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FMRP and Myelin Protein Expression in Oligodendrocytes

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Abstract

Fragile X syndrome (FXS) is caused by lack of expression of fragile X mental retardation protein (FMRP), the product of the *Fmr1* gene. In many cases FXS is associated with abnormalities in CNS myelination. Although FMRP is expressed in oligodendrocyte progenitor cells and immature oligodendrocytes (OLGs) previous studies have not detected it in mature, myelin-producing OLGs. FMRP represses translation of myelin basic protein (MBP) RNA *in vitro* and is believed to prevent premature MBP expression in immature OLGs. Lack of FMRP in FXS could lead to premature myelination and/or myelin abnormalities. Here we show that FMRP is expressed in mature, MBP-positive OLGs of rodents and in MBP-positive human OLGs. We confirm that FMRP is a translational repressor of MBP mRNA *in vitro*, but at concentrations likely too high to be physiologically relevant *in vivo*. We find MBP expression in cultured *Fmr1* KO OLGs to be similar to wild type, and expression of MBP and other myelin proteins in brain homogenates of the *Fmr1* KO mouse to be similar to wild type before, during, and after the period of active myelination. These results suggest that while FMRP is expressed in mature OLGs, myelin abnormalities caused by lack of FMRP expression in FXS are not recapitulated in rodents.

Introduction

Fragile X syndrome (FXS) is a protein loss-of-function disorder caused by lack of expression of the fragile X mental retardation protein (FMRP), the product of the *Fmr1* gene. FMRP is an RNA binding protein that regulates translation of several mRNAs *in vivo* and *in vitro* (Laggerbauer et al., 2001; Li et al. 2001). In neurons, several FMRP target RNAs encode synaptic proteins which may explain why FXS is associated with behavioral and cognitive abnormalities (Schapiro et al., 1995). Differences in white matter in FXS have been found using diffusion tensor imaging (DTI) and magnetic resonance imaging (MRI). These include both increases and decreases in white matter volume along with increased number of myelinated nerve fibers (Barnea-Garoly et al., 2003; Haas et al., 2009; Hoeft et

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al., 2010). These abnormalities may contribute to some of FXS symptoms since they affect regions or pathways in the brain that are involved in aspects of behavior or cognition that are altered in FXS (Hallahan et al., 2011; Hoeft et al., 2011).

Neuronal structure and function are affected by the lack of FMRP in FXS (Irwin et al., 2001) and in the *Fmr1* KO mouse, a model of FXS (Huber et al., 2002). Astrocyte function is also thought to be altered in the *Fmr1* KO (Jacobs et al., 2010). Therefore, myelin abnormalities in FXS patients could be due to altered signaling by neurons and/or astrocytes to oligodendrocytes (OLGs). Alternatively, primary dysfunction of OLGs could also lead directly to abnormalities in myelin composition and/or function. Dysregulated translation of myelin basic protein (MBP) mRNA in the absence of FMRP could affect OLG function, especially during the period of neonatal brain development since MBP is essential for the proper formation of CNS myelin (reviewed in Boggs, 2006). FMRP has been detected in rodent oligodendrocyte progenitor cells (OPCs) and immature OLGs (Pacey and Doering, 2007; Wang et al., 2004). *Fmr1* mRNA has also been detected in the corpus callosum of adult mouse brain, though the cell type expressing *Fmr1* mRNA was not identified (Hinds et al., 1993). A putative role for FMRP in regulating MBP expression has been suggested by several studies. FMRP binds MBP mRNA both *in vivo* and *in vitro* and inhibits MBP mRNA translation *in vitro* (Darnell et al., 2011; Li et al., 2001; Wang et al., 2004). In addition, the N20.1 OLG cell line that accumulates FMRP and MBP transcripts does not express MBP, and in the CG4 OLG cell line, FMRP expression declines concomitant with differentiation into MBP-positive cells. Furthermore, FMRP expression is reported to diminish during OLG maturation and previous studies did not detect FMRP in MBP-positive OLGs either *in vivo* or in culture. These results have been interpreted to indicate that FMRP represses translation of MBP mRNA in immature OLGs during normal neonatal brain development and that decrease of FMRP allows translation of MBP mRNA in mature OLGs later in development (Wang et al., 2004).

If FMRP regulates MBP mRNA translation, MBP expression may be altered in FXS and in the *Fmr1* KO mouse, especially early in development. Changes in MBP expression and myelination early in development, even if later rectified, could significantly modify neuronal transmission in the *Fmr1* KO mouse and FXS, which could contribute to the observed behavioral and cognitive deficits (The Dutch-Belgian Fragile X Consortium, 1994). Even changes in MBP expression that do not lead to gross changes in myelin morphology could lead to altered myelin function (Martin et al., 2006). In this study we investigate the expression of FMRP in mature OLGs, the role of FMRP in regulating MBP mRNA translation, and the expression of MBP and other myelin proteins in the *Fmr1* KO mouse.

Results

FMRP is expressed in mature, MBP-positive OLGs in culture

Developmental expression of FMRP and MBP during OLG lineage progression was analyzed by Western blotting. FMRP was not detected at day 1, when the majority of cells were OPCs (Fig. 1A), but was detected at day 3 and 5, when mature OLG differentiate, and was present at a similar level at both time points (Fig. 1A). MBP was detected at day 5 but not at day 1 or 3. These results indicate that FMRP expression does not decline as cells progress from immature OLGs to mature MBP-positive OLGs in culture.

