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Post-transcriptional Regulation of Myelin Basic Protein Expression

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Post-transcriptional Regulation of Myelin Basic Protein Expression

Anthony William Giampetruzzi, Ph.D.

University of Connecticut, 2014

Abstract

Myelin basic protein (MBP) is essential for the formation of myelin in the central nervous system (CNS). We have examined three factors that can potentially regulate its expression post-transcriptionally: the fragile X mental retardation protein (FMRP), the fragile X premutation transcript, and the tumor overexpressed gene (TOG) protein. We have revealed the presence of FMRP, a known translation inhibitor, in oligodendrocytes (OLGs) but found it had no effect on the translation of MBP mRNA. Correspondingly, the absence of FMRP in the *Fmr1* KO mouse did not result in increased MBP translation.

The presence in FXTAS patients of the fragile X premutation transcript, which contains expanded CGG repeats, has been associated with myelin changes in the CNS. We have found expanded CGG repeat RNA to be present in RNA granules that contain MBP transcripts. The presence of expanded CGG repeat RNA increased the expression of MBP mRNA in OLGs. In addition, increased translation of microinjected MBP mRNA occurred in fibroblasts from FXTAS individuals, suggesting that MBP mRNA translation may be upregulated in FXTAS.

Knocking out TOG in mouse OLGs results in the near absence of MBP, despite a normal level of MBP transcripts, and extensive CNS dysmyelination. Levels of other myelin proteins, proteolipid protein (PLP) and myelin-associated glycoprotein (MAG), were found to be similarly reduced. We have determined that TOG heterozygote (+/null) mice, which express half the normal level of TOG, have normal MBP levels before 45 days of age. This suggests that another mechanism, besides the originally proposed decrease in MBP mRNA translation, is the

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cause of MBP deficiencies in the 90 day old TOG heterozygotes. TOG is a microtubule-associated protein and may be necessary for the transport of MBP mRNA granules to the myelin compartment where translation occurs. The effect of the reduction of TOG on microtubule based transport was examined by measuring the transport parameters of mitochondria in the processes of cultured OLGs. The transport of mitochondria was only slightly altered in the TOG heterozygote. These results suggest that approximately half the amount of TOG allows for sufficient transport and proper translation of MBP mRNA.

Post-transcriptional Regulation of Myelin Basic Protein Expression

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B.A., Clark University, 2003

M.A.T., Clark University, 2004

A Dissertation

Submitted in Partial Fulfillment of the
Requirements for the Degree of Doctor of Philosophy
at the
University of Connecticut

2014

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Anthony William Giampetruzzi

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APPROVAL PAGE

Doctor of Philosophy Dissertation

Post-transcriptional Regulation of Myelin Basic Protein Expression

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University of Connecticut

2014

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List of Abbreviations

Arc- activity-regulated cytoskeleton-associated protein
cKO- conditional knockout
CNP- 3', 5'-cyclic-nucleotide phosphohydrolase
CNS- central nervous system
CPE- cytoplasmic polyadenylation element
CPEB- cytoplasmic polyadenylation element binding protein
DTI-diffusion tensor imaging
FMRP- fragile X mental retardation protein
FXS- fragile X syndrome
FXTAS- fragile X-associated tremor/ataxia syndrome
GFAP- glial fibrillary acidic protein
hnRNP- heterogeneous nuclear ribonucleoproteins
KO- knockout
MBP- myelin basic protein
MAG- myelin-associated glycoprotein
MOG- myelin oligodendrocytic glycoprotein
MOBP- myelin-associated oligodendrocytic basic protein
MRI- magnetic resonance imaging
OLG- oligodendrocyte
OPC- oligodendrocyte progenitor cell
PLP- proteolipid protein
sncRNA- small non-coding RNA
TOG- tumor overexpressed gene protein
UTR- untranslated region
WT- wild type

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Chapter 1: Introduction

The myelin membrane which surrounds axons plays a vital role in the nervous system. In the central nervous system (CNS), myelin basic protein is a major component of myelin. MBP expression can be regulated post-transcriptionally. Based on published literature, we examined three possible factors involved in the post-transcriptional regulation of MBP expression. These factors are the fragile X mental retardation protein (FMRP), the fragile X premutation RNA present in fragile X-associated tremor/ataxia syndrome (FXTAS), and the tumor overexpressed gene (TOG) protein. An overview of these factors, MBP, and myelin is presented in this chapter. Chapters 2-4 provide the results of our examination into the post-transcriptional regulation of MBP expression by the three factors.

Myelin

Myelin and Oligodendrocyte Functions

In the CNS, many axons are surrounded by a multilamellar membrane termed myelin, which is produced by oligodendrocytes (OLGs). Axons are myelinated by multiple OLGs and one OLG can myelinate numerous axons, up to approximately 40 (Quarles et al., 2006). Myelin wrapping of an axon facilitates saltatory conduction of action potentials down the axon. It does so by acting as an electrical insulator and leading to the clustering of sodium channels at the unmyelinated regions of the axons, known as the Nodes of Ranvier. The myelin membrane has a high lipid to protein ratio (70% to 30%) and low water content (40%), which contributes to its ability to be a good insulator. The thickness of the myelin sheath further improves its ability to be an insulator: on average the myelin sheath surrounding axons in the rodent brain stem is 320 nm (Chomiak and Hu, 2009). Saltatory conduction is much faster than conduction in non-myelinated fibers and allows the axon to use less energy and inhabit less space than an unmyelinated axon. For instance, if a myelinated frog axon with a diameter of 12 μm was conducting at 25 m/s, an unmyelinated axon of a giant squid would require approximately 15,000 times the space and 5,000 times the energy to conduct at the identical speed (Quarles et al., 2006).

Myelin and OLGs play other roles in support of axons. Myelination is involved in regulating phosphorylation of axonal neurofilaments and expression of axonal cytoskeletal components (Brady et al., 1999). Abnormalities in myelin sheaths cause alterations in axonal microtubules and change axonal transport rates (Bradl and Lassmann, 2010). Myelin also provides metabolic support for axons and protects against axonal degeneration (Fünfschilling et al., 2012; Yin et al., 2006). For example, lactate and pyruvate are transported from OLGs to myelinated axons, and used by axons when energy levels are low (Fünfschilling et al., 2012).

Myelin proteins are also involved in the inhibition of axon sprouting (Schwab and Thoenen, 1985; Dawe et al., 2006). The proposed role of this is to repress axon sprouting during development, therefore inhibiting the formation of new synapses after proper connections have been established (Fields, 2008). These proteins have the same role in preventing axonal outgrowth in response to axonal injury (Bregman et al., 1995; McKerracher et.al., 1994). Interestingly, OLGs serve functions that support the axon that do not act through myelin. OLGs provide trophic support to axons by synthesizing factors such as glial cell line-derived neurotrophic factor (GDNF), insulin-like growth factor 1 (IGF-1), and brain derived neurotrophic factor (BDNF) (Smith et al., 2013; Bradl and Lassman, 2010). Finally, the wrapping of axons by OLGs can regulate axon caliber size during development independently of myelin gene expression (Sanchez et al., 1996).

CNS Myelination

CNS myelination is a very precise process in which axons are ensheathed by myelin membrane produced by OLGs. Myelination occurs in a rostral to caudal direction in the spinal cord and in a caudal to rostral direction in the brain. In the cerebral hemispheres of rodent brain, myelination begins around postnatal day 12, with its most active period of myelination between days 15 and 40 (Fig. 1-1C) (Kanfer et al., 1989). In humans, myelination begins in the fetus, but reaches its peak in the brain during the first postnatal year (Baumann and Pham-Dinh, 2001). Myelination continues into the third decade of life in some areas of the human brain (Figure 1-1B) (Yakovlev and Lecours, 1966). This ability to myelinate late into life, which is not present in other species including rodents and chimpanzees, suggests myelin may play a significant role in optimizing information processing by experience (Figure 1-1B) (Miller et al., 2012; Fields 2008).

Myelination is typically studied in rodents due to their availability. In addition to the differences

in timing of myelination mentioned previously, there are several other main differences between rodents and humans that should be taken into consideration when extrapolating myelin studies from rodents to humans. First, some brain regions that can be myelinated in the human, such as neocortical regions, are lacking in mice brain. While others, such as the olfactory bulb, are overdeveloped in mice compared to humans. Additional sources of OLG progenitor cells (OPCs) in the brain may also be present in the human brain. As will be discussed further, humans and rodents express different isoforms of myelin basic protein (MBP) during adulthood. Finally, OLG lineage cells from humans and rodents have been shown to respond differently to certain factors (Bradl and Lassmann, 2010; Jakovcevski et al., 2009). In chapter 2, myelin changes present in fragile X syndrome (FXS) were found not to be mimicked in the mouse model. When evaluating these results it is important to be mindful of the differences in myelination between humans and mice.

Myelination is tightly regulated, both temporally and spatially. For instance, social deprivation during a critical period of development in the mouse leads to hypomyelination in the prefrontal cortex. Return of the mice to a social environment after that critical time does not restore myelination (Makinodan et al., 2012). In addition, the age of human fetuses can be accurately deduced based on which pathways are myelinated (Baumann and Pham-Dinh, 2001).

OLG differentiation occurs in distinct stages and begins with OPCs, which in the rodent cerebral hemispheres originate in the subventricular zone (SVZ). OPCs are bipolar, migratory, and proliferative. As they migrate from the SVZ, clusters of OPCs line along axonal tracts that will become myelinated. There is evidence that this alignment may be mediated by astrocytes *in vivo* (Meyer-Franke et al., 1999). Some of the OLG progenitors in the cluster will eventually adhere to axons and differentiate into myelinating OLGs. Axon signaling to the OLG is thought to regulate OLG differentiation leading to myelination of that axon. This process is discussed in the section below.

OLG differentiation occurs in stages which can be identified by the expression of specific proteins (Fig. 1-2). As previously stated, OLGs begin as OPCs. They then transform into pre-oligodendrocytes, which are still proliferative but much less mobile. *In vivo*, this transformation occurs to cells lined up along future white matter tracts. Upon proper signaling the pre-oligodendrocyte can become an immature OLG, with increased processes and the expression of the first myelin specific protein, 3', 5'-cyclic-nucleotide phosphohydrolase (CNP). These cells eventually will possess MBP mRNA but not express MBP (Zeller et al., 1985). One to two days after the first appearance of MBP mRNA, MBP, myelin-associated glycoprotein (MAG), and proteolipid protein (PLP) can be detected and the OLG will be considered a non-myelinating mature OLG. When OLGs form compact myelin sheaths along axons they are then mature, myelinating OLGs; these cells now express the myelin oligodendrocytic glycoprotein (MOG) (Baumann and Pham-Dinh, 2001). Differentiation of OLGs in culture mimics that of *in vivo*, even without the presence of neurons (Temple and Raff, 1986).

Electrical activity of neurons strongly affects myelination, both during the onset of myelination and throughout life. It appears to regulate whether or not an axon becomes myelinated, it may also regulate the extent of myelination on the axon. OPC proliferation in the rat optic nerve is dependent on neuronal activity, as is myelination (Demerens et al., 1996; Barres and Raff, 1993). Mice maintained without light since birth have a reduced number of myelinated axons in the optic nerve, and premature opening of eyes in neonatal rabbits causes premature myelination (Tauber et al., 1980; Gyllenstein and Malmfors, 1963). *In vitro*, the induction of neuronal activity promotes OLG survival, differentiation, and axonal myelination (Gary et al., 2012; Ishibashi et al., 2006; Stevens et al., 2002). Activity is thought to trigger myelination in the human brain as well. Magnetic resonance imaging (MRI) of humans has revealed that early experience in newborns leads to increased myelination (Als et al., 2004). On the other hand, a reduction in corpus callosum area is present in children who are victims of neglect (Teicher et

al., 2004). Comparison of brain tracts associated with musical performance in pianists and non-musicians showed that playing the piano accompanies an increase in myelination of those tracts and the number of hours practiced in childhood correlates with the extent of the myelin increase (Bengtsson et al., 2005).

Signals regulating myelin synthesis

Extracellular signaling to OLGs

In vitro, OLGs can differentiate and express MBP and other myelin proteins in the absence of axons. However, *in vivo* OLG differentiation and myelination is believed to be regulated by external signals, including the axon. Studies indicate that in the CNS the size of an axon may determine whether or not it is myelinated, which may be independent of any specific signaling from the neuron (Lee et al., 2012; Rosenberg et al., 2008). Experiments performed in zebrafish CNS stress the impact axons have on myelination. Zebrafish CNS has two very large axons, called the Mauthner axons, which are myelinated first. When genetically mutated zebrafish with an increased number of Mauthner axons were examined, the extra Mauthner axons were found to be myelinated properly and myelination was not accompanied by an increase in OLG number. Already existing OLGs were producing more myelin sheaths than normal, which shows the ability of axons to influence the myelinating potential of an OLG (Almeida et al., 2011). It is not known if Mauthner axons express signals which promote myelination, but signaling molecules have been identified on other axons that lead to myelination. One such protein is neuregulin-1. It communicates with OLGs by binding to ErbB receptors on OLGs. Hypomyelination occurs in mice with disruption in neuregulin-1-ErbB signaling and overexpression of neuregulin-1 can cause small caliber axons not typically myelinated to become myelinated (Makinodan et al., 2012; Brinkman 2008). Neuregulin-1 expression is

regulated by neuronal activity and myelination *in vitro* occurs preferentially on electrically active axons (Liu et al., 2011; Wake et al., 2011). Additional signaling molecules expressed on the cell surface have been shown to participate in communication between axons and OLGs and regulate myelination. These include the expression of polysialylated-neural cell adhesion molecule (PSA-NCAM) and Jagged1 on neurons and Notch1 and LINGO-1 on OLGs (Mi et al., 2005; Charles et al., 2000; Wang et al., 1998).

Several other signals influence the onset of myelination. For instance, the astrocytic tissue inhibitor of metalloproteinase-1 (TIMP-1) promotes the differentiation of OLGs and leukemia inhibitory factor (LIF) produced by astrocytes in response to neuron electrical activity promotes myelination in co-cultures (Moore et al., 2011; Ishibashi et al., 2006). In addition, extracellular matrix proteins, such as laminins, and growth factors secreted by neurons and astrocytes, such as fibroblast growth factors, also can influence differentiation (Relucio et al., 2009; Bansal 2002).

Intracellular Signaling to OLGs:

Intracellular pathways that incorporate the external signals that lead to myelination are present in OLGs. One intracellular signaling molecule that has been examined is Fyn kinase. Fyn is activated by phosphorylation and dephosphorylation events at different sites on the molecule (Laursen et al., 2009). There are numerous regulators of Fyn function, with glutamate released from electrically active axons as a potential additional regulator (Ahrendsen and Macklin, 2013; Wake et al., 2011). Mice deficient in Fyn do not develop myelin properly and have defects in MBP transcription and translation. The mitogen-activated protein kinase (MAPK)/extracellular related kinase (ERK) pathway along with the PI3 kinase/Akt/mTOR pathway in OLGs have also been proven to play a major role in myelination (Ahrendsen and Macklin, 2013).

