up to 4 days of differentiation suggests that translation of MBP is suppressed at early stages of oligodendrocyte development, and FMRP may contribute to such regulation, given that FMRP inhibits MBP translation in vitro (17).

**FMRP associates with polyribosomes in oligodendrocytes and interacts with oligodendrocyte-specific mRNAs in a selective manner**

Since FMRP has been reported to associate with translating polyribosomes and control the efficiency of polyribosome-association of its bound mRNA in several cell types, (3,15,29), we examined the possibility that FMRP may have a similar role in oligodendrocytes. Cytoplasmic extracts were derived from the N20.1 and C6 oligodendrocyte cell line and fractionated on a linear sucrose gradient. As shown in Figure 6, the majority of FMRP co-fractionated with polyribosomal complexes that contained translating polyribosomes in both cell lines. EDTA-treatment dissociated polyribosomes into subunits
and released mRNPs. As a result, FMRP was shifted into the top fractions containing mRNP complexes, suggesting that FMRP is associated with polyribosomes as an mRNP in oligodendrocytes.

We next questioned whether FMRP may selectively bind a subclass of oligodendrocyte-specific mRNAs. Using a well-established in vitro RNA-binding assay (7), we examined a few mRNAs that are expressed at high levels during oligodendrocyte development, including those of MBP, proteolipid protein (PLP) and cyclic-nucleotide 2',3'-phosphodiesterase (CNP) (26). As shown in Figure 7A, FMRP interacted with the full-length transcripts of MBP and PLP, but not with the transcript encoding CNP. We then focused on the MBP mRNA to further delineate mRNA interaction with FMRP, given the fact that FMRP inhibits MBP translation in vitro (17). As shown in Figure 7B, truncations into the MBP 3'UTR resulted in a marked reduction of FMRP-binding, and FMRP-binding was abrogated when the entire MBP 3'UTR was eliminated. We also show that the MBP 3'UTR was sufficient to bind FMRP by itself (Fig. 7B). 3'-truncations of the MBP 3'UTR resulted in attenuation of FMRP-binding in a similar manner as observed for the full length MBP transcript. Furthermore, the MBP 3'UTR was sufficient to mediate FMRP-binding in vitro when fused to a reporter mRNA encoding the green fluorescent protein (GFP), which lacks intrinsic FMRP-binding activity (Fig. 7C).

To determine if FMRP interacts with the MBP mRNA in vivo, we performed immunoprecipitation experiments to isolate FMRP–mRNA complexes from the P7 brain stem. Figure 8A shows the successful immunoprecipitation of FMRP from the wild type brain stem. RT–PCR analysis using a MBP-specific primer pair clearly detected the MBP mRNA in the FMRP–mRNP complexes isolated from the wild type brain (Fig. 8B), whereas no MBP mRNA was detected in the immunoprecipitates derived from the brain stem of age-matched Fmr1 ko mice in a parallel experiment. Furthermore, Southern hybridization analysis using a probe derived from a MBP cDNA fragment confirmed the identity of the MBP RT–PCR product derived from the immunoprecipitate. To confirm the specificity of FMRP–MBP mRNA interaction, we performed RT–PCR analysis using primers specific for the mRNA encoding for the growth-associated protein GAP-43. Despite detection of abundant GAP-43 mRNA in the input lysate, no GAP-43 mRNA was detected in the immunoprecipitated FMRP–mRNP complexes. These results clearly indicated that FMRP is associated with the MBP mRNA in young oligodendrocytes in vivo, potentially may regulate its translation during brain development.

MBP 3'UTR mediates translation inhibition by FMRP specifically in oligodendrocytes

The most critical question is whether FMRP may influence translation of its bound mRNA in oligodendrocytes. To address this question, we expressed Flag-FMRP together with