Immunocytochemistry verified that at day 5 the majority of cells in culture were mature, MBP-positive OLGs with membranous sheets (Fig. 1B). The presence of FMRP in mature OLGs was confirmed by immunocytochemistry in both mouse and rat cultures. FMRP antibody (Abcam 17722) specificity was validated by positive staining of OLGs from wild type (WT) (Fig. 1C) and absence of signal in *Fmr1* KO derived OLGs (Fig. 1D), as well as by Western blot analysis of homogenates from cerebrums of WT and *Fmr1* KO (data not

shown). Immunocytochemistry revealed co-localization of FMRP in MBP-positive cells in both WT mice and rat OLG cultures (Fig. 1C and 1E-E'', respectively). Quantification of FMRP immunostaining intensity in rat OLG cultures (Fig. 1E) showed no significant difference between mature, MBP-positive OLGs and immature, MBP-negative OLGs (Fig. 1F). These results indicate that in culture FMRP is expressed in mature rodent OLGs at levels exceeding those in OPCs and similar to those in immature OLGs.

FMRP is expressed in mature, MBP-positive OLGs *in vivo*

The presence of FMRP was also analyzed in OLGs of the corpus callosum in mice. Immunohistochemistry at P10 revealed that 2', 3'-cyclic nucleotide-3'-phosphohydrolase (CNP) and MBP-positive OLGs are FMRP-positive (Fig. 1G-G''). FMRP expression remained in MBP-positive OLGs at P24, which is during the peak period of myelination (Figure 1H-1H''). FMRP was also found in CNP-positive OLGs in adult mice (data not shown). Although FMRP was detected in MBP-positive OLGs, its level in OLGs was significantly lower than in neurons in adjacent cortex and hippocampus (data not shown). FMRP expression in OLGs in humans was also examined by immunohistochemistry of 22 week old gestational tissue. We found FMRP to be present in human MBP-positive OLGs of the subplate region (Fig 1I-I'', J-J''). These results indicate that FMRP expression by MBP-positive OLGs occurs *in vivo* and is not restricted to rodents.

FMRP is present in granules containing MBP mRNA

MBP mRNA is localized in OLG processes and their membranous sheets (Amur-Umarjee et al., 1997; Colman et al., 1982). It is transported to these locations as a component of RNA granules and is translated within or in proximity of individual granules (Ainger et al., 1993). If FMRP inhibits translation of MBP mRNA, it is likely present in the same RNA granules as MBP mRNA. To examine this possibility, co-localization of MBP mRNA and FMRP was analyzed in rat OLGs microinjected with Cy5-UTP labeled MBP mRNA, which assembles into granules (Ainger et al., 1993). Following microinjection, cells were incubated at 37°C overnight and then fixed and immunostained for FMRP. The staining intensity of FMRP in the cell body was too high to resolve individual granules or to assess co-localization with MBP mRNA. However, individual granules were resolved in the cell processes. Examination of the processes of microinjected cells revealed that $14.6 \pm 3.7\%$ (mean $\pm$ s.e.m.) of granules containing labeled MBP mRNA also contained FMRP. Some FMRP-positive and MBP mRNA-positive granules are identified with arrows in magnified views of the processes to the right of Fig. 2A-A''. These results indicate that FMRP is localized in a small subset of MBP mRNA containing granules, where MBP translation occurs.

MBP RNA translation *in vitro* is inhibited at high concentrations of FMRP

In vitro translation of Venus-MBP mRNA (full length rat MBP mRNA encoding 14 KDa MBP fused to Venus fluorescent protein) was analyzed in the presence of various concentrations of human FMRP. The concentration of Venus-MBP mRNA (8 nM) in the *in vitro* translation mixture is similar to the estimated concentration of endogenous MBP mRNA in individual granules *in vivo* and to the concentrations of other RNA in granules in neurons (Tatavary et al., 2012). *In vitro* translation of Venus-MBP was monitored at 10 min intervals for 130 min by fluorescence correlation spectroscopy (FCS). FMRP significantly inhibited translation of Venus-MBP mRNA at concentrations >168 nM but not at lower concentrations (Fig. 2B). A dose-dependent inhibition profile is shown in Fig. 2C. These results confirm previous findings (Li et al., 2001) that FMRP inhibits MBP mRNA translation *in vitro*. However, in our experiments a 12 fold molar excess of FMRP to MBP mRNA is required to inhibit translation, suggesting that this may not be physiologically relevant *in vivo*.

MBP expression in cultured *Fmr1* KO OLGs is similar to WT

If FMRP regulates translation of MBP mRNA *in vivo*, OLGs from the *Fmr1* KO mouse may express MBP earlier or may accumulate more MBP than their WT counterparts. OLG cultures derived from *Fmr1* KO and WT mouse brains were used to investigate these possibilities. In 4 days old cultures both genotypes had a similar percentage of Olig2-positive cells (a marker for all cells in the OLG lineage) that were MBP-positive (Fig. 3A, 3C). Densitometric analysis of the MBP-immunostained cells showed no differences in the level of MBP per cell in WT and *Fmr1* KO OLG cultures (Fig. 3B, 3D). These results show that the absence of FMRP does not accelerate or increase accumulation of MBP in OLGs in culture, suggesting that FMRP does not regulate MBP translation in mouse OLGs.