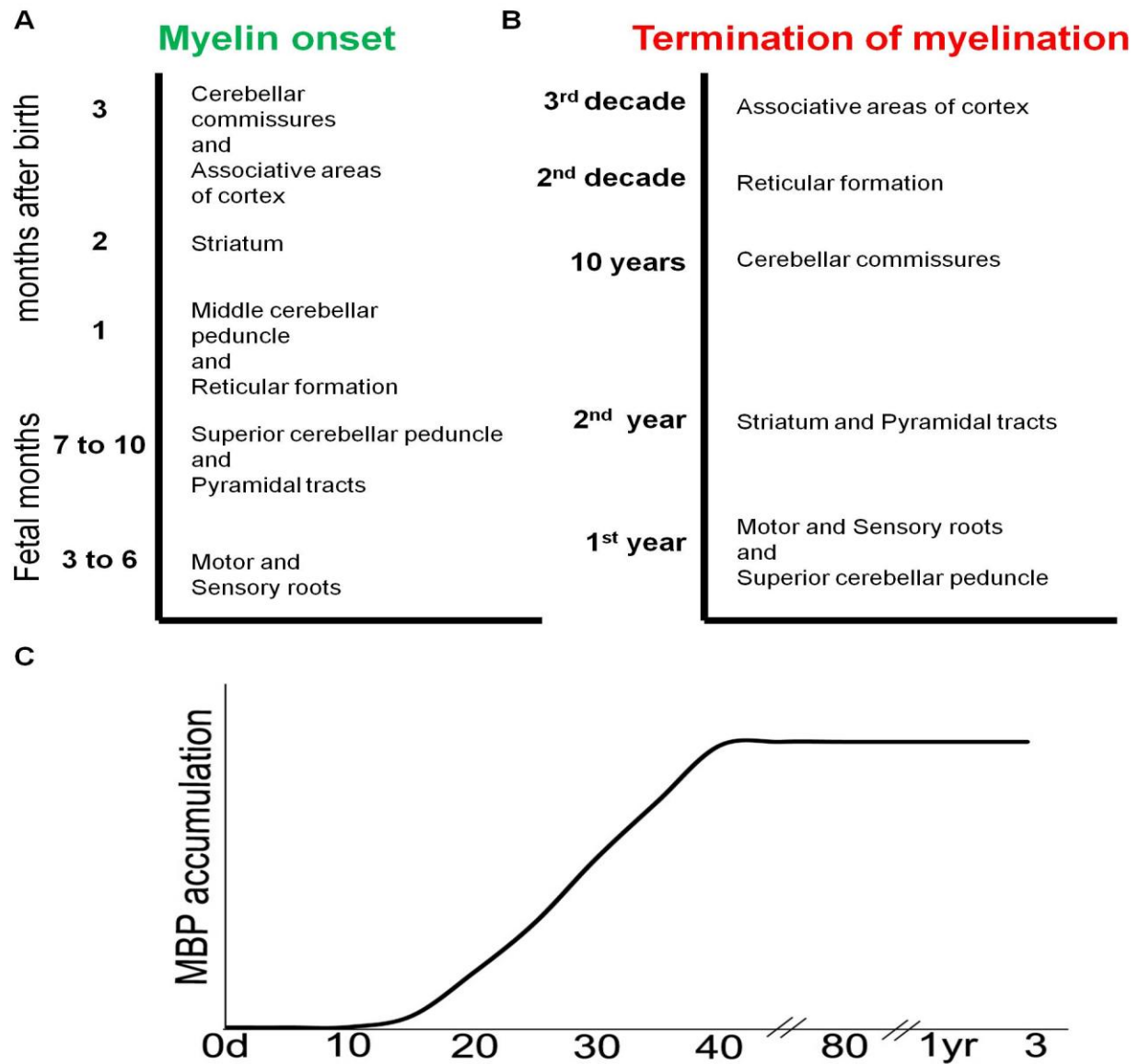


Figure 1-1: Timing of myelination in humans and rodents. (A) Timing of onset of myelination in different areas of human CNS (adapted from Yakovlev and Lecours, 1966). (B) Timing at which myelination terminates in different areas of the human CNS (adapted from Yakovlev and Lecours, 1966). (C) MBP accumulation, which correlates with myelination timing, in the rodent cerebral hemispheres (adapted from Delassalle et al., 1981).

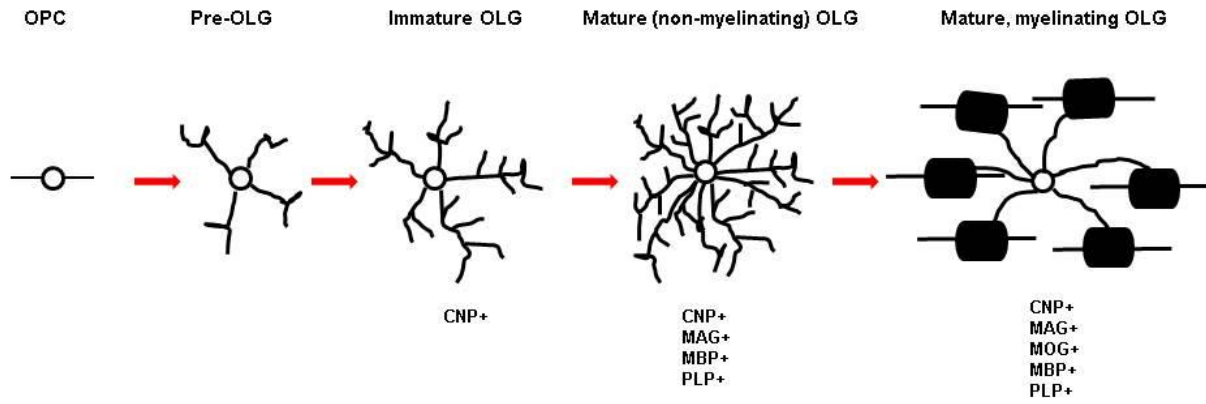


Figure 1-2: Developmental stages of OLG lineage cells. Myelin specific proteins that can be detected at each stage of development are listed. The pattern of OLG development is similar *in vitro* and *in vivo*.

Myelin and human disease

The importance of proper myelin function is evident in disorders of myelin. Myelin disorders in humans can lead to sensory-motor problems, paralysis, cognitive impairment, mental retardation, seizures, and death (Fields, 2008). They can be caused by the failure to form myelin, termed dysmyelination, or by demyelination, which is the breakdown of myelin. As expected, genetic mutations that directly affect OLGs result in myelin disorders. Such diseases include Canavan's Disease, 18q-syndrome, and Pelizaeus-Merzbacher Disease (Kumar et al., 2006; Mimault et al., 1999; Kolodny, 1993). White matter disorders can be caused by other factors in which OLG dysfunction is not primary. In Niemann–Pick Type C, neurons are dysfunctional due to impairment in internal cholesterol transport. Faulty signaling from the axon to the OLG is thought to be a cause of the myelin phenotype in that disorder (Takikita et al., 2004). Alexander's disease results in myelin abnormalities due to astrocytic impairment caused by a mutation in glial fibrillary acidic protein (GFAP) (Mignot et al., 2004). Breakdown of the blood brain barrier through inflammation or hypertension can also be responsible for myelin problems (van Dijk et al., 2004). Multiple sclerosis may be the most common known of all myelin disorders. It presents with demyelination in the CNS and is believed to be an autoimmune disorder. Neurological symptoms in multiple sclerosis usually correspond with the demyelinated region, such as optic nerve myelin lesions leading to vision loss or spinal cord lesions leading to bladder dysfunction (Keegan and Noseworthy, 2002).

Myelin and Cognition

Neuronal activity is a strong regulator of myelination and experiences which trigger neuronal activity help shape the myelin makeup of our brains. Thus, myelin is assumed to be important for learning and cognition. Recent evidence supports this assumption. For instance, MRI has revealed differences in myelin in individuals with autism, and a correlation between cognitive

processing speeds and myelin integrity was found in elderly individuals (Lu et al., 2013; Hoeft et al., 2010). In addition, polymorphisms in myelin genes and reduced levels of transcripts involved in OLG differentiation and survival have been linked to depression, schizophrenia, and other disorders (Fields, 2008). The proposed mechanism through which myelin influences cognition is by changing the conduction velocity of impulses through axons. A main idea in synaptic plasticity is that synaptic inputs that arrive together in the post-synaptic neuron are retained and strengthened, while non-coincident inputs are eliminated. Altering of the conduction velocity by changes in myelin could lead to differences in the timing at which inputs arrive together and therefore alter connectivity in the brain (Fields 2008; Nave 2010).

Myelin Basic Protein (MBP)

MBP is one of the two major proteins that make up myelin in the CNS, along with PLP. MBP is a myelin specific protein which constitutes about 30% of the total protein in CNS myelin and is expressed at much lower levels in the PNS (Baumann and Pham-Dinh 2001; Boggs, 2006a). MBP plays a more prominent role in the CNS than in the PNS. In the CNS, MBP is synthesized only by OLGs and its expression follows the timing of myelination, as described in the previous section (Fig. 1-1C). MBP mRNA appearance precedes that of MBP. At the very early stage of myelination MBP mRNA is restricted to the OLG cell body. However, as myelination proceeds MBP mRNA is present throughout the OLG processes (Verity and Campognoni, 1988).

MBP is transcribed from the *GOLLI* (Genes of Oligodendrocyte Lineage) gene complex that contains 10 exons in humans and 11 exons in mice, seven of which give rise to MBP (Givogri et al., 2001; Pribyl et al., 1993). The exons giving rise to MBP are highly conserved between mice and humans (Pribyl et al., 1993). Due to alternative splicing, different isoforms of MBP are present both in rodents and humans (Fig. 1-3). All MBP transcripts have exon 7, which contains the 3' untranslated region (UTR) that is crucial for proper MBP mRNA translation. Several experiments reported in the following chapters were performed using mRNA for the 14 kDa isoform of rat MBP mRNA. The 3' UTR of this mRNA is present in all isoforms. The expression of MBP isoforms changes during development, as the exon II containing isoforms are more highly expressed prior to the onset of myelination and the forms lacking exon II are more highly expressed after (Barbarese et al., 1978). In the rodent, the 14 kDa and 18.5 kDa isoforms are the isoforms predominantly expressed in adults while in humans the 18.5 kDa is most common (Quarles et al., 2006; Baumann and Pham-Dinh, 2001; Kamholz et al., 1986; Campagnoni and Macklin, 1988). All isoforms are present in compact myelin, though isoforms lacking exon II can also locate to the nucleus (de Vries et al., 1997; Pedraza, 1997). However, exon II is neither

required nor deleterious to compact myelin formation as transfection of exon II-negative MBP (14 kDa) or exon II-positive MBP (17.2 kDa) leads to similar restoration of myelination in a mouse model lacking MBP, the *shiverer* mouse (Kimura et al., 1998). The myelin level was lower in the transgenic mouse than in the heterozygote *shiverer* mouse, despite similar amounts of MBP mRNA, suggesting all MBP isoforms are important for proper myelin formation.

Analysis of rat brain has shown MBP to decay with rates of two different components. MBP that decays at the fast component rate has a half-life of approximately 3 weeks, while MBP decaying at the rate of the slow component is stable (Sabri et al., 1974). Measurements of MBP mRNA stability in P30 rodent brain slices showed no decay in MBP mRNA over 12 hours, indicating that its half-life is greater than 12 hours (Mathisen et al., 1997).

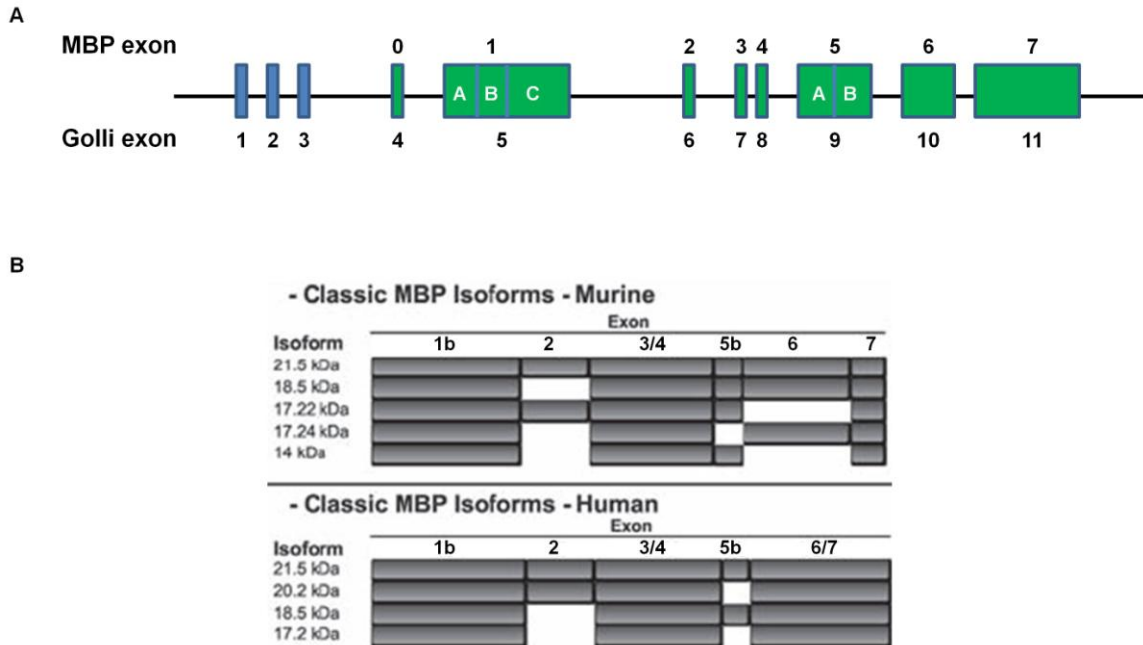


Figure 1-3: MBP isoforms created by alternative splicing. (A) Rodent *Golli* gene with the original exon numbering of MBP above and that for the *Golli* gene below (adapted from Quarles et al., 2006). (B) Alternate splice variants of MBP in murine and humans (adapted from Boggs, 2006a).

Functions of MBP

MBP's main function in CNS myelin is the formation of compact myelin. The *shiverer* mouse, in which MBP is not expressed due to a large deletion in the MBP gene, lacks compact CNS myelin (Readhead and Hood, 1990). The Long Evans shaker rat, which has undetectable levels of MBP due to a mutation in the MBP gene, also does not form compact CNS myelin (O'Connor et al., 1999). In the formation of compact myelin, the external faces of the plasma membrane appose each other to form the double intraperiod line. That is followed by the apposition of the cytoplasmic sides of the membrane to form the major dense line and then the cytosol is extruded (Baumann and Pham-Dinh, 2001). MBP, which is positively charged, is located on the cytoplasmic side of the membrane (Fig. 1-4). It is thought MBP may compact the membrane by coupling the cytoplasmic sides through its binding to the negative membrane

lipids (Harauz et al, 2004; Campagnoni and Skoff, 2001). Crosses of different mouse models with the disruption of the MBP gene were made to create mice with differing levels of MBP transcript and MBP. When MBP levels were below 50% of control, the thickness of the myelin sheath and the number of myelinated axons paralleled the level of MBP. At protein levels between 50% and 100% of control, the thickness of the myelin sheath and number of myelinated axons remained constant. This suggests that MBP levels 50% of normal are sufficient for proper myelin thickness and for the proper quantity of axons to be myelinated (Shine et al., 1992). Despite the normal appearance of myelin, MBP levels of 50% of control slightly alter myelin function (Martin et al., 2006).

MBP potentially plays many other roles besides compaction of the myelin membrane. MBP has been suggested to be involved in the neuronal dependent clustering of galactosylceramide (GalC) in plasma membranes of OLGs (Fitzner et al., 2006). Wild type OLGs when co-cultured with neurons display a clustering of GalC in the plasma membrane that is not observed in OLGs of *shiverer* mice, which lack MBP (Fitzner et al., 2006). PLP expression is also affected by MBP, as levels of PLP mRNA in the *shiverer* mouse are reduced to less than 55% of controls (Sorg et al., 1987). In addition, MBP colocalizes with voltage gated calcium channels in OLGs and may be involved in regulating calcium influx into OLGs (Smith et al., 2011). MBP is also capable of interacting with the cytoskeleton of OLGs. Actin can be bound by MBP *in vitro* and MBP has at least two actin binding sites (Hill and Harauz, 2005; Roth et al., 1993). MBP can also bind tubulin and cause bundling of microtubules and tubulin polymerization (Hill and Harauz, 2005; Modesti and Barra, 1986). *Shiverer* mice do not display colocalization of microtubular structures and actin filaments as found in wild type and they also display microtubules with a distorted sizes and smaller processes (Dyer et al., 1995). MBP is also involved in signaling in the OLG. For instance, cultured *shiverer* OLGs do not differentiate as well in the presence of growth factors as wild type (Seiwa et al., 2002). Treating cultured wild

type OLGs with GalC antibody resulted in decreased MBP phosphorylation and loss of microtubule structure, neither of which are found when treating *shiverer* OLGs (Dyer et al., 1994). Finally, MBP has been shown to partially colocalize with actin at the edge of OLG membrane sheaths, suggesting MBP may act as messenger between the two (Boggs et al., 2006b).