Figure 3. FMRP expression in oligodendroglia in vivo. (A) Oligodendrocyte progenitor cells are identified by the expression of NG2 (red) proteoglycan. FMRP positive (green) cells are evident throughout the section. The NG2-FMRP double-labeled cells are oligodendrocyte progenitors (arrowheads); the other FMRP positive cells are most likely neurons. (B) Detection of FMRP (green) and MBP (red) in immature oligodendrocytes in the neonatal cerebrum. Note that in a fully developed oligodendrocyte expressing high levels of MBP protein, FMRP is undetectable.
the GFP-MBP 3'UTR reporter by transfecting the C6 oligodendrocyte cell line (Fig. 9A), which allows relatively higher transfection efficiency as compared to other oligodendroglia cell lines. The GFP reporter construct that lacks the MBP 3'UTR was used in parallel transfection experiments to address whether FMRP's influence depends on the MBP 3'UTR (Fig. 7C). As shown in Figure 9A, Flag-FMRP significantly repressed the GFP-MBP 3'UTR reporter expression in transfected C6 cells in comparison to that in control cells. Due to the presence of a cross-reacting protein to anti-Flag antibody in C6 cells that co-migrates with FMRP, the FMRP transgene expression could not be demonstrated by the anti-Flag antibody. Nonetheless, the expression of exogenous FMRP was evidenced by the elevated levels of total FMRP in transfected cells (Fig. 7C). In contrast, similar levels of FMRP did not affect expression of the GFP reporter in parallel experiment, demonstrating that the inhibitory effect by FMRP is MBP 3'UTR-dependent. The same experiments were also performed in the HEK293T cells derived from human kidney origin. Interestingly, Flag-FMRP did not inhibit expression of either reporter genes, suggesting that the MBP 3'UTR mediated suppression by FMRP may specifically occur in oligodendroglia. Furthermore, RNase protection analysis (RPA, Fig. 9B) was performed from the same batch of transfected cells to detect the steady state level of the reporter mRNA. Translation of the reporter genes were calculated by normalizing the GFP level on immunoblot to the reporter mRNA level determined by RPA, and the result clearly indicated reduced translation of the GFP-MBP 3'UTR reporter by the exogenous FMRP (Fig. 9C).

**DISCUSSION**

The above studies provide the first evidence that FMRP is not restricted to neurons, but also expressed in cells of the oligodendroglia lineage in the developing brain. The rapid decline of FMRP during oligodendrocyte differentiation suggests that FMRP may play a transient role during oligodendroglia development. In addition, the selective interaction of FMRP with oligodendrocyte-specific mRNAs in vitro and in vivo, and the translation suppression by FMRP on its bound reporter mRNA specifically in oligodendrocytes support the hypothesis that FMRP regulates translation of a subgroup of mRNAs in the early stage of oligodendrocyte development.

**Developmentally-programmed FMRP expression suggests a critical time window for the functional requirement of FMRP**

The abundant FMRP expression in the brain during the first postnatal week (Fig. 1) suggests that the functional requirement of FMRP in brain development may be highest at this point. Although the cell/tissue pattern of FMRP expression during development has been studied by immunostaining (30), experiments in this report examined FMRP quantity for the first time in the whole tissue lysate of brain stem during early postnatal development. We have shown that in addition to neurons, FMRP is also expressed in oligodendroglia progenitors and immature oligodendrocytes during the window when high levels of FMRP are expressed in the brain (Figs 2 and 3). This is the first evidence suggesting that FMRP is expressed
in glia during brain development. Later, FMRP expression quickly declines upon oligodendroglia differentiation (Figs 2, 3 and 5), indicating that oligodendroglia contribute to the developmentally controlled decline of FMRP (Fig. 1). This also provides an explanation for the low level of FMRP in P28 brain stem, since brain stem contains more oligodendroglia than that in cerebral cortex. Therefore, FMRP may not be involved in myelination, consistent with the fact that myelination abnormality is not frequently found in fragile X patients.

How the transient expression of FMRP may influence oligodendroglia development remains elusive. The association of FMRP with mRNA and polyribosomes (Figs 6 and 7) and the localization of FMRP to the proximal oligodendrocyte processes (Figs 2 and 4) suggests that FMRP may play a role in regulating mRNA translation and/or localization during early oligodendrocyte development. In addition, FMRP is also possibly present in the nuclei of NG2 oligodendroglia progenitors in vivo (Fig. 3A). The sophisticated post-transcriptional regulation of oligodendrocyte-specific mRNAs at the level of translation and subcellular localization (33) suggests that FMRP may be involved in such regulation. Not all mRNAs in oligodendrocytes harbor FMRP-binding activity, since the CNP mRNA, one of the most abundant mRNAs in oligodendrocyte, does not bind FMRP (Fig. 7A). Therefore, it is likely that FMRP only affects a specific subclass of oligodendrocyte mRNAs. The exact amino acid domain in FMRP responsible for the interaction with the MBP 3'UTR still remains to be determined by future studies.