MBP expression is normal in *Fmr1* KO mouse brain

Although FMRP does not appear to regulate MBP expression directly in rodent OLGs in culture (Fig. 3), its absence in other neural cell types in the *Fmr1* KO CNS could lead to changes in expression of MBP and/or other myelin proteins. Using surface plasmon resonance (SPR) immunoassay, levels of MBP in cerebral hemisphere homogenates from *Fmr1* KO and WT were examined before the peak of myelination, at post-natal day 8, 12, and 15. MBP antibody was immobilized on a C5 sensor chip and homogenate from the cerebral hemispheres was flowed onto it. The concentration of MBP in homogenates from WT and *Fmr1* KO was negligible at P8, 2 nmoles/g total protein at P12, and approximately 9 nmoles/g total protein at P15 (Fig. 4A). To verify the validity of the SPR immunoassay in detecting MBP, the association and dissociation binding rates of the homogenates were compared to those of purified MBP and found to be similar (Fig. 4B). In addition, homogenate from the cerebral hemispheres of homozygous shiverer mice, which do not express MBP, was used as a negative control and showed only minimal binding (Fig. 4B). Western blots performed on homogenates of cerebral hemispheres from *Fmr1* KO and WT during the active period of myelination (P21) and after active myelination (6 weeks, 5 months) revealed no significant differences in the total amount of MBP in WT and the *Fmr1* KO (Fig. 4C). These results indicate that the absence of FMRP does not significantly affect MBP expression in mouse CNS, despite the presence of neurons and astrocytes that could potentially modulate the myelin expression program.

MBP mRNA translation in granules is not affected by FMRP in cultured OLGs

The comparable accumulation level of MBP in the *Fmr1* KO and WT, both in culture and *in vivo*, suggests that FMRP does not significantly inhibit MBP mRNA translation in OLGs *in vivo*. To investigate whether the presence of FMRP in MBP mRNA granules affects translation in OLGs, cultured rat OLGs were microinjected with Cy5-UTP labeled Venus-MBP mRNA. In these experiments, the fluorescent dye Cy5 served as a reporter for the localization of injected MBP mRNA in granules, while newly-synthesized fluorescent Venus fusion protein served as a reporter for MBP mRNA translation. Sixteen hours following injection, cells were fixed and immunostained to detect the presence of FMRP. The cells were examined by fluorescence microscopy to visualize Cy5-UTP-labeled Venus-MBP mRNA and the newly synthesized MBP (Venus-MBP) in individual granules. Analysis of translation output was done in morphologically well-resolved granules present in the OLG processes as seen in Fig. 2A". Translation output of MBP mRNA from FMRP positive and FMRP negative granules was measured by determining the fluorescence intensity of the reporter Venus in each granule. The value obtained for a granule was normalized to the amount of MBP mRNA present as determined by the fluorescence intensity of Cy5-UTP in that same granule. To examine if FMRP's effect on translation differs depending on the developmental stage of the OLG, analysis was performed at two time periods, at 2 and 3 DIC, when endogenous MBP mRNA is below its peak, and at 5–7 DIC, when MBP mRNA expression is at peak levels (Strait et al, 1997). The percentage of

granules expressing Venus-MBP was not significantly different between FMRP-positive and FMRP-negative granules at either time point (Fig. 4D). Furthermore, the level of translation was not significantly affected by the presence of FMRP in the granule (Fig. 4E). These results suggest that FMRP does not regulate MBP mRNA translation in cultured OLGs irrespective of their degree of maturation. This may occur because the amount of endogenous FMRP in individual granules is insufficient to inhibit MBP RNA translation.

To examine this possibility, the concentration of FMRP in individual granules was measured 3 hours after Venus-FMRP mRNA was injected into *Fmr1* KO OLGs. In the absence of endogenous FMRP, the amount of Venus-FMRP per granule provides an exact measure of total FMRP in the granules because all the FMRP in the granule comes exclusively from translation of the exogenous FMRP mRNA. FCS-photobleaching analysis was performed to determine the amount of Venus-FMRP in individual granules. The FCS observation volume was positioned to encompass a single granule and the decay in photon counts was measured as fluorescent FMRP in the immobile granule was photobleached. To calculate the concentration of Venus-FMRP per granule, the number of photon counts per granule was divided by the number of photon counts per Venus-FMRP molecule, previously determined by FCS of Venus-FMRP in solution. The average concentration of Venus-FMRP per granule is 13 nM (Fig. 4F). It should be noted that this concentration was not limited by the amount of Venus-FMRP present in the cell which was in excess. Next, the concentration of MBP mRNA per granule was measured in a similar way by FCS-photobleaching analysis in shiverer OLGs (which lack endogenous MBP mRNA) after injection of Cy5-UTP labeled MBP mRNA. The average concentration of MBP mRNA per granule is 5 nM (Fig. 4F). Thus the molar ratio of FMRP to MBP mRNA per granule in cultured OLGs (FMRP/MBP mRNA = 2.6), where FMRP does not inhibit MBP RNA translation, is much lower than the molar ratio (FMRP/MBP mRNA > 12) needed to inhibit MBP mRNA translation *in vitro*. This suggests that the amount of FMRP per granule may not be sufficient to inhibit MBP RNA translation in OLGs.