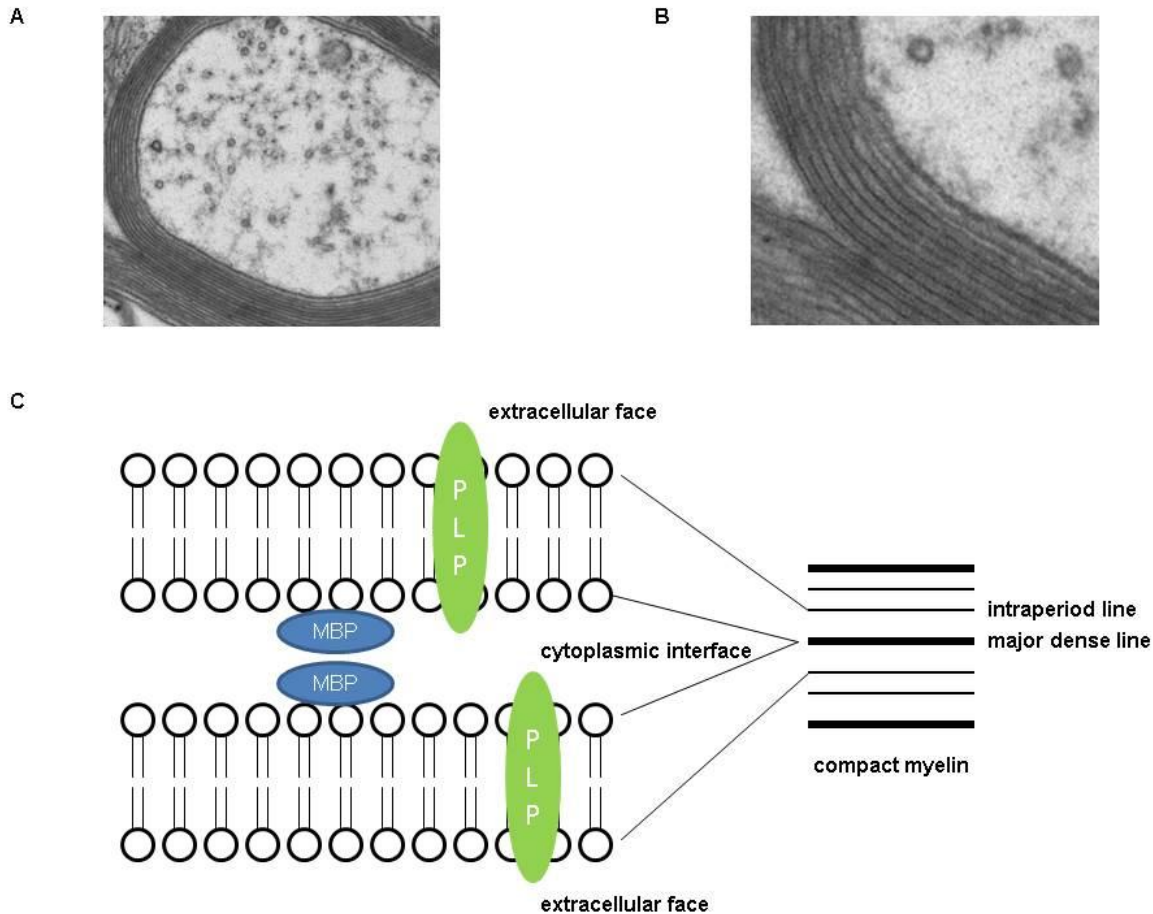


Figure 1-4: MBP and compact myelin. (A) Myelinated axon in the mouse optic nerve (image provided by Dr. Elisa Barbarese). (B) High magnification of compact myelin sheath shown in A. Darker lines are major dense lines. Major dense lines are formed by the compaction of the cytoplasmic side of the myelin membrane, where MBP is present. MBP is necessary for compact myelin in the CNS. (C) Cartoon depicting MBP and PLP in the CNS myelin membrane. The extracellular face of the membrane is called the intraperiod line. There are two intraperiod lines between each two major dense lines.

Mutations in the MBP gene

18q-syndrome

There is no known human disorder in which MBP is absent. However, some patients with 18q-syndrome have only one copy of the MBP gene. 18q-syndrome is caused by the deletion of part of the long arm of chromosome 18, which comprises the MBP gene. Gay et al. (1997) examined white matter of a 18q-syndrome patient with both copies of the MBP gene and that of patients with only one copy of the gene. Interestingly, they found that the patient with both copies of the gene did not exhibit the incomplete myelination that was present in those individuals with one copy of the gene (Gay et al., 1997). This suggests that reductions in MBP expression in humans can lead to myelin abnormalities. Whether or not those abnormalities relate to behavioral changes could not be ascertained from this study as patients displayed similar behaviors, such as mental retardation, regardless of the MBP gene copy number.

Mouse Models involving MBP mutations

The *shiverer* mouse is a result of a deletion in the MBP gene that leads to virtually no expression of MBP. The *shiverer* mouse presents with intention tremor starting around P11, is subject to seizures, displays learning deficits, and dies around five months of age (Readhead and Hood, 1990; Inagawa et al., 1988). *Shiverer* mice display hypomyelination and the absence of compact myelin. Their axons are smaller and changes to the organization of the axonal cytoskeleton are present (Brady et al., 1999). However, there is no axonal degradation (Griffiths et al., 1998). Though MBP is expressed in both the PNS and CNS, the absence of MBP from the PNS does not lead to noticeable myelin changes (Kirschner and Gansler, 1980). Mice that are heterozygous for the *shiverer* mutation (+/shi) have half the amount of MBP protein, but their myelin appears normal. They do not have obvious motor/locomotion abnormalities despite a slight decrease in conduction velocity (Martin et al., 2006; Shine et al. 1992).

MBP mRNA transport and translation

MBP mRNA and hnRNP A2 containing granules

In mature OLGs, subcellular fractionation and *in situ* hybridization has revealed that MBP mRNA is found in the myelin compartment of OLG processes where expression of MBP is much greater than in the perikaryon (Trapp et al., 1987; Colman et al., 1982). Microinjection of MBP mRNA has shown that MBP mRNA is localized to the myelin compartment via a multi-step pathway. That pathway begins with RNA assembling into granules in the perikaryon. Granules are then transported along microtubules of the processes until they are properly localized in the myelin compartment (Ainger et al., 1993). Granules are non-membranous complexes with a radius of approximately 0.7 μm (Barbarese et al., 1995). *In situ* hybridization and immunofluorescence of cultured OLGs has revealed that MBP mRNA containing granules contain, among others, the molecular motors kinesin and dynein and the machinery necessary for protein synthesis, such as ribosomes, aminoacyl t-RNA synthetases, and the elongation factor EF1 (Carson et al., 2001; Barbarese et al., 1995). Despite the presence of translational machinery, evidence suggests that the translation of RNAs in granules is suppressed until the RNAs reach their final destination (Kosturko et al., 2006). Granules travel to their destinations in a microtubule-dependent process, as transport of microinjected MBP mRNA was inhibited in OLGs treated with taxol to stabilize microtubules or nocodazole to disrupt microtubules. Transport does not necessitate microfilaments, as disruption with cytochalasin B had no effect on the translocation of MBP mRNA (Carson et al., 1997).

RNAs assemble into specific granules depending on their intended final destination. For instance, granules containing RNA for soluble proteins, such as globin, stay in the perikaryon. PLP mRNA or RNA of other membrane proteins are found in granules destined for the

endoplasmic reticulum (Carson et al., 2001). The type of granule a RNA incorporates into depends on the *cis*-acting elements in the RNA and the *trans*-acting elements in the cell (Carson et al., 2001). MBP mRNA contains two tandemly duplicated *cis*-acting sequences termed the A2 Response Element (A2RE) in its 3' UTR (Fig. 1-5). Heterogeneous nuclear ribonucleoprotein A2 (hnRNP A2) binds to the A2RE and allows MBP mRNA to assemble into hnRNP A2 containing granules (Munro et al., 1999; Ainger et al., 1997; Hoek et al., 1998). HnRNP A2 containing granules have been estimated to contain around 30 molecules of RNA (Mouland et al., 2001). The MBP mRNA A2RE in humans is homologous to that of rodents and the sequence of hnRNP A2 is highly conserved in human, rat, and frog (Ainger et al., 1997; Brumwell et al., 2002).

HnRNP A2 has four different isoforms (A2, A2b, B1, and B1b). The A2b isoform is the predominant cytoplasmic isoform in OLGs and neuronal cells and is the major isoform responsible for the transport of A2RE containing mRNAs (Han et al., 2010). HnRNP A2 can dimerize through its glycine-rich dimerization domain and contains a nuclear localization domain and two RNA binding domains (RBD1 and RBD2), with the RBD2 domain necessary for proper targeting of hnRNP A2 to the OLG periphery (Brumwell et al., 2002). The specific binding of hnRNP A2 to the A2RE is formed by the tandem RBDs and its proximal C terminal region. HnRNP A2 is also capable of binding more weakly and non-specifically to RNA through a region different than A2RE (Shan et al., 2000). In neurons, hnRNP A2 colocalizes in granules with A2RE containing RNAs 75% of the time and non-A2RE containing RNAs 25% of the time (Gao et al., 2008). HnRNP A2 is present in the nucleus, the perikaryon, and in the periphery of OLGs, though its expression in the nucleus is over 20 times greater than in the cytoplasm (Brumwell et al., 2002).

Several studies have emphasized the importance of MBP mRNA binding to hnRNP A2 through the A2RE for proper localization of MBP mRNA to OLG processes. First, microinjection of MBP

mRNA with and without the A2RE demonstrated the necessity of the A2RE for localization of MBP mRNA (Ainger et al., 1997). Second, the addition of the A2RE sequence to the 3' UTR of green fluorescent protein (GFP) RNA led to a significant increase in GFP RNA traveling to OLG processes (Ainger et al., 1997). Third, mutations in the A2RE which hinder A2 binding caused decreased localization of A2RE-GFP RNA to OLG processes, and OLGs treated with hnRNP A2 siRNA showed MBP mRNA confined to the cell body (Laursen et al., 2011; Munro et al., 1999).

There are several other proteins in the brain whose RNAs are known to contain A2RE sequences. In OLGs, one of several transcripts for myelin-associated oligodendrocytic basic protein (MOBP) contains an A2RE-like sequence and localizes to the myelin compartment (Carson et al., 2001). RNAs transported to dendrites in neurons, such as activity-regulated cytoskeleton-associated protein (Arc) and α -cam kinase II, also have A2RE sequences. Their proper localization has been shown to be dependent on their A2RE and binding of hnRNP A2 (Gao et al., 2008).

Regulation of MBP mRNA translation through the 3'UTR

In addition to the binding of hnRNP A2, the 3' UTR of MBP mRNA can regulate MBP translation in several other ways. First, the 3'UTR of MBP mRNA contains a polyadenylation sequence AAUAA and cytoplasmic polyadenylation element (CPE), which are thought to be necessary for cytoplasmic polyadenylation (Fig. 1-5) (Mendez et al., 2000). The cleavage and polyadenylation specificity factor (CPSF) protein binds the AAUAA and is responsible for recruitment of poly(A) polymerase (Dickson et al., 1999). The cytoplasmic polyadenylation element binding protein (CPEB) binds to the CPE and phosphorylation of the CPEB leads to polyadenylation and translation of RNA in oocytes (Mendez et al., 2000). Examination of neurons suggests that cytoplasmic polyadenylation through CPEB phosphorylation occurs similarly in neuronal RNAs

with a CPE sequence (Du and Richter, 2005). Though further work is needed to assess this possibility in OLGs, regulation of cytoplasmic polyadenylation of the MBP transcript through sequences in its 3' UTR may act in concert with other elements to modulate MBP translation.

The 3'UTR contains several repeats of an hnRNP-K sequence that binds hnRNP K (Laursen et al., 2011). Knockdown of hnRNP-K in cultured OLGs results in reduced MBP mRNA expression while MAG expression is unchanged, suggesting hnRNP-K specifically inhibits the translation of MBP mRNA (Laursen et al., 2011). It has also been reported that a small non-coding RNA 715 (sncRNA 715) binds to MBP mRNA 3' UTR and inhibits its translation (Figure 1-5). Increases in the levels of sncRNA 715 have been detected in multiple sclerosis patients (Bauer et al., 2012). The fragile X mental retardation protein (FMRP) binds to the 3' UTR of MBP mRNA and has also been suggested to inhibit MBP mRNA translation *in vivo* (Fig. 1-5)(Darnell et al., 2011; Li et al., 2001; Wang et al., 2004).

Ribonucleoproteins that bind hnRNP A2 have also been implicated in regulating the translation of MBP mRNA. HnRNP F associates with MBP mRNA through binding to hnRNP A2, though it has been suggested it may bind directly to MBP mRNA (White et al., 2012). Knockdown of hnRNP F leads to decreased MBP expression in primary OLG cultures. Knockdown or overexpression of hnRNP F in an OLG cell line caused increased amounts of MBP mRNA in the processes and decreased amounts of MBP translation (White et al., 2012). HnRNP F is phosphorylated by Fyn kinase, which is the proposed mechanism to lift the inhibition of MBP mRNA translation by HnRNP F. HnRNP E1 also binds to hnRNP A2 and inhibits the translation of MBP mRNA. The two ribonucleoproteins bind to each other *in vitro* and hnRNP E1 colocalizes with A2RE-RNA and hnRNP A2 in OLGs. Translation of GFP RNA containing an A2RE is inhibited by hnRNPE1 overexpression in B104 cells (a rat neuroblastoma cell line), while inhibition of translation of GFP RNA without an A2RE does not occur when hnRNPE1 is overexpressed (Kosturko et al., 2006).

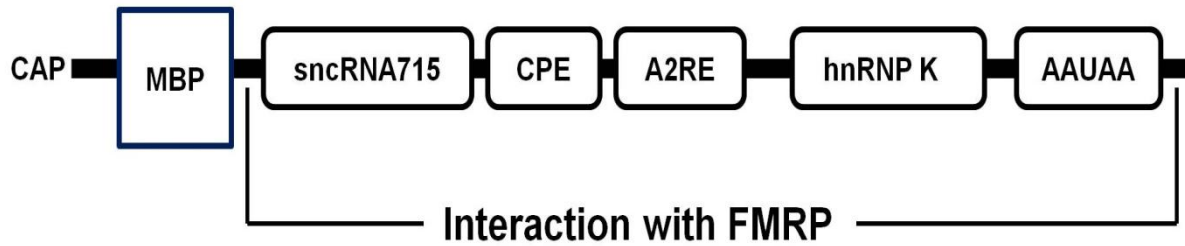


Figure 1-5: MBP mRNA and its 3' UTR. The 3' UTR of MBP contains a CPE sequence, two tandemly located A2RE sequences, several hnRNP K binding sequences, and a polyadenylation sequence. FMRP also binds the 3'UTR of MBP mRNA, though where FMRP binds in the 3' UTR is unknown.

Fragile X Syndrome (FXS) and fragile X-associated tremor/ataxia syndrome (FXTAS):

Trinucleotide Repeat Disorders

Trinucleotide repeat disorders are neurological disorders that are caused by the expansion of trinucleotide repeats in some genes. The presence of the expansion can lead to gene silencing, mRNA toxicity by the altered transcript, or toxicity from the protein encoded by the gene (Li and Bonini, 2010; Orr and Zoghbi, 2007). FXS and FXTAS are trinucleotide repeat disorders due to the expansion of CGG repeats in the 5' untranslated region (UTR) of the *FMR1* gene, which encodes the fragile X mental retardation protein (FMRP). The *FMR1* gene usually contains between 5 and 55 CGG repeats (Cornish et al., 2009). FXS is caused by the expansion of repeats to more than 200. Individuals in which the expansion in the *FMR1* gene leads to CGG repeats numbering between 55 and 200 are considered to have the fragile X premutation. Many of these individuals, greater than 50% of males age 70 or older, will develop symptoms of FXTAS (Oostra and Willemsen, 2009).

CGG repeats in 5' UTR of <i>FMR1</i> gene	<i>FMR1</i> mRNA levels	FMRP Protein level	Phenotype
< 55	normal	normal	normal
55-200	increased	normal or slightly reduced	FXTAS
>200	significantly reduced to not present	significantly reduced to not present	Fragile X Syndrome

Table 1-1: The effects of CGG repeat expansion in the 5' UTR of the *FMR1* gene.

FMRP and Fragile X Syndrome

FXS is present in approximately 1 in 5000 males and 1 in 10,000 females worldwide (Coffee et al., 2009). Transcription of the *FMR1* gene, which is located to the X chromosome, does not occur or is significantly decreased in FXS because the presence of 200 or more CGG repeats in its 5' UTR leads to hypermethylation of the repeats and the upstream CpG island in the promoter region (Pieretti et al., 1999). Individuals with FXS display behavioral and cognitive abnormalities along with abnormalities in physical features, such as macroorchidism (Lachiewicz and Dawson, 1994). Mental retardation and deficits in short term memory, visuo-spatial processing, arithmetic, and executive functions are all associated with FXS (Schapiro et al., 1995). Behavioral abnormalities include anxiety, social-interaction deficits, and hyperactivity (Tsiouris and Brown, 2004). In addition, around 30% of males are diagnosed with full autism, and many more are diagnosed with an autism spectrum disorder (Harris et al., 2008).

Differences in white matter in both adult and toddlers with FXS have been found using diffusion tensor imaging and MRI. These differences include increases and decreases in white matter along with an increased number of myelinated nerve fibers (Hallahan et al., 2011; Hoeft et al., 2011; Barnea-Garoly et al., 2003). Many of these differences correlate with regions or pathways in the brain that are involved in behavior or cognition that are deficient in FXS patients. For instance, it is believed that one function of the ventral frontostriatal pathway is cognitive inhibition. Deficits in cognitive inhibition are found in many FXS patients, and more myelinated fibers in that pathway have been reported in toddlers with FXS (Haas et al., 2009).