**Figure 5.** FMRP is down-regulated in the CG4 oligodendroglia cell line during differentiation. (A) Morphology of CG4 cells that undergo vigorous proliferation (day 0) and differentiation (Dif) at various days. (B) Down-regulation of FMRP upon induced differentiation of the CG4 oligodendroglia cells. The days following induced differentiation is indicated on top of each lane. The same blot was re-probed with antibody against MBP and the house-keeping protein elf5a as indicated on the left. Similar observations were observed in three repeated experiments. (C) Detection of the MBP mRNA by RT–PCR in proliferating and differentiating CG4 cells. The days following induced differentiation are marked on top of each lane. Rat brain (P10) total RNA was used as a positive control and reaction containing rat brain RNA lacking reverse-transcriptase (-RT) was carried out as negative control. The doublet banding pattern represents alternatively spliced MBP mRNAs.

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Interestingly, the most vigorous synaptogenesis in normal brain (31) and the most severe dendritic spine abnormality in Fmr1 ko brain (24) were found at the end of the first postnatal week when abundant FMRP expression occurs (Fig. 1). Since glia play important roles in synaptic development (25,32), developmentally controlled FMRP expression in glia may influence synaptic development of the adjacent neurons. Whether the dendritic spine abnormality in the fragile X brain may result from combined deficits in both neurons and glia remains an intriguing and challenging question for future studies.

**FMRP may function transiently during early oligodendroglia development**

The detection of FMRP in oligodendroglia precursors and immature oligodendrocytes (Figs 2 and 3), together with the decline of FMRP expression upon oligodendrocyte differentiation (Figs 2, 3 and 5), suggests that FMRP may act transiently in early oligodendrocyte development. The major function of mature oligodendrocytes is known to form myelin which enhances nerve impulse conduction along the axon (26). However, myelin formation occurs at later ages, as compared to the time window for high level expression of FMRP. In mice, the most active period of myelination occurs in the third postnatal week and FMRP is unlikely expressed in these mature oligodendrocytes. Therefore, FMRP may not be involved in myelination, consistent with the fact that myelination abnormality is not frequently found in fragile X patients.

The interaction of FMRP with the 3'UTR of MBP mRNA in vitro and in vivo (Figs 7 and 8) suggests that MBP mRNA may be one of the targets for FMRP during oligodendrocyte development, although no G-quartet structure is present in the MBP mRNA. The MBP mRNA is deposited to the distal oligodendrocyte processes and extended myelin sheath for localized translation (34). The 3'UTR of MBP mediates regulation of both localization and translation of the MBP mRNA (33,35,36), perhaps via interaction with various RNA-binding proteins including FMRP. Several lines of evidence suggest that the MBP mRNA may undergo translational
inhibition. In developing oligodendrocytes, the appearance of MBP mRNA but absence of MBP protein in the perikarya suggested that the MBP mRNA was translationally inert in the soma (37). The presence of MBP mRNA but no detectable MBP protein in the N20.1 cells (Fig. 4) as well as in proliferating CG4 cells (Fig. 5) also suggests translational suppression of the MBP mRNA, perhaps in a defined stage of early oligodendrocyte development. Indeed, reporter gene studies indicated that FMRP inhibits translation in a MBP 3'UTR-dependent manner (Fig. 9). Furthermore, such phenomenon was observed in oligodendroglia cell lines but not in HEK293T cells (Fig. 9). One possible explanation is that other oligodendrocyte specific RNA-binding proteins may be required for FMRP to exert its inhibitory influence on translation mediated by the MBP 3'UTR in vivo.

It is important to point out that although this report focused on FMRP's influence mediated by the MBP 3'UTR, potential regulation by FMRP in oligodendrocytes is not limited to the MBP mRNA. In fact, the MBP 3'UTR is also present in one of the splicing isoforms of the Golli transcript, another oligodendrocyte-specific gene implicated in controlling process extension (38,39). In addition to myelin structural protein mRNAs analyzed in this paper, FMRP may also interact with other mRNAs that encode regulatory factors controlling oligodendrocyte development that are yet to be identified. Microarray hybridization analysis of FMRP associated mRNAs (3), antibody mediated in situ identification of FMRP-bound mRNA in cells (5) and the identification of the G-quartet RNA structure for interaction with FMRP (4) have provided promising approaches for future studies to identify targets of FMRP specifically for regulating oligodendroglia development.