Expression of other myelin proteins in the *Fmr1* KO is similar to WT

Proteolipid Protein (PLP) mRNA is an FMRP target *in vitro* and *in vivo* (Darnell et al., 2011; Wang et al., 2004). Dysregulated expression of PLP, a major component of CNS myelin, could like MBP result in myelin abnormalities. The level of PLP in *Fmr1* KO and WT brain homogenates, as well as that of other important CNS myelin components, CNP, myelin associated glycoprotein (MAG), and myelin oligodendrocyte glycoprotein (MOG), was determined by Western blot. No differences were observed in the level of PLP, MAG or MOG during myelin formation in the brain (21 days) or at any later times examined (PLP, MAG, MOG, and CNP) (Fig. 5 A–D).

Discussion

It has previously been reported that FMRP is expressed in OLG progenitors and in immature OLGs, but not in mature MBP-positive OLGs. The decline in FMRP expression in mature OLGs was interpreted to indicate that FMRP inhibits MBP mRNA translation in immature OLGs, and that its absence in mature OLGs allows translation of MBP mRNA to proceed (Wang et al., 2004). Our results do not support this interpretation. Firstly, we detected FMRP expression in mature OLGs both in culture and *in vivo*. This finding is not specific to rodents as FMRP was detected in MBP-positive OLGs in human tissue. Secondly, we found that FMRP levels do not decrease as OLGs mature into MBP expressing cells in culture. Thirdly, we showed that FMRP has no effect on MBP mRNA translation in cultured OLGs and overall on MBP expression in the rodent brain. Interestingly, we found that the level of PLP, whose mRNA is reported to be a target of FMRP, was also unaffected in *Fmr1* KO CNS.

Presence of FMRP in mature OLGs is consistent with the finding that *Fmr1* RNA is present in the adult mouse corpus callosum (Hinds et al., 1993). That study did not identify the cell type harboring the transcripts, but our results suggest that at least some of these cells could be OLGs. The low level of FMRP present in OLGs as compared to that of nearby cortical neurons could be due to the correspondingly low level of *Fmr1* transcripts found in the corpus callosum (Hinds et al., 1993). The discrepancy between our results and previous reports in detecting FMRP in mature OLGs could be related to the use of an antigen retrieval method and an anti-FMRP antibody different than the ones used in previous studies.

Although FMRP is present in mature OLGs, its concentration may be too low to inhibit overall expression of some of its target mRNAs, including MBP and PLP. Examination of the intensity of FMRP immunostaining in mouse brain tissue indicates that FMRP levels in neurons of the cortex and hippocampus exceed the levels in OLGs, a finding similar to that reported for human tissue (Devys et al., 1993). We have determined that the average concentration of FMRP in granules in cultured OLGs is approximately 3 times greater than that of MBP mRNA. However, >12 fold molar excess of FMRP over MBP mRNA is necessary to inhibit translation *in vitro*.

In their study, Li et al., (2001) report that *in vitro* translation of MBP mRNA is inhibited by an equimolar concentration of FMRP. Differences in the *in vitro* translation protocol used may contribute to the differences in our results. We used a wheat germ lysate translation system while Li et al., (2001) used a rabbit reticulocyte lysate. One important distinction between the two systems is that rabbit reticulocyte lysate contains endogenous FMRP, while wheat germ lysate does not (Bardoni et al., 1999). This may have increased the actual concentration of FMRP in the Li et al., (2001) system by up to two fold. In addition, we did not pre-incubate MBP mRNA with FMRP. Pre-incubation of FMRP with target mRNA prior to its addition to the translation system has been demonstrated to significantly increase the degree of translation inhibition caused by FMRP (Li et al., 2001). Pre-incubation with FMRP prevents FMRP from binding to other components of the translation system and also gives FMRP access to MBP mRNA not bound by any ribonucleoproteins that may influence FMRP's ability to bind to MBP mRNA and inhibit its translation. Furthermore, our translation protocol used concentrations of MBP mRNA similar to the concentration found in granules from which MBP mRNA is translated *in vivo*. With the caveat that no *in vitro* translation system can exactly model *in vivo* situations, we believe that our experimental procedure more closely reflects the action of FMRP on MBP mRNA translation *in vivo*. Failure of FMRP to inhibit MBP mRNA translation in culture may also be due to its phosphorylation state in OLGs. Work done with neurons indicates that phosphorylated FMRP can inhibit translation while dephosphorylated FMRP cannot (Ceman, 2003; Narayanan, 2007). The phosphorylation state of FMRP under basal conditions in OLGs is not known.

Though the timing and the level of MBP accumulation does not appear to be regulated by the presence of FMRP in OLGs under normal conditions, the presence of FMRP in mature OLGs is significant. It suggests that FMRP may not regulate translation of all the RNAs to which it binds. In addition, *Fmr1* gene expression may play a role in other neurological disorders. For example, Fragile X Tremor Ataxia Syndrome (FXTAS) is a disorder thought to arise from a toxic gain of function of *Fmr1* mRNA. Primary dysfunction of OLGs could contribute to the demyelination found in FXTAS when *Fmr1* mRNA is expressed in OLGs.