FMRP: a translational inhibitor

Twelve alternatively spliced isoforms of FMRP have been identified in the rodent brain (Brackett et al., 2013). Limited studies have been performed on the functions of the different isoforms and most work focuses on the main isoform of FMRP, a 632 amino acid protein that contains three common RNA-binding domains, a nuclear localization signal (NLS) and a nuclear export signal (NES) (Fig. 1-6) (Bhakar et al., 2012). FMRP expression is highest in the brain and testes, with neurons being the highest expressing cell type in the brain (Devys et al., 1993; Hinds et al., 1993). FMRP binds to 4% of mRNA in the human brain and is found mostly in the cytoplasm, though a small percentage of FMRP may be present in the nucleus (Feng et al., 1997; Ashley et al., 1993). The role of FMRP in the nucleus has not been extensively studied, but it is important as individuals with a missense mutation in the NLS have developmental delay (Collins et al., 2010).

The role of FMRP in the cytoplasm has been examined in greater detail. FMRP is present in mRNA containing granules in the cytoplasm and it has been demonstrated that FMRP represses the translation of several mRNAs *in vivo*. Transcripts for Arc and α Cam Kinase II contain A2RE sequences and are two of which that can be repressed (Zalfa et al., 2003). The majority of the studies examining FMRP's function have focused on neurons, in particular their dendritic spines. In neurons, FMRP's function has been shown to be mediated by signaling through NMDA receptors, muscarinic acetylcholine receptor, and group 1 metabotropic glutamate receptors (mGluR) (Lee et al., 2011; Volk et al., 2007; Bear et al., 2004). Many of the phenotypes present in the *Fmr1* KO mouse can be attenuated by group 1 mGluR inhibition and the "mGluR theory of fragile X" is very prominent in the field. The "mGluR theory of fragile X" states that increased translation of proteins downstream of the mGluR signaling at synapses lead to the neurological and psychiatric dysfunction found in FXS (Bear et al., 2004)

The stage of translation that is affected by FMRP is undetermined, but there is evidence FMRP may inhibit at both the initiation and elongation stage. The evidence that FMRP may exhibit translational repression at the initiation phase began with *in vitro* translation assays performed by Lagerbauer et al. (2001). The assays showed that the presence of FMRP significantly decreased the amount of RNA that associated with 80s ribosomes. Further data supporting FMRP playing an inhibitory role during translation initiation was found in cleavage-stage *Drosophila* embryos. In these embryos, FMRP associates with mRNPs but not polyribosomes (Monzo et al., 2006). FMRP's capability to recruit and/or stabilize CYFIP1 on the 5' end of specific mRNA has been suggested as a mechanism to repress translation initiation. CYFIP1 inhibits translation by binding to eIF4e associated with the mRNA. This prevents eIF4e from binding to eIF4g, which is needed for the initiation of cap dependent translation (Napoli et al., 2008). However, this inhibition may only occur in a subset of RNAs that FMRP binds to. For instance, FMRP is known to bind its own RNA, yet that RNA was not found to be in any CYFIP1 containing mRNPs (Napoli et al., 2008).

FMRP has been found in multiple studies to be associated with polyribosomes, which provides evidence that FMRP may stall translation at the elongation step (Darnell et al., 2011; Stefani et al., 2004; Ceman et al., 2003; Weiler et al., 1997). Rare cases of FXS are found in individuals with a point mutation in the KH domain of FMRP, I304N. These individuals express normal amounts of FMRP, but the mutation inhibits the ability of FMRP to bind to polyribosomes (Feng et al., 1997b). FMRP's inhibition of translation on polyribosomes is mediated by its phosphorylation. Phosphorylated FMRP associates with stalled polyribosomes and unphosphorylated FMRP is found on those that are actively translating (Ceman et al., 2003).

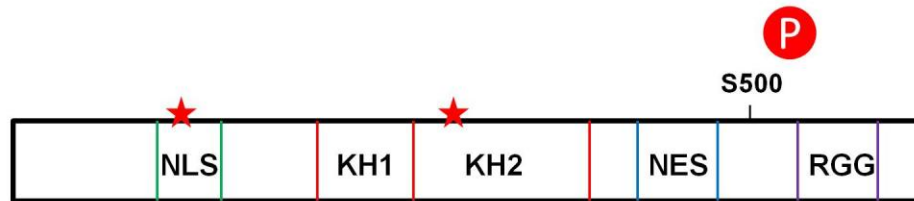


Figure 1-6: FMRP protein and its functional domains. The two K-homology domains (KH1 and KH2) and the arginine-glycine-glycine (RGG) box are RNA binding domains. Mutation in the NLS, R138Q, results in developmental delay in humans. Mutation in the KH2 domain, I304N, abolishes FMRPs ability to associate with polyribosomes and results in FXS, despite normal quantities of FMRP. S500 is a major phosphorylation site (adapted from Bhakar et al., 2012).

FMRP, Oligodendrocytes, and MBP translation

As previously stated, the study of FMRP has mainly focused on neurons. Recent studies on astrocytes have demonstrated they express FMRP early in development and can influence neuronal phenotypes (Pacey and Doering, 2007; Jacobs and Doering, 2010). Studies on FMRP's effect on OLGs and myelin have been very limited. Though limited, there is evidence to suggest a role for FMRP in OLGs, specifically in regulating MBP mRNA translation. As described previously, white matter differences have been found in FXS. Since myelination can be influenced by many factors, including some of neuronal and astrocytic origin, it is possible that dysfunction of these cell types leads to myelin abnormalities. However, OLGs are the myelinating cells in the CNS so it is logical that primary OLG dysfunction could also lead to these changes.

There are several pieces of evidence that suggest primary dysfunction in cells of the OLG lineage may occur in FXS. First, it is thought that FMRP is expressed in at least some cells of the OLG lineage because *Fmr1* mRNA has been detected in the OLG rich corpus callosum of adult mice and FMRP has been reported in OPCs and MBP-negative immature OLGs (Pacey and Doering, 2007; Wang et al., 2004; Hinds et al., 1993). Second, receptors that are involved in signaling that regulates FMRP function in neurons (muscarinic acetylcholine receptors, NMDA receptors, and mGluR receptors) are all expressed on cells of the OLG lineage (Cao and Yao, 2013; Cui et al., 2006; Luyt et al., 2006). For instance, NMDA receptors on OLGs are involved in mediating the differentiation and migration of OLGs (Cao and Yao, 2013). Finally, there is preliminary evidence that FMRP may inhibit MBP mRNA translation in OLGs.

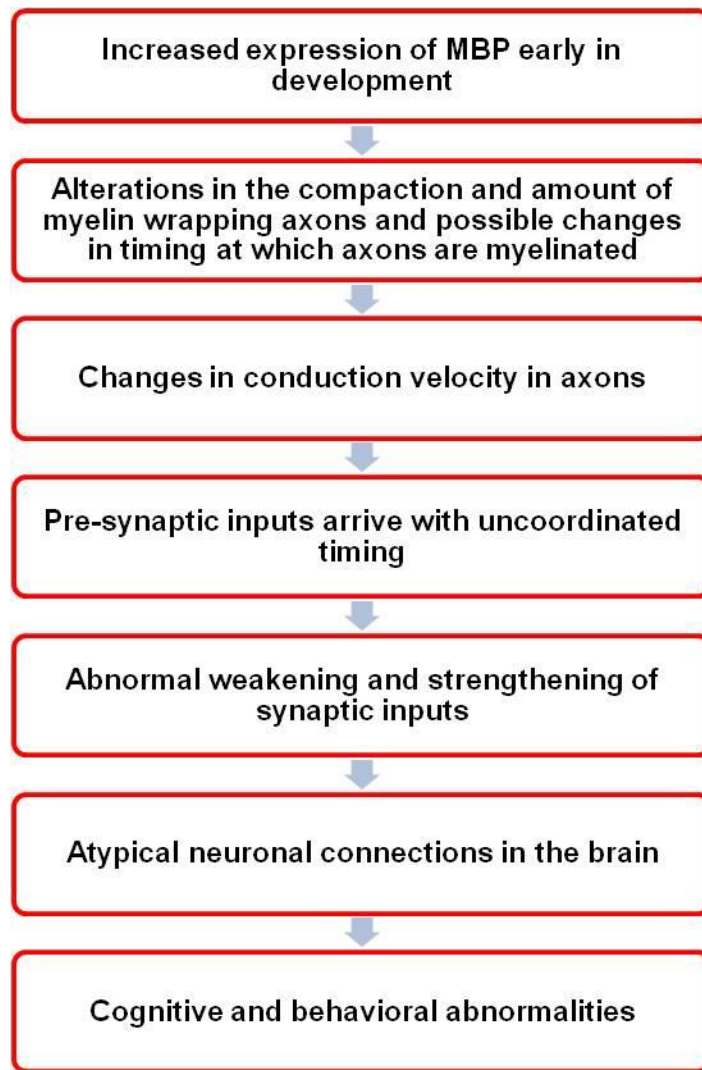


Figure 1-7: Mechanism by which premature MBP expression may lead to cognitive and behavioral abnormalities in FXS and *Fmr1* KO mice.

As discussed previously, MBP is critical for the formation of CNS compact myelin and constitutes 30% of all myelin proteins. FMRP binds to MBP mRNA, both *in vitro* and *in vivo*, and can inhibit MBP translation *in vitro* (Darnell et al., 2011; Wang et al., 2004; Li et al., 2001). The ability of FMRP to inhibit translation *in vitro* and to bind to MBP mRNA is dependent on the 3'UTR of MBP mRNA (Wang et al., 2004; Li et al., 2001). The temporally coordinated appearance of MBP and disappearance of FMRP in OLGs further suggested that FMRP inhibits MBP mRNA translation in OLGs.

Wang et al. (2004) reported that in cultured rat OLGs, OLG cell lines, and OLGs of P2 mouse brain that FMRP was

present only in OLGs not expressing MBP. These findings have led to the interpretation that during development FMRP inhibits the translation of MBP mRNA in immature OLGs and that a reduction in FMRP allows for the translation of MBP mRNA in mature, MBP expressing OLGs (Wang et al., 2004). If this is indeed true, the expression of MBP may occur prematurely in FXS due to the absence of the translational repression of FMRP. This may lead to not only myelin differences in FXS but could also alter connectivity in the brain by changes in conduction velocity caused by the myelin changes (Fig. 1-7).

***Fmr1* KO mouse model**

The *FMR1* gene is highly conserved among species and rodent FMRP has a 97% amino acid sequence homology with the human protein and is structurally conserved (Ashley et al., 1993). The pattern of FMRP expression in tissues is also very similar in the human and mouse. The *Fmr1* KO mouse was created by inserting a neomycin cassette in exon 5. These mice lack normal *Fmr1* mRNA and FMRP protein. The *Fmr1* KO mouse is a commonly used model to study FXS and shares many characteristics with FXS patients, such as macroorchidism, hyperactivity, and learning deficits (Dutch-Belgian Fragile X Consortium, 1994). The *Fmr1* KO mice used for our studies are on a C57/B6 background, *Fmr1* KO mice on different genetic backgrounds have different behavioral phenotypes (Spencer et al., 2011).

Myelination and in particular MBP expression has not been extensively studied in the *Fmr1* KO. Myelin in P30 *Fmr1* KO mouse had been examined by Ellegood et al., (2010) by diffusion tensor imaging and MRI and no difference was found at that time point. MBP and myelination in the cerebellum of *Fmr1* KO was only very recently examined. This study found decreased amounts of MBP and CNP in the cerebellum at day 7 and MBP reduction continued at day15, while CNP levels returned to normal. MBP levels returned to normal at P30, but then became higher than normal in adulthood. Electron microscopy at P7 revealed differences in the number of myelinated axons and g-ratio, but these were not present at P15. A decrease in the number of progenitors was present at P7 revealing a possible reason for the myelin alterations in the cerebellum of the *Fmr1* KO (Pacey et al., 2013). Our work, presented in Chapter 2, is the only published examination of the expression of MBP and other myelin proteins in the cerebral hemispheres of the *Fmr1* KO mouse over the course of development.

Fragile X premutation and FXTAS

The fragile X premutation is present when the *FMR1* gene contains between 55 and 200 CGG repeats, which are unmethylated (Cornish et al., 2009). Individuals with the fragile X premutation have increased levels of *FMR1* mRNA and normal to slightly reduced levels of FMRP (Peprah et al., 2010; Tassone et al., 2000). Of those, around 20% of women are diagnosed with premature ovarian insufficiency and one third of all pre-mutation carriers have symptoms of FXTAS by the age of 50 (Oostra and Willemsen, 2009). The presence of *FMR1* mRNA in intranuclear inclusions in FXTAS patients, increased levels of *FMR1* mRNA, and slight or no change in the presence of FMRP has led to the belief that mRNA toxicity is the cause of the neurological deficits associated with FXTAS and the fragile X premutation.

Those affected with FXTAS present with intention tremor and cerebellar gait ataxia that is frequently accompanied by autonomic dysfunction, Parkinsonism, and cognitive decline (Hagerman and Hagerman, 2004). Purkinje cell loss and solitary ubiquitin-positive intranuclear inclusions in both neurons and astrocytes in post-mortem tissue are hallmarks of FXTAS. The number of inclusions correlates with the number of CGG repeats (Greco et al., 2006). Mouse models of the fragile X premutation also include ubiquitin-positive intranuclear inclusions in neurons and astrocytes and display some cognitive and motor difficulties (Hunsaker et al., 2009; Van Dam et al., 2005). FXTAS is generally perceived to be a disease with an onset late in life, however there is evidence that FXTAS may be the manifestation of a process that begins early in development. For instance, cultured P1 neurons and embryonic fibroblasts from the fragile X premutation knock-in mouse display abnormalities (Garcia-Arocena and Hagerman, 2010). Also, behavior and cognitive difficulties and an increased susceptibility to seizures are present in youth with the fragile X premutation, demonstrating the premutation transcript may exert toxicity early in life (Aziz et al., 2003).

FXTAS and Myelin

Symptoms of FXTAS, such as intention tremor, indicate myelin plays a strong role in the disorder. Indeed, MRI and examination of post mortem tissue of FXTAS patients have shown the presence of cerebral and cerebellar white matter lesions, especially in the middle cerebellar peduncles, and significant astrocytic pathology in the cerebral white matter (Garcia-Arocena and Hagerman, 2010; Cohen et al., 2006; Greco et al., 2006). Changes in myelin in the presence of the premutation may be occurring throughout life as asymptomatic premutation carriers around 45 years of age show white matter reductions (Battistella et al., 2013). These changes may be due to improper myelination during development, improper maintenance of myelin, and/or failure to properly remyelinate following demyelination. There are no obvious intranuclear inclusions in OLGs, though isolation of intranuclear inclusions from a FXTAS patient contained MBP (Iwahashi et al., 2006). The cause of these lesions in FXTAS may be due to dysfunction of the cell types that display intranuclear inclusions in FXTAS, which are neurons and astrocytes. However, primary dysfunction in OLGs may also contribute.

A mechanism by which primary OLG dysfunction may occur is through altered translation of MBP mRNA. The presence of hnRNP A2 in intranuclear inclusions suggests that hnRNP A2 may be able to bind to the fragile X premutation transcript through its CGG repeats (Iwahashi et al., 2006). The binding of hnRNP A2 to the premutation transcript suggests several possibilities by which MBP expression can be altered, as MBP mRNA is transported and translated from hnRNP A2 containing granules. First, excess premutation transcript may become part of hnRNP A2 granules, altering the dynamics of the granule and affecting translation of its mRNAs. As part of these granules, excess premutation transcript may affect MBP mRNA translation by outcompeting MBP mRNA for access to the granule. It is also possible that the excess CGG repeats may recruit translational activators or repressors to the granule. Furthermore, recent work from the Carson lab has demonstrated that the CGG repeats of the premutation transcript

may alter translation by binding to CGG repeats of other mRNAs in a granule that encode proteins which regulate translation (personal communication with Dr. John H. Carson). Through this mechanism, the expression of translational regulators in hnRNP A2 granules would be altered in FXTAS. This would affect the translation of all RNAs found in hnRNP A2 containing granules, including MBP mRNA. It is also plausible that due to binding to the excess premutation transcript, hnRNP A2 may become sequestered in complexes containing premutation transcript and not be able to be present in granules. In Chapter 3, work is presented in which we investigated the presence of expanded CGG repeat RNA, as found in the fragile X premutation, in hnRNP A2 containing granules. Data is also presented in that chapter on the translation of MBP mRNA in the presence of expanded CGG repeat RNA.

A further possibility is that the presence of the premutation transcript does not affect specifically MBP mRNA translation but rather the overall health of the OLG. For instance, mitochondrial dysfunction has been identified in the CNS of FXTAS patients and this could eventually affect overall OLG function (Ross-Inta et al., 2010). Changes in OLG health and function could lead to demyelination or hinder the ability of the OLG to remyelinate and result in white matter lesions found in FXTAS.