**MATERIALS AND METHODS**

**Cell culture and animals**

The N20.1 cell line (kindly provided by Dr Campagnoni, UCLA, Los Angeles, CA) was cultured in DMEM/Ham’s F12 with 10% Nuserm as previously described (27). CG4 cells (generously provided by Dr Lopresti, Northwestern University) were expanded in DMEM plus 1% heat inactivated FBS, insulin (5 μg/ml), transferrin (50 μg/ml), putrescine (100 mM), progesterone (20 nM), selenium (30 nM), biotin (10 ng/ml), PDGFAA (10 ng/ml) and bFGF (10 ng/ml). Differentiation of CG4 cells was induced by switching cells from proliferation medium to differentiation medium containing DMEM, insulin, transferrin, tri-iodothyronine (50 nM) and 0.5% FBS as previously described (28). C6 cells were raised in DMEM containing 10% FBS and CAD cells were raised in DMEM/F12 with 8% FBS. Primary cultures of oligodendrocyte progenitors were isolated from neonatal rat brain (P1) following published procedures (40). Their differentiation was initiated by the addition of a serum-free, chemically defined media 24 h post plating. The stage of differentiation at each time point was determined by analysis of stage specific marker expression (41).

Wild type C57bl/6 and Fmr1 ko mice were raised at the Emory University animal facility and treated in accordance with NIH regulations and under approval of the Emory University Animal Care Committee.
University IACUC. Littermates of wild type and Fmr1 ko were produced by breeding heterozygous females with Fmr1 ko males. For tissue collection, mice at various ages were anesthetized and the brain regions of interest were immediately dissected on ice. For preparing brain tissue sections, P7 mice were anesthetized and perfused with 4% paraformaldehyde in ice-cold PBS. The brain was then removed and fixed overnight at 4°C, then immersed in 30% (w/w) sucrose in 0.1 M PB for 24 h for cryoprotection. P2 mice brains were fixed in paraformaldehyde overnight and immersed in 10% (w/w) sucrose in 0.1 M PB at 4°C. Brains were sectioned (40 μm sections) on a cryostat.

Plasmid constructs

The PC-Flag2.17 construct was described previously (9). In vitro synthesis of Flag-FMRP was carried out in TNT translation system (Promega, Madison, WI). GFP-MBP 3′UTR was constructed by subcloning the full-length MBP 3′UTR into the NotI site of pEGFP-N3 (Clontech, Palo Alto, CA). The correct orientation of MBP 3′UTR was verified by restriction enzyme digestion. The full-length cDNA containing GFP and GFP-MBP-3′UTR were subcloned into Bluescript II KS and PCDNA3 (Invitrogen) and the biotinylated transcripts of GFP and GFP-MBP-3′UTR were generated by in vitro transcription of linearized plasmids (Invitrogen). Plasmids harboring MBP, CNP and PLP full-length cDNA were generously provided by Dr Campagnoni (UCLA).
Protein sample preparation

For whole tissue lysate preparation, brain tissues were dissected, minced and immediately lysed by sonication in 1X Laemmlli buffer as described previously (42). For linear sucrose gradient fraction, N20.1 and C6 cells were incubated with cycloheximide (100 μg/ml) for 15 min to arrest polyribosome migration. Cells were then lysed to isolate postmitochondrial extract, which was fractionated on 15–45% (w/v) gradient sucrose sedimentation monitored at OD254 (29). EDTA-lysate was fractionated on a parallel gradient lacking MgCl2 but containing 1 mM EDTA to dissociate ribosomes into subunits. Twelve fractions were derived from each gradient tube and 2% of each fraction was subjected to immunoblot analysis.

Antibodies and immunodetection

For immunoblot analysis, the protein quantity of each sample was estimated by Bradford assay following manufacturer's instruction (Bio-Rad, Hercules, CA) before subjected to SDS–PAGE. After overnight transfer, the blots were subjected to Ponceau S staining (Sigma, St Louis, MO) to confirm equal protein loading before carrying out immunoblot as described previously (42). The primary antibodies were diluted as following: 1C3, 1 : 1000; eIF5α, 1 : 5000 (Santa Cruz, CA); MBP, 1 : 500 (Chemicon, Temecula, CA).