Our study is the first to examine myelin proteins in the *Fmr1* KO mouse. In addition to the expression of MBP and PLP, other myelin components investigated were found to be unaffected in the cerebral hemispheres of the *Fmr1* KO. The possibility remains that brain region-specific differences in myelin are present that could not be detected by our assays or

that proteins, other than myelin specific proteins, that are involved in lipid metabolism for example, are affected and results in subtle changes in myelin structure. However, our results and the results of the DTI study of myelin in the P30 *Fmr1* KO (Ellegood et al., 2010) suggest that major CNS myelin specific proteins are unaffected in the *Fmr1* KO. The fact that myelin abnormalities are observed in FXS suggests that FMRP may play a more significant role in regulating expression of MBP and other myelin proteins in humans than in rodents or that myelin abnormalities found in FXS patients are due to other factors in addition to the absence of FMRP.

Materials and Methods

Animals

Mice: C57/B16 *Fmr1* knockout mice (*Fmr1* KO) were generously provided by Dr. William Greenough (University of Illinois, Urbana, IL) and maintained at the University of Connecticut Health Center (Greenough et al., 2001). Wild type of the same strain were obtained from the Jackson Laboratories and used as control. Shiverer mice (C3Fe.SWV-*Mbp^{shij}*/J, stock # 001428) were also obtained from the Jackson Laboratories. Untimed pregnant Sprague Dawley Rats were obtained from Charles River Laboratories. Procedures for animal use were approved by the Animal Care Committee of the University of Connecticut Health Center and followed federal guidelines for research with animals.

Fluorescence correlation spectroscopy *in vitro* translation (FCS-IVT)

Wheat germ lysate translation system (Promega), human recombinant FMRP (Origene, TP322699, 21–185 nM), and bovine serum albumin (BSA) (120–200 nM) were used in the *in vitro* translation assay. FCS-IVT was performed as described by Tatavirt et al. (2012). The lysate was centrifuged (10,000×g, 30 min) to remove fluorescent particulate material prior to the addition of other components. FMRP was added to the cleared lysate followed by addition of Venus-MBP mRNA. Reactions were carried out in a 96 well glass bottom dish. Newly synthesized Venus-MBP protein molecules were detected by FCS using a Zeiss Confocor 3 system with a 40X, 1.2 NA water immersion objective. FCS measurements of total photon counts in each well were taken at 10 minute intervals for 130 minutes.

Culture: Mouse oligodendrocytes

P0–P2 mice were sacrificed and mixed glia cultures were prepared from cerebral cortices as described by O'Meara et al., (2011). Cells were plated in T-75 flasks coated with poly-L-Lysine (0.1 mg/mL in borate buffer). Cortices from 3 to 4 mice were used per flask. The cells were grown in 10% fetal bovine serum/DMEM-F12 containing antibiotic and antimycotic mixture solution (Gibco 15420). OPCs were mechanically harvested and plated in growth medium on poly-L-Ornithine (.05 mg/mL in borate buffer) coated coverslips for 90 minutes. After attachment the growth medium was replaced with OLG differentiation medium (ODM) [2 mM GlutaMAX-I supplement (Gibco 35050), 30 ng/mL T3 (Sigma T6397) and 2% B-27 Supplement (Gibco 17504-044) in Neurobasal medium (Gibco 21103-049)] containing antibiotic and antimycotic mixture solution (Gibco 15420).

Rat Oligodendrocytes

Primary glia cultures were prepared from the brains of rats at postnatal days 1–3 and OLGs were isolated from these cultures as described by McCarthy and de Vellis (1980). OLGs were plated on poly-L-Ornithine (.05 mg/mL in borate buffer) coated coverslips or tissue culture dishes in the same ODM as for mouse OLGs.

Immunocytochemistry

Cells grown on coverslips were washed two times with PBS, fixed in 4% paraformaldehyde in PBS for 20 minutes and rinsed 3 times with PBS. Cells were then permeabilized with 0.5% NP-40 for 1 minute and rinsed with PBS 3 times. Cells were incubated in blocking buffer (10% goat serum in PBS) for 20 minutes followed by a solution of primary antibody diluted in blocking buffer for 30 minutes. After several rinses, secondary antibodies diluted in blocking buffer were added for an additional 30 minutes. After removal of the secondary antibodies, coverslips were washed 3 times with PBS and covered with Prolong gold anti-fade reagent. All steps were performed at room temperature. Primary antibodies included rabbit anti-FMRP (1:1000, Abcam 17722), rat anti-MBP (1:500, Millipore 386), mouse anti-CNPase (1:200, Sigma C5922) and rabbit anti-Olig2 (1:250, Millipore 9610). Fluorochrome-conjugated secondary antibodies were from Molecular Probes (Carlsbad, CA). TO-PRO-3 staining (1/500) (Invitrogen) was also performed on select coverslips.