Tumor Overexpressed Gene (TOG)

TOG is a 218 KDa protein that is encoded by the cytoskeleton associated protein 5 (*CKAP5*) gene and is ubiquitously expressed in all tissues, with highest expression in the brain.

Homologues of TOG are present in every eukaryotic organism that has been investigated (Gard et al., 2004). There are two isoforms of TOG, ch-TOG and TOG2, which differ by 60 amino acids. TOG2 is the only isoform expressed in OLGs and neurons (Kosturko et al., 2005). TOG and its homologues are microtubule binding plus end tracking proteins which positively regulate microtubule growth. Under usual conditions, TOG catalyzes the addition of tubulin dimers to the growing plus end of microtubules, though in certain circumstances TOG can catalyze microtubule shrinkage (Widlund et al., 2011; Brouhard et al., 2008). TOG is most commonly known for its crucial role in centrosome and microtubule stabilization and spindle assembly during mitosis (Cassimeris and Morabito, 2004; Gergely et al., 2003; Lee et al., 2001; Charrasse et al., 1998). Because of this function of TOG it is necessary for embryonic development. This is evident in that crosses of TOG heterozygous mice produced no TOG null pups out of 33 pups examined from 7 litters (Barbarese et al., 2013).

TOG is a protein which contains multiple TOG domains, there is evidence that there are between 5 and 7 TOG domains (personal communication with Dr. John H. Carson ; Hood et al., 2013; Andrade et al., 2001). The C-terminus also contains a sequence for the binding of the TACC domain of proteins (Lee et al., 2001). Each TOG domain consist of 6 HEAT (huntingtin, elongation factor 3, the PR65/A subunit of protein phosphatase 2A, and the lipid kinase Tor) repeats, which are known to mediate important protein-protein interactions (Andrade et al., 2001).

TOG and RNA granules

In OLGs, as in other cell types, TOG associates with microtubules, and immunostaining showed that TOG colocalized with cytoplasmic linker protein 115 (CLIP 115), a protein preferentially located at microtubule plus ends (Kosturko et al., 2005). In neurons and OLGs, TOG interacts with hnRNP A2 in granules. TOG's interaction with hnRNP A2 was first suspected when a yeast two-hybrid screen performed to identify the partners of hnRNP A2 from a human brain cDNA library revealed the longer isoform of TOG (TOG2) as a binding partner. This binding was confirmed by the co-immunoprecipitation of TOG2 and hnRNP A2 both *in vitro* and in B104 cells (Kosturko et al., 2005). Immunostaining for TOG and hnRNP A2 revealed they do indeed interact in OLGs as the two proteins colocalize in 90% of granules examined in the medial and distal OLG processes and the proteins are present in a defined stoichiometry.

HnRNP A2 granules also contain MBP mRNA and the effects of TOG on MBP mRNA transport and translation have been examined. Knockdown of TOG, using shRNA, in maturing cultured OLGs showed no MBP expression when examined by immunostaining. However, expression of MOG, which does not contain an A2RE, was unaffected (Francone et al., 2007). This suggests that decrease in MBP expression was not in the inability of the OLG to differentiate but specifically in the ability of the cell to express MBP, as MOG is expressed in the late stages of OLG differentiation (Fig. 1-2). Though the translation of MBP mRNA was inhibited, immunostaining for hnRNP A2 and *in situ* hybridization for MBP mRNA revealed no differences in the location or amount of granules in the OLGs in which TOG was knocked down (Francone et al., 2007). To examine if TOG's influence on translation is specific to hnRNP A2 granules TOG was knocked down in B104 cells and the translation of microinjected GFP RNA with and without a functional A2RE was analyzed. The knockdown led to decreased GFP expression of A2RE-GFP RNA but did not alter the expression of GFP RNA containing a mutated A2RE that inhibits the binding of hnRNP A2. The experiments of Francone et al., (2007) imply that TOG,

through binding to hnRNP A2, is necessary for proper translation of MBP mRNA but not for granule formation or transport. These findings led to the creation of mouse models with reduction in TOG expression to examine the role of TOG *in vivo*.

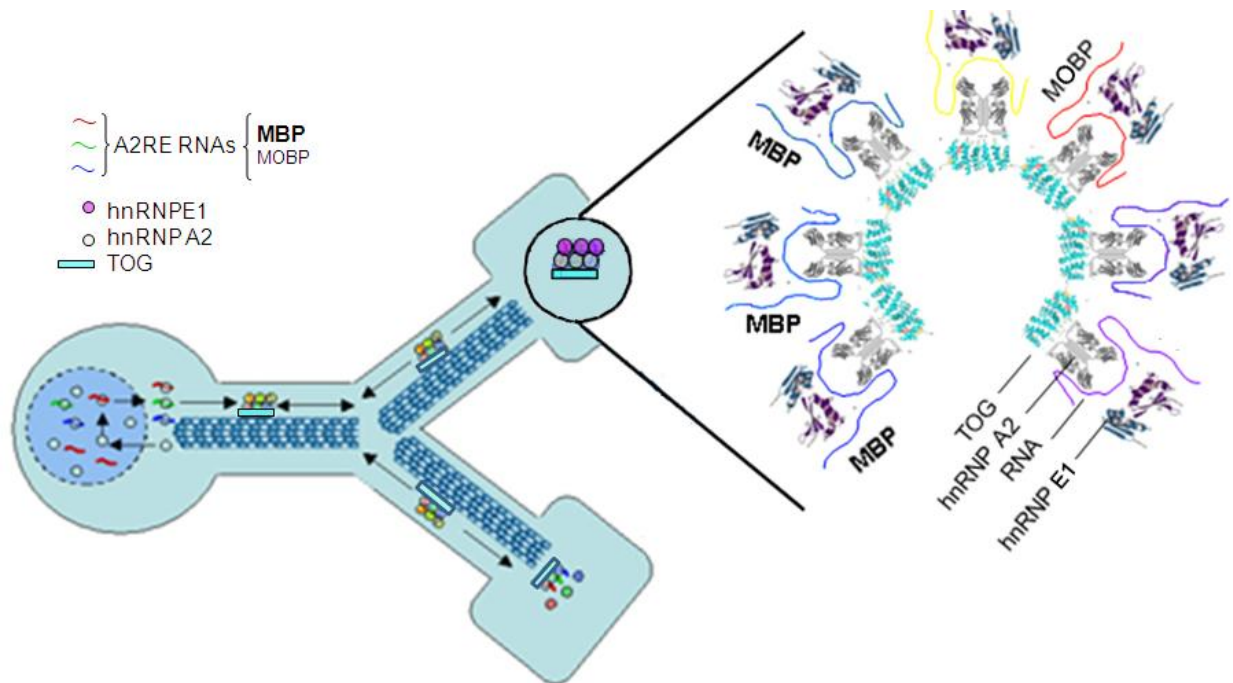


Figure 1-8: Transport of MBP mRNA in hnRNP A2 granules. In OLGs, MBP mRNA is transported on microtubules in hnRNP A2 granules, which also contain TOG. Enlarged image displays RNAs, including MBP mRNA, and one TOG protein with 7 TOG domains each bound to hnRNP A2 in a granule found in the OLG process (image provided by Dr. John H. Carson).

Examining TOG's function through mouse models

CNP TOG cKO

The effects of TOG on MBP expression previously described led our lab to investigate the consequences of conditionally knocking out TOG in OLGs. To achieve this goal, floxed TOG animals were created by introducing a lox P site into intron 3 and a frt-PGKNeo-frt-loxP cassette in intron 6 of the *CKAP5* gene that encodes TOG (Barbarese et al., 2013). These mice were crossed with mice in which the Cre recombinase sequence was placed downstream of the CNP promoter, replacing the CNP gene (Lappe-Siefke et al., 2003). Offspring with both TOG alleles floxed and containing the gene for Cre recombinase downstream of the CNP promoter were analyzed and termed CNP TOG cKO. CNP is expressed exclusively in myelinating cells, OLGs (CNS) and Schwann Cells (PNS) (Sprinkle, 1989). CNP is the earliest myelin specific protein to be expressed, *in vivo* its expression in rodents precedes that of MBP by two to three days (Reynolds and Wilkin, 1988). Therefore, the CNP TOG cKO animal has normal expression of TOG in all cell types except for OLGs and Schwann cells. TOG was knocked out using the CNP promoter because knockout of TOG in OLG precursor cells (OPCs) earlier in development would probably affect their proliferation and process elongation, due to TOG's role in mitosis and in regulating microtubule dynamics. This would make it more difficult to specifically examine TOG's influence on MBP mRNA translation as myelin and MBP expression in those animals would most likely be altered by changes to OPC proliferation and process elongation.

CNP TOG cKO preliminary data

Analysis of the CNP TOG cKO revealed the animals contain 20% of the control level of MBP at approximately two months of age. Importantly, the quantity of MBP transcript in CNP TOG cKO is the same as in wild type mice, supporting the work by Francone et al. (2007) that TOG affects MBP mRNA translation. Levels of MOG, a myelin specific protein expressed at late stages of OLG maturation, are decreased to a level of 80% of control. This slight decrease reveals that in TOG's absence the OLG is still able to differentiate. The change in MOG levels may not be directly due to the absence of TOG, but due to the lack of expression of MBP, as the *shiverer* mouse (discussed on page 18) contains only 30% the amount of MOG as controls. The decrease in MBP and MOG is not due to a decrease in OLGs. Expression of additional myelin proteins were examined and are presented in Chapter 4. Staining in the corpus callosum for the OLG lineage cell marker Olig2 revealed similar levels of Olig2+ cells in the CNP TOG cKO as in control. As previously described, TOG is a microtubule associated protein and MBP mRNA requires intact microtubules for transport. Therefore MBP translation may be altered due to changes in microtubule dynamics caused by the absence of TOG. To estimate microtubule dynamics, the microtubule-dependent movement of mitochondria in the processes of cultured OLGs was measured by monitoring the velocity of fluorescent live mitochondria. Preliminary data shows decreased mitochondrial movement in the CNP TOG cKO, indicating altered microtubule dynamics.

Examination of the optic nerve revealed that 25% of CNP TOG cKO axons were myelinated, as compared to approximately 100% of controls. No differences in the axon diameter were present in the CNP TOG cKO when compared to control. This indicates that the hypomyelination in these animals is not due to axonal defects, but lies in the inability of the OLG to produce myelin. An increase in gliosis is also present in the TOG cKO. This is probably due to the decrease of MBP and the resulting hypomyelination, as gliosis is present in young adult *shiverer*

(Westenbroek et al., 1998). Changes in PNS myelin were evaluated by electron microscopic examination of the sciatic nerve. No differences in the number of myelinated axons or the thickness of the myelin sheaths were observed in the CNP TOG KO. This is due most likely to the diminished role of MBP in PNS myelin formation. Behavior tests of the CNP TOG cKO revealed severe deficiencies in motor functions using the rotorod apparatus and measuring the gait and stride. Similar locomotion, strength, and coordination abnormalities are seen in the *shiverer* mouse, evidence that these changes are due to hypomyelination. It is important to note that in the CNP TOG cKO, one CNP allele has been replaced by the gene that encodes the Cre recombinase. This reduction of CNP probably does not contribute significantly to the phenotypes found in the CNP TOG cKO as mice that do not express CNP develop normally and overall myelin structure and MBP level are unaffected in those mice (Lappe-Siefke et al., 2003).

TOG +/-null

The TOG +/-null animal has only one wild type *CKAP5* allele (Barbarese et al., 2013). Deletion of one *CKAP5* allele is not conditional and therefore is present in all cells and every cell type throughout development in the TOG +/-null. The deletion leads to decreased amounts of TOG, which are ~ 55% of control levels. A complete knockout of TOG in humans would likely be embryonic lethal because of the role of TOG in mitosis. However, there are cases where the level of TOG may be reduced. The TOG +/-null is a model that can be used to study such situations. One such situation is when the expression of the microRNA miR-155 is increased. TOG/CKAP5 mRNA is a target of the microRNA miR-155, which inhibits its translation (Lossner et al., 2011). Elevated levels of miR-155 may therefore lead to reduction in TOG expression under certain conditions. This could occur in the brains of Down's syndrome patients, which overexpress miR-155 (Wang et al., 2013). MiR-155 expression may also increase during inflammation and is increased in brains of multiple sclerosis patients (Junker et al. 2009). TOG expression may also be decreased in some cases of Potocki-Shaffer syndrome. Potocki-

Shaffer syndrome results from a large multi-genes deletion on chromosome 11. In some cases the deletion encompasses the *CKAP5* gene which results in TOG haploinsufficiency. Those individuals with a deletion that encompasses the *CKAP5* gene show increased cognitive disability when compared to those without the deletion, suggesting that a reduction in TOG may negatively affect cognitive abilities (Swarr et al., 2010).

Preliminary data show that MBP levels are decreased by approximately 40% in the brain of the +/null mouse. The rotorod test and analysis of the gait and stride of the TOG +/null revealed no substantive changes in their motor behaviors despite the decrease in MBP. In work presented in Chapter 4, we have measured the level of MBP transcripts, the expression of other myelin proteins, gliosis, the migration and proliferation of OLG lineage cells, and mitochondria movement in OLGs in the +/null mice to characterize the effects of TOG on MBP expression and brain development.

Chapter 2: FMRP and myelin protein expression in oligodendrocytes

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A.G., J.H.C., and E.B. designed the study; A.G. performed the experiments; A.G. analyzed the data; A.G. wrote the manuscript with modifications provided by E.B.

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 and 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 and 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 and 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 and 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 and 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, 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.

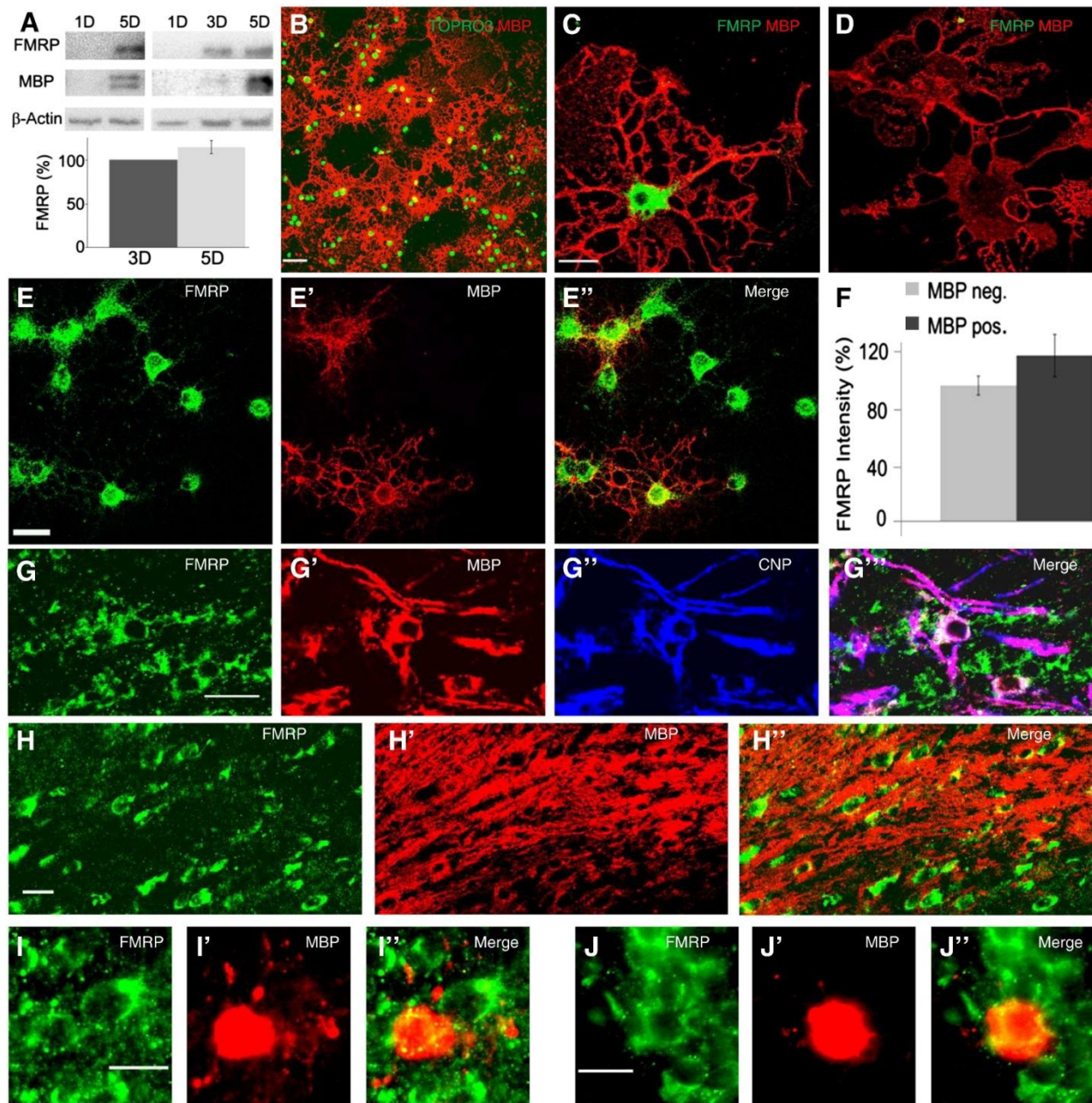


Figure 2-1: FMRP is expressed in mature, MBP-positive OLGs. (A) Western blots of rat OLG cultures and densitometric analysis of FMRP levels at 1 day (D), 3D and 5D in culture. Values were standardized to β -actin and means normalized to 3D. There is no significant differences in levels of FMRP between 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 μm . (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 μm . (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 μm .