For indirect immunofluorescent staining, N20.1 cells were fixed in 4% paraformaldehyde, permeabilized with 0.3% Triton X-100 in PBS and incubated in 0.3% H2O2 to block peroxidase. Frozen P7 sections were permeabilized and blocked in the same manner. After washing, the slides were blocked in PBS containing 5% normal goat serum, 3% BSA and 0.3% Triton X-100 for 1 h at room temperature followed by incubation with primary antibodies (1 : 200 dilution) at 4°C overnight. The monoclonal antibody 1C3 (13) was used to detect FMRP. FITC and Texas Red conjugated secondary antibodies were added the next day (1 : 200). After 30 min incubation at room temperature, fluorescence was detected using Olympus IX51 fluorescent microscope.

Primary cultures of oligodendrocytes were incubated with A2B5 or O1 antibodies (hybridoma supernatant) for 30 min at room temperature. Cultures were fixed in 4% paraformaldehyde for 30 min at room temperature and rinsed with PBS. To visualize FMRP, the cells were permeabilized with 0.25% Triton X 100 in PBS for 5 min at room temperature. After
rinsing with PBS, FMRP antibody was diluted 1:200 in antibody media (40) and incubated overnight at 4°C. Fluorescent secondary antibodies (1:100) were incubated for 1 h at room temperature. As a control for non-specific staining, parallel cultures were incubated with secondary antibodies only. Immunofluorescence was visualized using a Zeiss Axiocam imaging system.

Frozen sections of P2 brain were thawed, permeabilized with 0.1% Triton X-100 in PBS, and sequentially incubated with NG2 and FMRP antibodies overnight in a humidified chamber at 4°C. The sections were rinsed and incubated with biotinylated secondary antibodies, followed by incubation with FITC or RTIC avidin. The sections were mounted in Vectashield and imaged with a BioRad confocal microscope.

Immunoprecipitation of FMRP–mRNP complexes

For immunoprecipitation of endogenous FMRP, P7 brain stem was homogenized individually in a lysis buffer containing 200 mM NaCl, 50 mM Tris, 0.3% Triton X-100, complete protease inhibitor (Boehringer Mannheim, Indianapolis, IN), and 400 U/ml RNasin (Promega) on ice. Nuclei and mitochondria were removed by spinning the homogenate at 10,000 g for 10 min at 4°C. The supernatant was first preclared with SPA protein beads, 2% of the input supernatant was subjected to immunoblot analysis and the remaining supernatant was mixed with SPA-bound 7G1-1 and incubated with constant mixing for 2 h at 4°C. After washing, the immunoprecipitated FMRP–mRNA complex was eluted using the 7G1-1#1 peptide as described previously (3). Ten percent of the eluate was used for immunoblot analysis and the rest of the eluate was subjected to RNA extraction.

RNA extraction and analysis

Total RNA was extracted using Trizol reagent according to the manufacturer’s protocol (Invitrogen). Cytoplasmic RNA was extracted by phenol-chloroform followed by ethanol precipitation. The quantity of RNA from each sample was determined by OD260 reading and further confirmed by ethidium bromide staining agarose gel electrophoresis. Reverse transcription (RT) was carried out following the manufacturer’s protocol (Invitrogen). Four microliters of each RT-reaction was subjected to standard PCR analysis of 30 cycles. The primers for PCR analysis are listed below: U1, CAGCACGCGCA-CCCCAGCTGG and UR2, GTTTAGTGTAACCAATGGG for MBP; GAGATGGCTCTGTACTACC and CCTGTGC-GGCACCTTCTTCTAG for GAP-43. The RNase protection analysis for the MBP mRNA was carried out as described previously (42). For detecting rat MBP mRNA, primers in the MBP coding region was used in RT–PCR reactions as described previously (42).

RNA-binding assay

35S-Methionine labeled FMRP was produced by in vitro translation using TNT (Promega) and incubated with biotinylated RNA synthesized by in vitro transcription as described previously (7). RNA binding reaction was carried out and bound 35S-FMRP was captured by streptavidin conjugated magnetic beads (Dynal, A.S., Oslo, Norway) as described previously (42). After washing, the captured 35S-FMRP was eluted in 1 x Laemmli buffer followed by scintillation counting. An aliquot of 35S-FMRP input as well as the eluted FMRP-RNA complex was also fractionated by SDS–PAGE to visualize the 35S-FMRP.

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