Immunohistochemistry

Mouse brains were fixed in 4% paraformaldehyde overnight and immersed in 30% sucrose in PBS overnight at 4°C. Brains were then frozen at -80 °C and 30 µm slices were cut and attached to coated microscope slides. Antigen retrieval was performed by microwave heating tissue for 25 minutes in 0.01M citrate acid buffer, pH 6.3. Immunohistochemistry was performed as described by Morgan et al. (1992). Human brain tissue (22 week old) fixed in 4% paraformaldehyde in 0.1 M PBS, pH7.4, overnight and sectioned on a cryostat was obtained from the laboratory of Dr. Nada Zecevic. Antigen retrieval was performed on the tissue by microwave heating tissue for 25 minutes in 0.01M citrate acid buffer, pH 6.3. Tissue was washed with PBS and blocked for 1 hour with blocking buffer (1 % Tween-20, 1% BSA, and 10 % normal goat serum in PBS). Following blocking, primary antibodies diluted in blocking buffer were added to tissue overnight. Tissue was then washed with PBS and the appropriate secondary antibodies diluted in PBS were added for 2 hours. Primary antibodies used were rabbit anti-FMRP (1:1000, Abcam 17722) and rat anti-MBP (1:500, Millipore 386). Secondary conjugated antibodies were from Molecular Probes (Carlsbad, CA).

Generation of Venus-MBP expression vector

This vector was obtained from Dr. Victor Francone (Quinnipiac University). Rat MBP cDNA was PCR amplified using the primers: forward 5'-GCGAGATCTATGGCATCACAGAAGAGACCCTC-3'; reverse: 5'-CGCCCCGGGCAAGTTTATTATTTTATTTTC-3', which contain the Bgl II and SMA I restriction sites respectively. MBP DNA fragment was ligated into pEGFP-C1 (Clontech, Mountain View, CA) using Bgl II and SMA I restriction sites. GFP cDNA was replaced by Venus cDNA using modified restriction-free cloning (Geiser et al., 2001). Venus cDNA fragment was PCR amplified using the following primers: forward 5'-CACCA TGGTGAGCAAGGGCGAGGA-3'; reverse: 5'-CTTGTACAGCTCGTCCATGC-3'. Venus PCR amplification product was gel purified and used in a linear amplification reaction with recipient MBP pelf-CI plasmid under the following conditions: denaturing at 95°C for 30 sec followed by 18 cycles of 30 sec at 95°C, 30 sec at 55°C, and 12 min at 68°C, all in a final volume of 50µl. Two and a half units of Pfu Turbo DNA polymerase was used for elongation. After amplification the methylated parental plasmid was digested with 10 U DpnI at 37°C for 2h. TOPO competent cells were transformed with a 2µl aliquot of the reaction mix, and positive clones were confirmed by sequencing.

Venus-FMRP expression Vector

Obtained from Dr. John H. Carson (UCHC) (Tatavarty et al., 2012).

Synthesis of RNA

Venus-MBP mRNA was synthesized by *in vitro* transcription of linearized template DNA, capped and polyadenylated using mScript Kit (Epicentre) according to the manufacturer's protocol. Fluorescently labeled RNA was prepared by addition of Cy5-conjugated UTP (Amersham biosciences) to the transcription reaction in a 5:3 ratio with unlabeled UTP. The resulting RNA was purified by precipitation with 5 M ammonium acetate and ethanol, and was dissolved in RNase free water.

Microinjection

Cy5-UTP labeled Venus-MBP RNA (1 mg/mL) was injected into the perikaryon of cultured rat OLGs as described previously (Ainger et al., 1993), with the exception that cells remained in ODM during injection. Following injection at room temperature, cells were returned to incubate at 37°C until fixed for immunocytochemistry or analyzed by FCS-photobleaching.

Image Analysis

To measure staining intensity, images of immunostained OLGs were analyzed using Adobe Photoshop. To quantify MBP staining intensity of OLGs from *Fmr1* KO and WT cultures each cell of interest was outlined using the wand function after determining threshold between the interior and exterior of the cell. The mean intensity inside the selected area was determined using the histogram command. The mean intensity of background outside the OLG was subtracted from the mean intensity inside the OLG to give the mean intensity value of MBP staining. The average of the mean intensity of MBP staining for WT cells was normalized to 100 % MBP staining intensity. For analysis of FMRP levels in non-MBP expressing and MBP expressing rat OLGs, FMRP staining intensity was measured in non-MBP expressing OLGs and MBP expressing OLGs from the same culture as described above for MBP. The average FMRP staining intensity of the non-MBP expressing OLGs was normalized to 100%.

Translational output of Cy5-UTP labeled Venus-MBP mRNA in FMRP positive and FMRP negative granules were determined using Image J. A circle encompassing an area of 1.56 μm^2 was positioned with Cy5-UTP labeled Venus-MBP mRNA at the center. The intensity of Cy5 and of Venus was measured in the delineated area and a ratio of Venus to Cy5 was calculated for each granule. The average ratio of Venus to Cy5 for granules without FMRP (FMRP negative) was normalized to a translational output of 1 for each cell.