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'-(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 and 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.

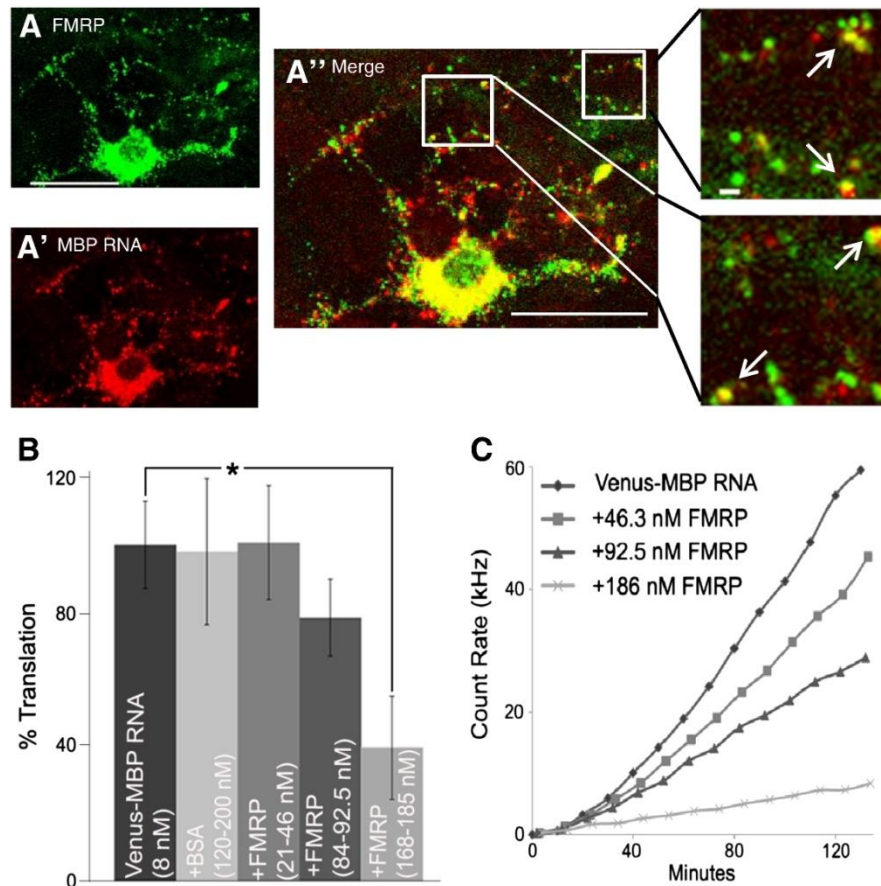


Figure 2-2: FMRP localizes to granules and inhibits translation of MBP RNA *in vitro*.

(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 8 nM Venus-MBP RNA in wheat germ lysate was measured every ten minutes for 130 min using FCS. Translation was monitored by measuring the photon count rate after Venus excitation. (B) Photon count rates for each condition at 130 min (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 min intervals.

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.

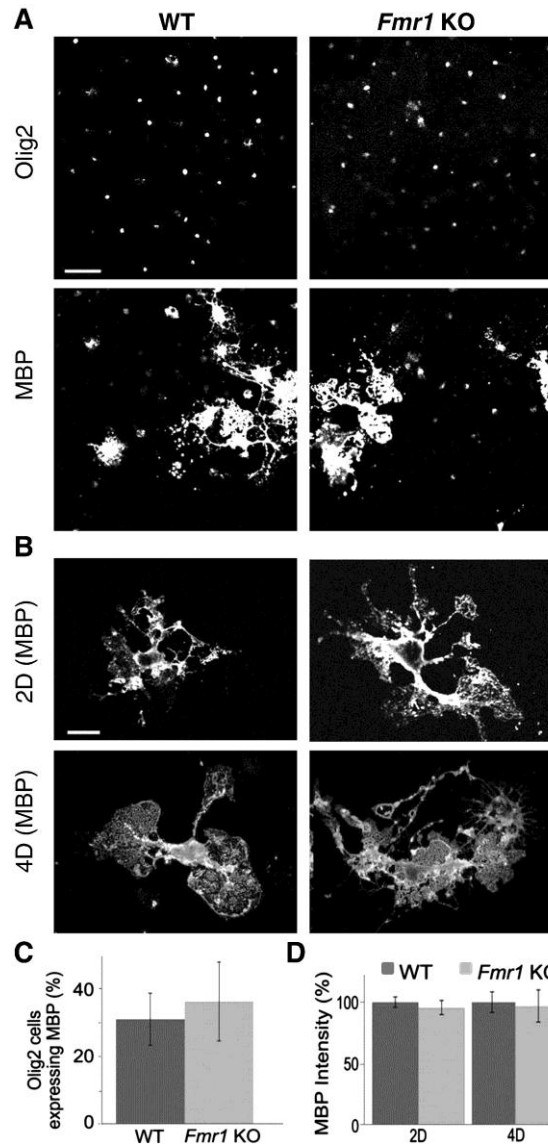


Figure 2-3: MBP expression in OLG cultures from *Fmr1* KO is similar to WT. (A) OLG cultures derived from WT and *Fmr1* KO were fixed after 4 days in ODM and immunostained for Olig2 and MBP. Scale bar, 20 μ m. (B) Immunocytochemistry for MBP in WT and *Fmr1* KO OLGs at 2 days and 4 days in ODM. Scale 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).

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 of neurons and astrocytes that could potentially modulate the myelin expression program.

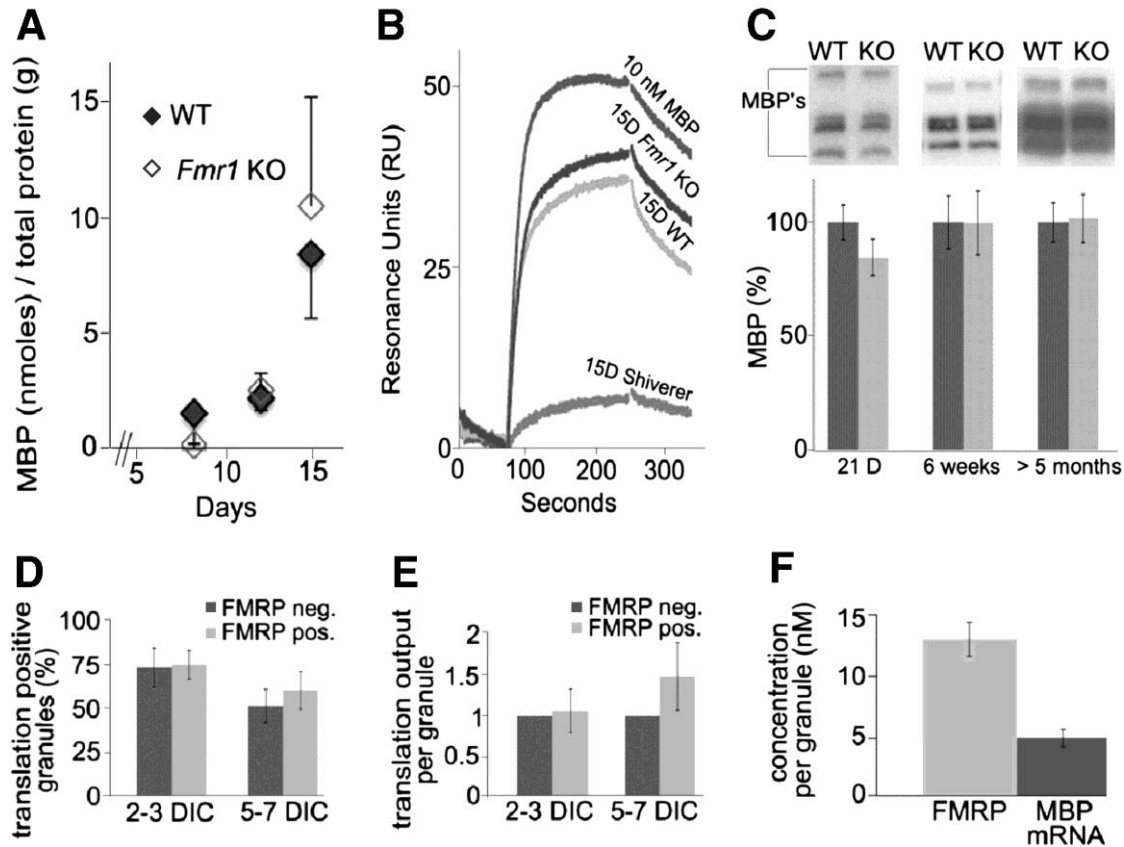


Figure 2-4: FMRP does not affect the *in vivo* accumulation and translation of MBP. (A) Accumulation of MBP (mean \pm s.e.m.) in the cerebral hemispheres of P8 ($n = 2$ for WT, $n = 3$ for KO), P12 ($n = 3$ for WT, $n = 3$ for KO), and P15 ($n = 3$ for WT, $n = 3$ for KO) mice measured by SPR does not 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 days, $n = 3$ WT, 3 KO at 6 weeks, $n = 6$ KO, 5 WT at > 5 months). Values were standardized to β -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.

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 h 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 and 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. 5A–D).

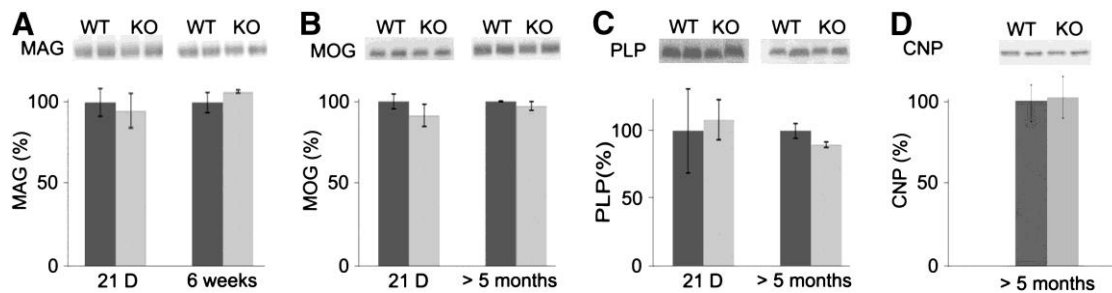


Figure 2-5: Levels of myelin proteins are similar in WT and *Fmr1* KO during and after the peak of myelination. Western blots of brain homogenates at time periods during and after the peak of myelination. (A) MAG (n = 2 WT, 3 KO at 21 days, n = 2 WT, 3 KO at 6 weeks); (B) MOG (n = 2 WT, 4 KO at 21 days, n = 2 WT, 3 KO at > 5 months); (C) PLP (n = 2 WT, 2 KO at 21 days, 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).

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 et al., 2003 and Narayanan et al., 2007). The phosphorylation state of FMRP under basal conditions in OLGs is not known.

Though the timing and the level of MBP accumulation do 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.

Experimental methods

Animals

Mice: C57/Bl6 *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 mice of the same strain were obtained from the Jackson Laboratories and used as control. *Shiverer* mice (C3Fe.SWV-*Mbp*^{shi}/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 Tatavarty 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 40 ×, 1.2 NA water immersion objective. FCS measurements of total photon counts in each well were taken at 10 minute intervals for 130 min.

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 min. 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 min and rinsed 3 times with PBS. Cells were then permeabilized with 0.5% NP-40 for 1 min and rinsed with PBS 3 times. Cells were incubated in blocking buffer (10% goat serum in PBS) for 20 min followed by a solution of primary antibody diluted in blocking buffer for 30 min. After several rinses, secondary antibodies diluted in blocking buffer were added for an additional 30 min. 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 min in 0.01 M 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, pH 7.4, 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 min in 0.01 M citrate acid buffer, pH 6.3. Tissue was washed with PBS and blocked for 1 h 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 h. 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'-CGCCCCGGGCAAGTTTATTATTTTATTTC-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 s followed by 18 cycles of 30 s at 95 °C, 30 s at 55 °C, and 12 min at 68 °C, all in a final volume of 50 µl. Two and a half units of Pfu Turbo DNA polymerase were used for elongation. After amplification the methylated parental plasmid was digested with 10 U DpnI at 37 °C for 2 h. TOPO competent cells were transformed with a 2 µl 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 was 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 Myszkka (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 μ L/min for 180 s. The RU values increased as MBP analyte binds to antibody ligand. Post-injection dissociation was monitored for 180 s at a flow rate of 30 μ L/min. The surface was regenerated between injections with 10 mM glycine-HCl (pH 2.0) at a flow rate of 30 μ L/min for 10 s. 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- β -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 3 h. FCS was performed using a Zeiss Confocor 3 system with a 63 \times , 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 s, which lead to photobleaching of the granule. The photon count rate decay throughout the 10 s 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.

Acknowledgments

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Chapter 3: Analyzing myelin basic protein mRNA translation in the presence of the fragile X premutation RNA

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A.G., J.H.C., and E.B. designed the study; A.G. performed the experiments; A.G., J.H.C. and E.B. analyzed the data; A.G. wrote the manuscript with modifications provided by J.H.C and E.B.

Abstract

The fragile X premutation transcript, with expanded CGG repeats, is found at elevated levels in fragile X-associated tremor/ataxia syndrome (FXTAS). Altered translation of MBP mRNA caused by the fragile X premutation may contribute to the white matter pathology present in FXTAS. The effect of the fragile X premutation transcript on translation of myelin basic protein (MBP) mRNA was examined in oligodendrocytes and fibroblasts. Due to its expanded CGG repeats, which bind to hnRNP A2, the fragile X premutation was hypothesized to be present in MBP mRNA containing granules, where it could affect the translation of MBP mRNA. We found that indeed RNA with expanded CGG repeats can be present in granules with MBP mRNA. The presence of the expanded CGG repeat RNA in OLGs was found to increase translation of microinjected MBP mRNA in granules. In addition, increased translation of microinjected MBP mRNA was observed in fibroblasts from FXTAS individuals when compared to controls. Taken together these results imply that the presence of the fragile X premutation transcript may increase the translation of MBP mRNA in individuals with FXTAS.

Introduction

The fragile X premutation is present when the *FMR1* gene contains between 55 and 200 CGG repeats (Cornish et al., 2009). Individuals with the fragile X premutation have increased levels of *FMR1* mRNA and normal to slightly reduced levels of FMRP (Peprah et al., 2010; Tassone et al., 2000). Approximately 20% of female premutation carriers are diagnosed with premature ovarian insufficiency and approximately one third of all premutation carriers have symptoms of FXTAS by the age of 50 (Oostra and Willemsen, 2009). The presence of *FMR1* mRNA in intranuclear inclusions, increased levels of *FMR1* mRNA, and slight or no change in the level of FMRP in FXTAS patients has led to the belief that mRNA toxicity is the cause of the neurological deficits associated with the fragile X premutation.

The expanded number of CGG repeats is believed to be the source of the toxicity in premutation RNA. The number of CGG repeats correlates with the severity of motor dysfunction, the age of onset of tremor and ataxia, the number of intracellular inclusions, and the age of death of FXTAS patients (Leehey et al., 2008; Tassone et al., 2007; Greco et al., 2006). Also, the presence of an expanded number of CGG repeats without the remainder of the *FMR1* gene is toxic to cells both *in vitro* and *in vivo* (Hashem et al., 2009; Handa et al., 2005; Jin et al., 2003).

The predominant hypothesis is that the CGG repeats induce their toxicity by sequestration of proteins that perform important functions in the cell. Intranuclear inclusions in FXTAS and in cells with expanded CGG repeat RNA independent of the *FMR1* transcript contain various RNA binding proteins that have significant roles in the cell. Overexpression of some of these sequestered proteins can suppress the neurodegenerative phenotype in *Drosophila* models with expanded CGG repeat RNA (Sellier et al., 2010; Jin et al., 2007; Sofola et al., 2007; Iwahashi et al., 2006). Another emerging hypothesis is that repeat-associated non-AUG-initiated (RAN) translation of polyglycine from expanded CGG repeat RNA in the cells can also be toxic (Todd et al., 2013).

