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FMRP and myelin protein expression in oligodendrocytes

Anthony Giampetruzzi [a], John H. Carson [b,c], Elisa Barbarese [a,*]

[a] Department of Neuroscience, University of Connecticut Health Center, 263 Farmington Avenue, Farmington, CT 06030-3401, USA
[b] Department of Molecular, Microbial and Structural Biology, University of Connecticut Health Center, 263 Farmington Avenue, Farmington, CT 06030, USA
[c] Center for Cell Analysis and Modeling, University of Connecticut Health Center, 400 Farmington Avenue, Farmington, CT 06030, USA

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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-Goraly et al., 2003; Haas et al., 2009; Hoeft et 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 concomitantly 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,

* Corresponding author. Fax: +1 860 679 8766.
E-mail addresses: Giampetruzzi@student.uchc.edu (A. Giampetruzzi), JCarson@nso2.uchc.edu (J.H. Carson), barbarese@nso2.uchc.edu (E. Barbarese).

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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 days 3 and 5, when mature OLGs 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 E–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. Immunohistochemisty 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 (Fig. 1H–H″). 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″ and 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 ± 3.7% (mean ± 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 (Tatavarty 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 day 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 and C). 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 and D). 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 days 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 nmol/g total protein at P12, and approximately 9 nmol/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