Surface Plasmon Resonance (SPR) Immunoassay

SPR measurements were performed using the BiacoreT100 system (GE Healthcare) as described in Rich and Myszka (2007). Ligand consisting of rat antibody to MBP (Millipore MAB 386, 1:80 in sodium acetate, pH 4.5) was immobilized on a CM5 sensor chip (Biacore) at a concentration corresponding to 6000 resonance units (RUs). Analyte consisting of purified bovine MBP (Sigma M1891) diluted in HEPES buffered saline buffer (10 mM HEPES, 3 mM EDTA, 0.25 M NaCl, and 0.05% surfactant p20, pH 7.5) or cerebral hemisphere homogenate (0.4 mg/mL to 2.2 mg/mL) diluted in HEPES buffered saline buffer was injected over the sensor surface at a flow rate of 30 $\mu\text{L}/\text{min}$ for 180 s. The RU values increased as MBP analyte binds to antibody ligand. Post-injection dissociation was monitored for 180s at a flow rate of 30 $\mu\text{L}/\text{min}$. The surface was regenerated between injections with 10mM glycine-HCl (pH2.0) at a flow rate of 30 $\mu\text{L}/\text{min}$ for 10s. The concentration of MBP in each homogenate sample was calculated based on the maximum RU minus the background for shiverer homogenate, which lacks MBP. Purified bovine MBP analyte was used as a standard to determine the molar MBP concentration in cerebral

hemisphere homogenate samples. Using the MBP molar concentration and total protein concentration for each homogenate, the concentration of MBP per gram of total protein for each homogenate was calculated.

Western Blots

All samples were homogenized in 0.3 M Sucrose/50 mM Tris-HCl, pH 7.5. Samples used to detect PLP were not heated. Samples used to detect MAG were electrophoresed under non-reducing conditions. All samples were run on 12% polyacrylamide/Bis-Tris gels and transferred electrophoretically onto Immobilon-P membranes (Millipore, Bedford, MA). Immunodetection was performed with: mouse anti-MBP (SMI-94 Covance, 1:3000), rat anti-MBP (Millipore 386, 1:3000), mouse anti-PLP (Millipore mAb 388, 1:3000), mouse anti-MAG (Millipore mAb 1567, 1:3000), mouse anti-MOG (Millipore mAb 5680, 1:3000), mouse anti- α -actin (Sigma A5441, 1:5000), mouse anti-GAPDH (Millipore MAB 374, 1:5000), and appropriate secondary antibodies conjugated to peroxidase. Western blots were visualized using chemiluminescence detection (Pierce).

Fluorescence Correlation Spectroscopy (FCS)-Photobleaching

To perform FCS-photobleaching of granules, Venus-FMRP mRNA or Cy5-UTP labeled Venus-MBP mRNA (1 mg/mL) was microinjected into the perikaryon of OLGs. After microinjection, cells were returned to 37° for three hours. FCS was performed using a Zeiss Confocal 3 system with a 63X, 1.4 NA oil immersion objective. FCS-photobleaching was then performed by positioning the FCS observation volume to encompass individual immobile fluorescent granules in the microinjected OLGs. The observation volume was excited with the proper wavelength of light for the granule being analyzed (488 nm for Venus-FMRP RNA, 633 nm for Cy5-UTP labeled Venus-MBP mRNA) for 10 seconds, which lead to photobleaching of the granule. The photon count rate decay throughout the 10 seconds was recorded. Subtracting the photon count rate after photobleaching from the count rate before photobleaching provided the total photon count rate per granule. To determine the number of molecules per granule, the total photon count rate per granule was divided by the photon count rate per molecule of RNA or protein determined by FCS of the labeled RNA or labeled protein in solution in a 96 well glass bottom dish. The number of molecules per granule was then converted to concentration by dividing the number of molecules per granule by the volume of the FCS observation volume.

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Figure 1. FMRP is expressed in mature, MBP-positive OLGs

(A) Western blots of rat OLG cultures and densitometric analysis of FMRP levels at 1 day (D), 3D and 5D in culture. Values were standardized to β -actin and means normalized to 3D. There is no significant differences in levels of FMRP between 3D and 5D ($n = 2$, mean $\pm$ sem.; t -test, $p > 0.05$). (B) TO-PRO-3 (green), MBP (red) immunostaining of rat OLGs 5 days in ODM. Scale bar, 20 μ m. (C,D) Immunocytochemistry for FMRP (green) and MBP (red) in WT (C) and *Fmr1* KO (D) OLGs. Scale bar, 20 μ m. (E-E'') Immunocytochemistry for FMRP (green) (E) and MBP (red) (E') in immature and mature rat OLGs in culture; merge (E'') Scale bar, 20 μ m. (F) Densitometric analysis of FMRP staining intensity (%) (mean $\pm$ s.e.m) in cultured rat OLGs, normalized to the intensity of MBP negative OLGs.

There is no significant difference in FMRP staining intensity between MBP negative OLGs (n= 11) and MBP positive OLGs (n= 11). (t -test, $p > 0.05$). (G-G''') Immunohistochemistry of P10 WT mouse corpus callosum with anti-FMRP (green) (G), anti-MBP (red) (G'), and anti-CNP (blue) (G''); merged images (G'''). Scale bar, 20 μ m. (H-H'')

Immunohistochemistry of P24 WT mouse corpus callosum with anti-FMRP (green) (H) and anti-MBP (red) (H'); merged images (H''). Scale bar, 20 μ m. (I-I'', J-J'')

Immunohistochemistry of the subplate region of human 22 week old gestational tissue with anti-FMRP (green) (I,J) and anti-MBP (red) (I', J'); merged images (I'',J''). Scale bar, 10 μ m.