A novel hypothesis has been proposed that CGG repeat RNA in FXTAS may alter translation of mRNAs containing the A2 Response element (A2RE) (Dr. John H. Carson and Rene Norman, personal communication). The A2RE sequence is a sequence for which heterogeneous ribonucleoprotein A2 (hnRNP A2) has high affinity (Hoek et al., 1998). The binding of hnRNP A2 to A2RE mRNAs allows those mRNAs to assemble into hnRNP A2 containing granules in the cell body and travel to their destination, either dendrites or OLG processes, to be translated (Munro et al., 1999; Hoek et al., 1998; Ainger et al., 1997). Expanded CGG repeat RNA has also been demonstrated to bind to hnRNP A2, and expanded CGG repeats have an increased binding affinity (Sofola et al., 2007). The presence of expanded CGG repeat RNA in hnRNP A2/RNA granules may alter translation of A2RE mRNAs in the same granules. Proteins whose RNAs contain A2RE sequences include activity regulated cytoskeletal-associated protein (Arc), α Cam Kinase II, and myelin basic protein (MBP) (Gao et al., 2008; Ainger et al., 1997). Preliminary evidence suggests that the translation of Arc mRNA is decreased in the presence of expanded CGG repeats (personal communication, Rene Norman), supporting the hypothesis that expanded CGG repeats can affect the translation of A2RE containing mRNAs.

White matter disease is prevalent in individuals diagnosed with FXTAS (Garcia-Arocena and Hagerman, 2010a; Cohen et al., 2006). Though no intranuclear inclusions have been identified in oligodendrocytes (OLGs) in this disorder, it is possible that altered translation of MBP mRNA from hnRNP A2/RNA granules contributes to the white matter disease (Greco et al., 2002). Altered translation of MBP mRNA occurring during development, during myelin maintenance, or during remyelination in response to injury could all lead to the white matter disease present in FXTAS. In this work we investigated if the presence of expanded CGG repeat RNA affected the translation of MBP mRNA. This was performed by examining the translation of microinjected Venus-MBP mRNA in rat OLGs coinjected with 99 CGG repeat RNA and in fibroblasts of FXTAS patients.

Results

CGG repeat expansion RNA is found in MBP mRNA granules

CGG repeat RNA is capable of binding to hnRNP A2 (Sofola, 2007). MBP mRNA is transported and translated in hnRNP A2 containing RNA granules (Munro et al., 1999; Hoek et al., 1998; Ainger et al., 1997). Therefore, we examined if expanded CGG repeat RNA is present in granules containing MBP mRNA. Labeled RNA containing 99 CGG repeats (Cy3-UTP 99 CGG repeat) was coinjected into mature rat OLGs with differentially labeled MBP mRNA (Cy5-UTP Venus-MBP mRNA). Cy3-UTP 99 CGG repeat RNA was found in granules in the cellular processes. It colocalized with Cy5-UTP Venus-MBP mRNA in a population of granules, 67% of labeled MBP mRNA granules also contained labeled 99 CGG repeat RNA (n = 108) and 94% of labeled CGG 99 repeat RNA granules also contained labeled MBP mRNA (n=77) (Fig. 3-1 A-E). Injection of Cy3-UTP RNA with an identical sequence to 99 CGG repeat RNA, but with no CGG repeats (0 CGG repeat RNA), was diffuse and did not form granules or colocalize with Cy5-UTP Venus-MBP mRNA.

Expanded CGG repeat RNA does not alter the level of MBP mRNA that localizes in granules

One mechanism by which expanded CGG repeat RNA could affect granule assembly is by outcompeting MBP mRNA for access to the granule. To examine this possibility we assessed if the presence of the 99 CGG repeat RNA altered the quantity of MBP mRNA in granules. OLGs were coinjected with Cy5-UTP Venus-MBP mRNA and either Cy3-UTP 99 CGG repeat RNA or Cy3-UTP 0 CGG repeat RNA. The average intensity of Cy5 in labeled MBP mRNA containing granules did not differ between OLGs injected with labeled 99 CGG repeat RNA or labeled 0 CGG repeat RNA (Fig. 3-1F). These results indicate that the presence of expanded CGG repeat RNA does not affect the level of MBP mRNA that is incorporated into a granule.

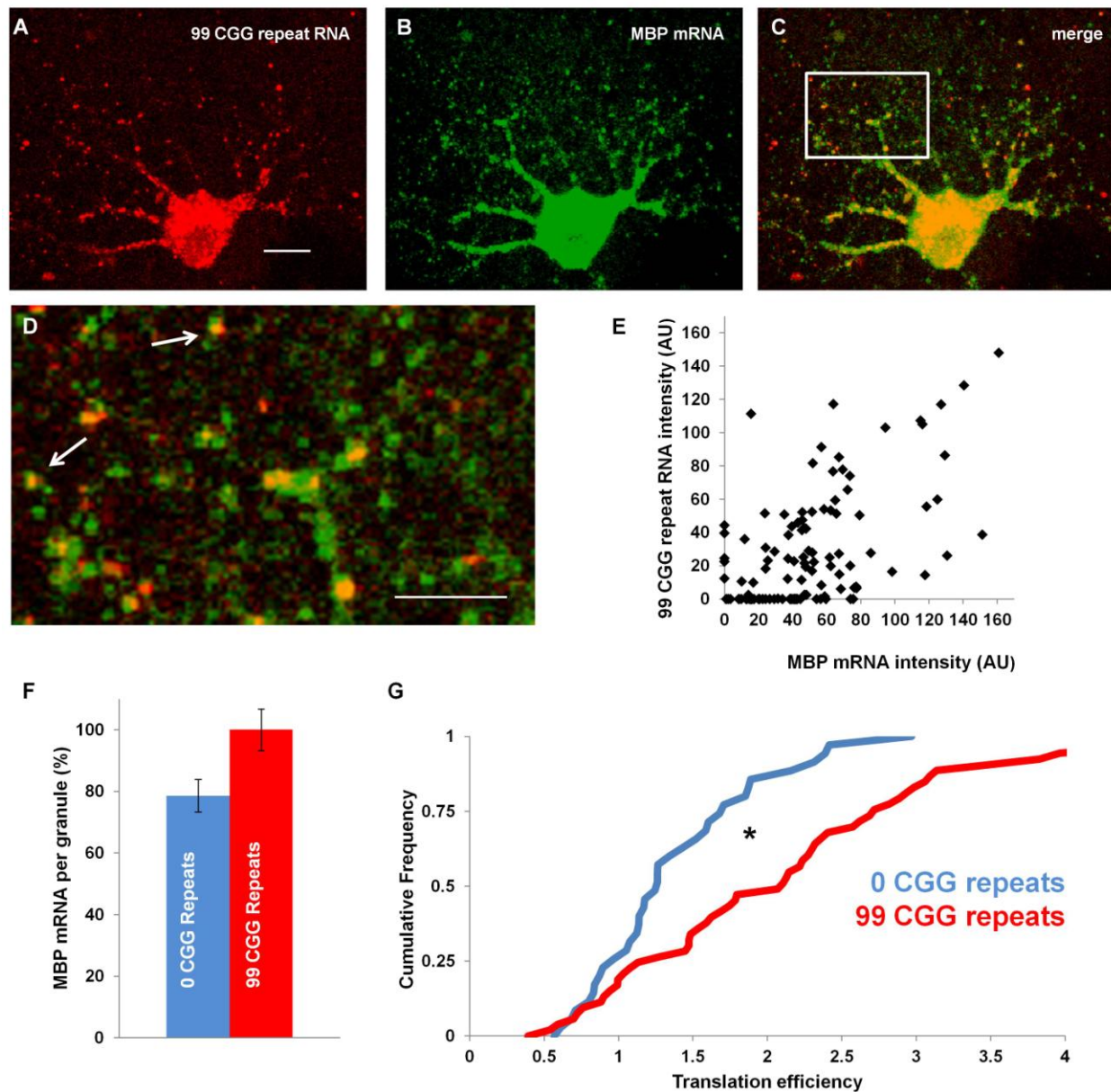


Figure 3-1: Expanded CGG repeat RNA increases MBP mRNA translation. (A-C) Rat OLGs were coinjected with Cy3-UTP 99 CGG repeat RNA (red; A and C) and Cy5-UTP Venus-MBP mRNA (green; B and C). Scale bar, 10 μm . (D) Higher magnification of area identified in C, arrows denote granules in which Cy3-UTP 99 CGG repeat RNA (red) and Cy5-UTP Venus-MBP mRNA (green) are colocalized (scale bar, 5 μm). (E) Intensity of Cy5-UTP Venus-MBP mRNA and Cy3-UTP 99 CGG repeat RNA in granules of OLGs coinjected with both RNAs. (F) Intensity of Cy5-UTP Venus MBP mRNA in granules from OLGs coinjected with Cy3-UTP 99 CGG repeat RNA (n = 54 granules from 4 cells) and those coinjected with Cy3-UTP 0 CGG repeat RNA (n = 39 granules from 3 cells). No significant difference was present when comparing the intensity of Cy5-UTP in granules coinjected with 99 CGG repeat RNA or 0 CGG repeat RNA (mean \pm s.e.m; Nested ANOVA, $p > 0.05$). (G) Cumulative frequency plot of

translation efficiency (Venus Intensity/Cy5 Intensity) in rat OLGs coinjected with Cy5-UTP Venus-MBP mRNA and either Cy3-UTP 0 CGG repeat RNA or Cy3-UTP 99 CGG repeat RNA. Venus-MBP mRNA translation efficiency is increased in OLGs coinjected with Cy3-UTP 99 CGG RNA when compared to OLGs coinjected with Cy3-UTP 0 CGG repeat RNA (Kolmogorov-Smirnov Test, $*p < 0.01$).

Transient presence of expanded CGG repeats increases MBP mRNA translation in OLG granules

Microinjected, fluorophore conjugated mRNA is capable of assembling into granules, is properly transported, and can be translated (Tatavarty et al., 2012). To examine if the presence of expanded CGG repeat RNA in granules could alter MBP mRNA translation in the same granules, translation of Cy5-UTP Venus-MBP mRNA coinjected with Cy3-UTP 99 CGG repeat RNA was analyzed and compared to OLGs coinjected with Cy3-UTP 0 CGG RNA. In these experiments, the molar concentration of Cy3-UTP 99 CGG repeat RNA was more than twice that of the Cy5-UTP Venus-MBP mRNA injected. OLGs were fixed 3.5 hours after microinjection and the translation efficiency in individual granules was calculated by capturing the intensity of the reporter Venus, whose open reading frame (ORF) was 5' of MBP's ORF, and dividing it by the intensity of Cy5, which labeled MBP mRNA. Since expanded CGG repeats were only present in the OLG for 3.5 hours, differences in MBP mRNA translation would most likely be due to effects of CGG repeats on granule translation. In this short time period, it is unlikely that changes in translation would be due to global effects of 99 CGG repeat RNA on the cell physiology, such as deterioration in viability or changes in gene expression. Those OLGs coinjected with labeled 99 CGG repeat RNA displayed increased translation of MBP mRNA in granules compared to those coinjected with labeled 0 CGG repeat RNA (Fig. 3-1G).

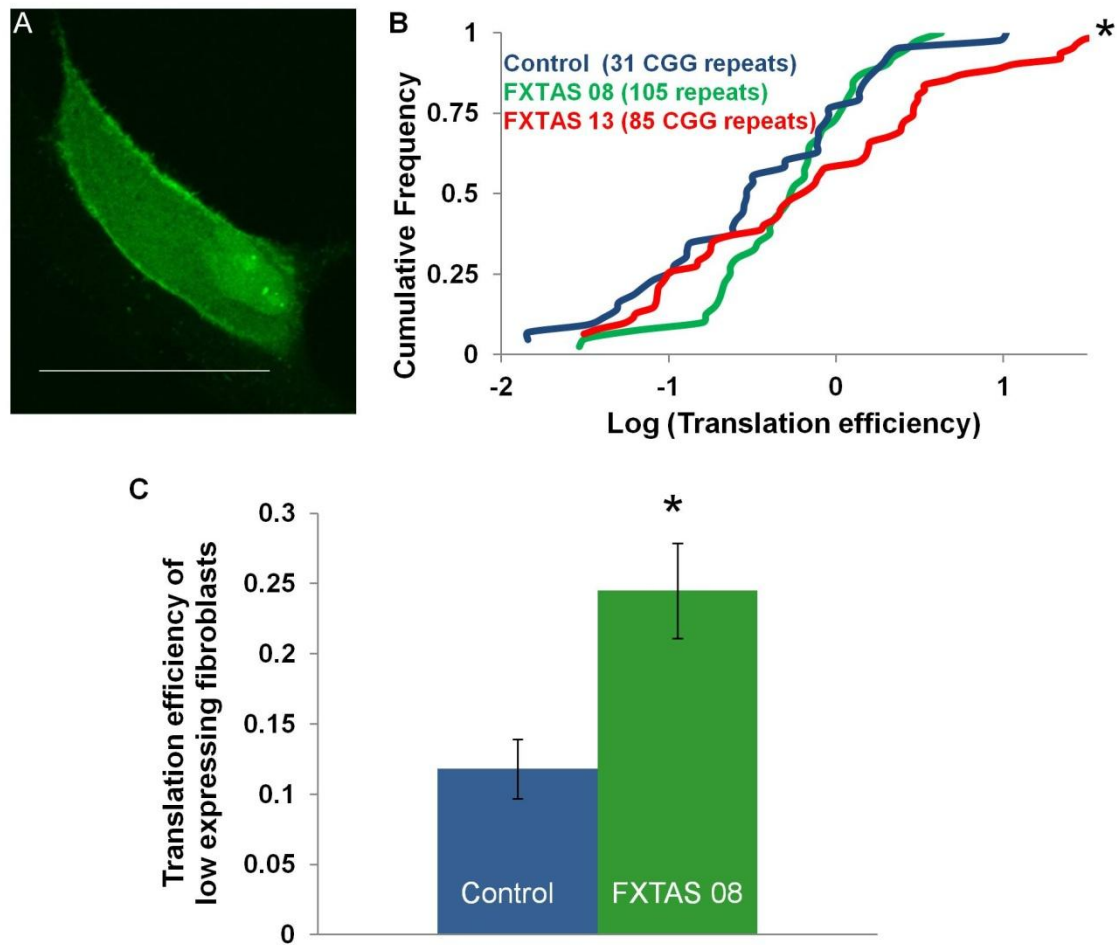


Figure 3-2: Increased translation of Venus-MBP mRNA in FXTAS fibroblasts. (A) Venus-MBP in control fibroblast microinjected with Cy5-UTP Venus-MBP mRNA. Scale bar, 50 μ m. (B) Cumulative frequency plot of translation efficiency (Venus Intensity/Cy5 Intensity) in fibroblasts injected with Cy5-UTP Venus-MBP mRNA. Translation of Venus-MBP mRNA is increased significantly between FXTAS 13 and control in high expressing fibroblasts (Kolmogorov-Smirnov Test, $*p < 0.01$). (C) Mean translation efficiency of Cy5-UTP Venus-MBP mRNA is significantly increased in low expressing fibroblasts of FXTAS 08 when compared to controls (t -test, $*p < 0.01$).

FXTAS fibroblasts have increased MBP mRNA translation

Translation of microinjected Cy5-UTP Venus-MBP mRNA was examined in human fibroblasts obtained from several different FXTAS patients (FXTAS 08 and FXTAS 13) and from a control individual (control 603). Though investigation of MBP mRNA translation in OLGs expressing the fragile X premutation would be best, we believe fibroblasts are a suitable cell type to study the effects of the fragile X premutation transcripts on translation of MBP mRNA. Fibroblasts contain hnRNP A2 and fibroblasts of FXTAS patients express increased amounts of fragile X premutation transcript compared to control (Garcia-Arocena et al., 2010b; Zhu et al., 2002). In addition, we found that when microinjected Venus-MBP mRNA was translated, newly synthesized Venus-MBP was localized to the membrane (Fig. 3-2A). This suggests that fibroblasts have similar transport and translation mechanisms as OLGs. Examination of Venus-MBP mRNA expression five hours after microinjection revealed that FXTAS 13 fibroblasts have a statistically significant increase in translational efficiency when compared to control (Fig. 3-2B). This increase in efficiency was prevalent in the population of higher expressing fibroblasts. Examination of the population of lower expressing fibroblasts, those whose translation was below the median, revealed Venus-MBP mRNA expression was increased in the lower expressing fibroblasts of FXTAS 08 (Fig. 3-2C). These results imply that the presence of the fragile X premutation in cells can increase the translation of MBP mRNA.