Figure 2. FMRP localizes to granules and inhibits translation of MBP RNA *in vitro* (A-A'') Immunocytochemistry of rat OLGs for FMRP (green) (A) and microinjected Cy5-UTP labeled MBP RNA (red) (A'); merged image (A''). Cell processes are magnified (right panels) and show granules positive for both FMRP and MBP mRNA (arrows). Scale bar, 20 μ m; scale bar for A'' enlarged images, 1 μ m. (B,C) Translation of 8nM Venus-MBP RNA in wheat germ lysate was measured every ten minutes for 130 minutes using FCS. Translation was monitored by measuring the photon count rate after Venus excitation. (B) Photon count rates for each condition at 130 minutes (mean $\pm$ s.e.m) (n= 3 for all conditions except: +BSA [n= 2], and + 21–46 nM FMRP [n= 4]); means were normalized to the mean count rate of Venus-MBP without FMRP addition. FMRP significantly inhibits the translation of Venus-

MBP mRNA at concentrations greater than 168 nM (one-way *ANOVA* followed by LSD test, * $p < 0.05$). (C) Example of Venus-MBP RNA translation profiles in the presence of increasing concentrations of FMRP monitored at 10 minutes intervals.

Figure 3. MBP expression in OLG cultures from *Fmr1* KO is similar to WT

(A) OLG cultures derived from WT and *Fmr1* KO were fixed after 4 days in ODM and immunostained for Olig2 and MBP. Scale bar, 20 μ m. (B) Immunocytochemistry for MBP in WT and *Fmr1* KO OLGs at 2 days and 4 days in ODM. Scale bar, 20 μ m. (C) Average of the percentage of Olig2⁺ cells that are MBP⁺ per culture after 4 days in ODM (n= 3 cultures per genotype). There is no significant difference in the percentage of MBP⁺ cells in the *Fmr1* KO when compared to WT (*t*-test, *p* > 0.05). (D) The intensity of MBP staining (mean $\pm$ s.e.m.) of OLGs at 2D (n= 15 cells from 1 culture for WT, n= 13 cells from 1 culture for KO) and 4D (n= 38 cells from 4 cultures per genotype) is not significantly different in OLGs derived from WT and *Fmr1* KO at either time point (*t*-test, *p* > 0.05).

Figure 4. FMRP does not affect the *in vivo* accumulation and translation of MBP

(A) Accumulation of MBP (mean $\pm$ s.e.m.) in the cerebral hemispheres of P8 ($n=2$ for WT, $n=3$ for KO), P12 ($n=3$ for WT, $n=3$ for KO), and P15 ($n=3$ for WT, $n=3$ for KO) mice measured by SPR does not significantly differ at any time point between genotypes (t -test, $p > 0.05$). (B) The specificity of the SPR immunoassay for quantifying MBP was validated by measuring the association and dissociation rates of antigen binding from brain homogenates as compared to purified MBP and by examining the binding of brain homogenate from shiverer mice. (C) MBP Western blots of brain cerebrum homogenates at time periods during and after the peak of myelination (mean $\pm$ s.e.m.) ($n=6$ WT, 5 KO at 21 D, $n=3$ WT, 3 KO at 6 weeks, $n=6$ KO, 5 WT at > 5 months). Values were standardized to α -actin and means normalized to WT at each time point. There is no significant difference at any time point in levels of MBP between WT and *Fmr1* KO (t -test, $p > 0.05$). (D) Percentage of Cy5-UTP labeled MBP mRNA granules that translate Venus-MBP in granules with and without positive staining for endogenous FMRP at 2–3 DIC ($n=9$ cells from 2 cultures) and 5–7 DIC ($n=11$ cells from 2 cultures). (E) Translation output per cell (Venus-MBP intensity/ labeled MBP mRNA intensity) from granules identified as translation positive. For each cell, the average translation output per granule (mean $\pm$ s.e.m.) was normalized to the average output from FMRP-negative granules. No significant difference in translation output was found when comparing translation positive granules with and without FMRP at both time points (t -test, $p > 0.05$, $n=9$ cells from 2 cultures for 2–3 DIC, $n=10$ cells from 2 cultures for 5–7 DIC). (F) FCS photobleaching analysis of Venus-FMRP in granules ($n=82$ granules) of cultured *Fmr1* KO OLGs ($n=7$) and of Cy5-Venus MBP mRNA in granules

(n=47 granules) of cultured shiverer OLGs (n= 7) was used to determine the concentrations of FMRP (mean $\pm$ s.e.m.) and MBP mRNA (mean $\pm$ s.e.m.) in granules.

Figure 5. Levels of myelin proteins are similar in WT and *Fmr1* KO during and after the peak of myelination

Western blots of brain homogenates at time periods during and after the peak of myelination. (A) MAG (n= 2 WT, 3 KO at 21 D, n= 2 WT, 3 KO at 6 weeks); (B) MOG (n= 2 WT, 4 KO at 21 D, n= 2 WT, 3 KO at > 5 months); (C) PLP (n= 2 WT, 2 KO at 21 D, n= 2 WT, 2 KO at > 5 months); (D) CNP (n= 4 WT, 4 KO). Values were standardized to β -actin and means normalized to WT at each time point. There are no significant differences in levels of the myelin proteins between *Fmr1* KO and WT at any of the time points (mean $\pm$ s.e.m.; *t*-test, $p > 0.05$).