Discussion

Coinjection of labeled MBP mRNA and labeled 99 CGG repeat RNA into OLGs revealed that the two RNAs colocalize in many granules. Interestingly, granules containing only one species of labeled RNA have a lower intensity of that labeled RNA than many of the granules containing both species of RNA (Fig. 3-1E). This may occur because granules with only one species of labeled RNA detected may be granules which cannot accommodate as much RNA. Less capacity for RNA in these granules may lead to less overall RNA in the granule, perhaps leading to the exclusion of a species of RNA. Decreased concentration of RNA in these granules may be due to decreased quantities of hnRNP A2 in the granule. It is also possible that granules with only one species of labeled RNA detected are not granules with decreased accommodation for RNA, but are granules in which there is a high level of endogenous, unlabeled RNA. Though the injected OLGs do not have endogenous 99 CGG repeat RNA, they do contain *Fmr1* transcript which does contain CGG repeats.

Microinjection of MBP mRNA into fibroblasts obtained from both FXTAS patients and a control displayed differences in translation of MBP mRNA. Future experiments should be performed to confirm these results. First, fibroblasts from only one control individual were examined. It is possible that due to other genetic factors fibroblasts from the control individual used in our assays may translate more or less than the average population. An additional caveat in these experiments is that because of random X inactivation in female fibroblasts the fragile X premutation transcript is presumably expressed in approximately 50% of the FXTAS patient derived fibroblasts. This may have decreased our ability to detect the full extent of the effects of the premutation transcript on MBP mRNA translation. Future experiments should be performed with fibroblasts from additional control and FXTAS subjects. It would be most beneficial to perform additional experiments with fibroblasts derived from male patients, so that all FXTAS fibroblasts examined express the fragile X premutation. Ideally, the results of the fibroblast and

OLG experiments should also be validated in OLGs derived from iPSCs of FXTAS patients and/or in OLGs derived from the *FMR1* CGG knock-in mouse, which is a commonly used mouse model to study FXTAS (Berman et al., 2009).

It has been theorized that the expanded CGG repeat RNA of the fragile X premutation transcript may bind to other CGG repeat containing RNAs involved in translation regulation, inhibiting their translation (Fig. 3-3, Dr. John H. Carson and Rene Norman, personal communication). Whether the expression of inhibitors or enhancers of translation regulation are more affected by the presence of the fragile X premutation transcript would determine if the translation of other mRNAs, such as MBP mRNA is increased or decreased. Our results suggest that expanded CGG repeat RNA has a greater effect on reducing the translation of mRNA of proteins that inhibit MBP mRNA translation. Arc mRNA, which also has an A2RE sequence, has increased translation both in fibroblasts and neurons in the presence of expanded CGG repeats. If indeed CGG repeat RNA of the fragile X premutation transcript binds to and inhibits CGG repeat containing RNAs involved in translation regulation, our results imply that different translation regulatory proteins control the translation of Arc mRNA and MBP mRNA.

Overall, the results of our experiments provide evidence that MBP mRNA translation is increased by the presence of the fragile X premutation. In the peripheral nervous system, hypermyelination of axons early in development has been found to lead to demyelination later on (Adlkofer et al., 1995). It is possible that in FXTAS the myelin lesions found are due to demyelination caused by hypermyelination earlier in life. Examination of myelin expression in the *FMR1* CGG repeat knock-in mouse and imaging of youth with the fragile X premutation will reveal if hypermyelination contributes to the myelin lesions present in FXTAS.

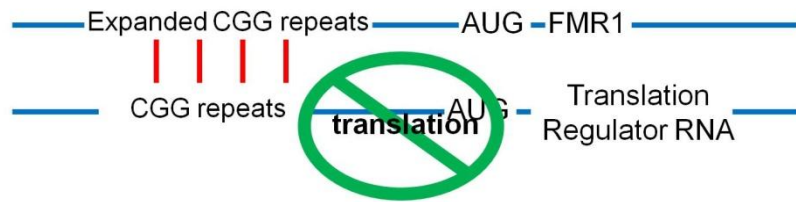


Figure 3-3: Suggested mechanism by which the fragile X premutation influences the expression of proteins which regulate translation. Expanded CGG repeats in the fragile X premutation RNA bind to CGG repeats present in transcripts for proteins which regulate translation. This binding prevents the translation of mRNAs that encode proteins that regulate mRNA translation, therefore altering the translation of other mRNAs, such as MBP mRNA.

Experimental Methods

Fibroblast Cell lines

Fibroblast cell lines were obtained from Dr. Anita Bhattacharyya at the University of Wisconsin-Madison. FX 08-2 (referred to as FXTAS 08 in text and figures) was derived from a female with one allele with 31 CGG repeats and one allele with 105 CGG repeats present in the 5'UTR of the *FMR1* gene. FX 13-2 (referred to as FXTAS 13 in text and figures) was derived from a female with one allele with 33 CGG repeats and one allele with 85 CGG repeats present in the 5'UTR of the *FMR1* gene. Control 603 was derived from a male whose sole allele contained 31 repeats in the 5'UTR of the *FMR1* gene.

Animals

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.

Rat OLGs

Primary glia cultures were prepared from the brains of rats at postnatal days 1–3 and OLG progenitor cells (OPCs) were isolated from these cultures as described by McCarthy and de Vellis (1980). OPCs were plated on poly-L-Ornithine (.05 mg/mL in borate buffer) coated coverslips in 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).

Venus-MBP expression vector

The vector was obtained from Dr. Victor Francone (Quinnipiac University). Rat MBP cDNA was PCR amplified using the primers: forward 5'-GCGAGATCTATGGCATCACAGAAGAGACCCTC-3'; reverse: 5'-CGCCCCGGGCAAGTTTATTATTTTATTTC-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 s followed by 18 cycles of 30 s at 95 °C, 30 s at 55 °C, and 12 min at 68 °C, all in a final volume of 50 µl. Two and a half units of Pfu Turbo DNA polymerase were used for elongation. After amplification the methylated parental plasmid was digested with 10 U DpnI at 37 °C for 2 h. TOPO competent cells were transformed with a 2 µl aliquot of the reaction mix, and positive clones were confirmed by sequencing.

CGG repeat expression vector

Plasmids containing CGG repeats along with firefly luciferase (pT7-*FMR1*-5'UTR(CGG)_n-FL, n=0, 30, 62, 99) were gifts from Dr. Michael Fry (Khateb et al., 2007). Briefly, a T7 promoter sequence was ligated to the Bsp1 site 235 bases upstream of human *FMR1* 5'UTR (pE5.1; GenBank accession number L29074) fused to firefly luciferase. The CGG repeats were located 117 bases upstream of the *FMR1* ATG initiation codon and a Xho I site was located approximately 10 bases after the final CGG repeat (and approximately 60 bases upstream of

the initiation codon). Pre-transcription, these plasmids were linearized with Xho I, eliminating the firefly luciferase reporter sequence.

Synthesis of RNA

Cy5-UTP Venus-MBP mRNA, Cy3-UTP 0 CGG mRNA, and Cy3-UTP 0 CGG mRNA were synthesized by *in vitro* transcription of linearized template DNA. The DNA was transcribed, capped, and polyadenylated using mScript Kit (Epicentre) according to the manufacturer's protocol, with the exception that Cy5-conjugated UTP or Cy3-conjugated UTP (Amersham biosciences) were added 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 of Rat OLGs

Cy5-UTP labeled MBP mRNA (0.5 mg/mL) was microinjected into the perikaryon of mature rat OLGs in primary cultures at least 5 days in differentiation medium. The Cy5-UTP labeled MBP mRNA was coinjected with Cy3-UTP 0 CGG RNA (0.1 to 1 mg/mL) or Cy3-UTP 99 CGG RNA (0.1 mg/mL). Following injection at room temperature, cells were returned to incubate at 37°C until fixed 3.5 hours later.

Comparing Cy5-UTP MBP mRNA quantity in granules of RAT OLGs

The quantity of Cy5-UTP labeled Venus-MBP mRNA in granules of microinjected rat OLGs was determined using NIH Image J software. Using the oval tool, the granule was delineated and

the intensity of Cy5 enclosed area was measured and the background subtracted. For OLGs in which granules with expanded 99 CGG repeats and without expanded 99 CGG repeats were examined from the same cell the average Cy5 intensity of granules without the 99 CGG repeats was normalized to 1. For the experiment in which the concentration of Cy5-UTP MBP mRNA from granules of OLGs coinjected with either 99 CGG repeat RNA or 0 CGG repeat RNA were compared, the average Cy5 intensity of granules from the 99 CGG repeat RNA was normalized to 100%.

Measuring Translation of MBP mRNA in granules of microinjected rat OLGs

Translational output of Cy5-UTP labeled Venus-MBP mRNA in granules was determined using Image J. Using the oval tool, the granule was delineated and the intensity of Cy5 and of Venus was measured in the enclosed area and the background subtracted. The ratio of net Venus intensity to net Cy5 intensity was calculated for each granule, and was classified as translational output.

Measuring whole cell translation of Venus MBP mRNA in fibroblasts

Cy5-UTP Venus MBP mRNA (0.3 mg/mL or 1 mg/mL) was coinjected into the perikaryon of fibroblasts with dextran. Following injection, cells were returned to incubator at 37°C and images were acquired five hours post injection or cells were fixed with 4% paraformaldehyde at five hours and images acquired at a later time point. Cells that were imaged were chosen by the detection of dextran in the cell. Image J was used to analyze the images. Using the polygon tool, the outline of the fibroblast was drawn. The intensity of Cy5 and of Venus was measured in the encompassed area and the background subtracted. The net intensity of Venus was used as a measure of translation for the fibroblasts.

Chapter 4: The Tumor Overexpressed Gene protein and its regulation of the expression of Myelin Basic Protein

Anthony Giampetruzzi and Elisa Barbarese

A.G. and E.B. designed the study; A.G. performed the experiments; A.G. analyzed the data, A.G. wrote the manuscript with modifications provided by E.B.

Abstract

Knocking out the tumor overexpressed gene (TOG) protein in mouse OLGs results in the near absence of myelin basic protein (MBP) and extensive CNS dysmyelination. We found levels of other myelin proteins, proteolipid protein (PLP) and myelin-associated glycoprotein (MAG), to be similarly reduced when TOG is knocked out in OLGs. We also examined TOG heterozygote (+/null) mice, which express half the normal level of TOG. Previously, MBP levels at 90 days had been found to be decreased in these mice. We discovered that MBP levels do not differ from wild type in the cerebral hemispheres of TOG heterozygote mice at 22 and 45 days, and levels of other myelin proteins also do not differ at 45 days. This suggests that another mechanism, besides the originally proposed decrease in MBP mRNA translation, is the cause of MBP deficiencies in the 90 day old TOG heterozygotes. TOG is a microtubule-associated protein and may be necessary for the transport of MBP mRNA granules to the myelin compartment where translation occurs. The effect of the reduction of TOG on microtubule based transport was examined by measuring mitochondrial transport. The transport of mitochondria was only slightly altered in the TOG heterozygote. These results suggest that approximately half the amount of TOG allows for sufficient transport of MBP mRNA and proper translation of MBP mRNA.

Introduction

In the central nervous system (CNS), myelin basic protein (MBP) is expressed exclusively by oligodendrocytes (OLGs), the myelinating cells of the CNS. MBP is expressed at much greater levels in the OLG processes than in the perikaryon, and MBP mRNA preferentially localizes to OLG processes where it will be translated (Trapp et al., 1987; Colman et al., 1982). MBP mRNA is present in ribonucleoprotein complexes termed granules that move into OLG processes by a microtubule-based mechanism. RNA granules contain molecular motors and machinery necessary for protein synthesis (Carson et al., 2001; Carson et al., 1997; Barbarese et al., 1995). MBP mRNA binds to heterogeneous ribonucleoprotein A2 (hnRNP A2) via two tandemly duplicated *cis*-acting sequences termed A2 Response Elements (A2REs) in its 3' UTR (Hoek et al., 1998). This interaction allows MBP mRNA to assemble into hnRNP A2 containing granules (Munro et al., 1999; Hoek et al., 1998; Ainger et al., 1997). These granules also contain the Tumor Overexpressed Gene (TOG) protein, most likely because TOG binds to hnRNP A2 (Kosturko et al., 2005).

TOG is the product of the Cytoskeleton Associated Protein (*CKAP*) 5 gene, a single copy gene with homologues in species ranging from yeast to vertebrates (Gard et al., 2004). TOG is a microtubule-associated protein most commonly studied in dividing cells for its crucial role in centrosome and microtubule stabilization and spindle assembly (Cassimeris and Morabito, 2004; Gergely et al., 2003; Lee et al., 2001; Charrasse et al., 1998). An additional role for TOG, as a component of hnRNP A2 containing granules in OLGs and neurons, has been uncovered. Conditional knockout of TOG in hippocampal neurons led to decreased incorporation of the activity-regulated cytoskeleton-associated (Arc) protein mRNA into RNA granules, decreased granule transport in dendrites, and decreased translation of Arc mRNA, which contains an A2RE sequence (Barbarese et al., 2013; Gao et al., 2008). In addition, TOG knockdown in

B104 cells (a rat neuronal cell line) led to decreased translation of GFP RNA containing an A2RE sequence but not of GFP RNA (Francone et al., 2007).

TOG's role in OLGs has been examined by knockdown approach, using shRNA, in developing OLGs in culture. TOG knockdown resulted in the absence of MBP expression. The knockdown did not alter the expression of a mature myelin protein, myelin oligodendrocyte glycoprotein (MOG), whose mRNA does not contain an A2RE sequence. The TOG knockdown did not alter the ability of MBP mRNA to incorporate into granules and be properly transported, suggesting that unlike neurons TOG may only play a role in translation and not granule assembly and transport in OLGs (Francone et al., 2007).

MBP is required for myelin formation in the CNS (Readhead and Hood, 1990). Therefore, we have investigated the function of TOG in MBP expression by examining the level of MBP and its transcripts and the presence of myelin in animals in which the cre/lox system was used to conditionally knockout TOG in OLGs. Myelin abnormalities in the CNP TOG cKO are obvious as mice have chronic intention tremors, seizures, and die prematurely. Analysis of the CNP TOG cKO revealed the animals contain 20% of the control level of MBP at approximately two months of age, without a change in MBP transcript level. Accumulation of MOG, a myelin specific protein expressed at late stages of OLG maturation, is decreased to a level of 80% of control. This slight decrease reveals that in TOG's absence the OLG is still able to differentiate. The change in MOG levels may not be directly due to the absence of TOG, but due to the lack of expression of MBP, as the *shiverer* mouse (discussed in chapter 1) contains only 30% the amount of MOG as controls. The decrease in MBP and MOG is not due to a decrease in OLGs. Staining in the corpus callosum for the OLG lineage cell marker Olig2 revealed similar levels of Olig2+ cells in the CNP TOG cKO as in control. Changes in microtubule dynamics in the OLG may play a role in the decreased expression of MBP, as the TOG CNP cKO exhibits decreased mitochondrial movement, suggesting alterations in microtubule dynamics.

Preliminary data strongly suggest that complete knockout of TOG is embryonic lethal (Barbarese et al., 2013). In order to examine a situation more applicable to human conditions we have investigated MBP expression in the TOG heterozygote (+/null) mouse. This animal possesses 55% of control TOG levels in the CNS. Because TOG is ubiquitously expressed it is assumed that TOG is reduced in all cell types (Gard et al., 2004). The decline in TOG expression is throughout development, unlike in the TOG CNP cKO. Analysis of MBP levels in the TOG heterozygote at 90 days reveals MBP levels ~ 60% of control.

The objective of the work presented in this chapter was to expand the analysis of OLGs and myelination in the CNP TOG cKO and in the TOG heterozygote. In the CNP TOG cKO, the expression of additional myelin proteins was examined. In the TOG heterozygote, distribution and quantity of OLG lineage cells was examined to investigate if a reduction in TOG could affect OLGs during development. In addition, MBP expression was analyzed at time points earlier than 90 days and the expression of several other myelin proteins was also examined. Finally, to examine if microtubule dynamics may be altered as in OLGs of the CNP TOG cKO, we indirectly studied microtubule dynamics in the TOG heterozygote OLGs by analyzing mitochondrial movement.

Results

Myelin deficiencies include proteolipid protein (PLP) and myelin-associated glycoprotein (MAG) in the CNP TOG cKO

Previous work performed in our lab found reductions in MBP and MOG in the CNP TOG cKO at 2 months of age. Two additional major myelin proteins were investigated, PLP and MAG. Significant reductions were present in both of those proteins in the CNP TOG cKO (Fig. 4-1). These reductions are most likely secondary to the absence of MBP as reductions in those proteins are also found in the *shiverer* mouse (Shiota et al., 1991; Sheedlo et al., 1986).

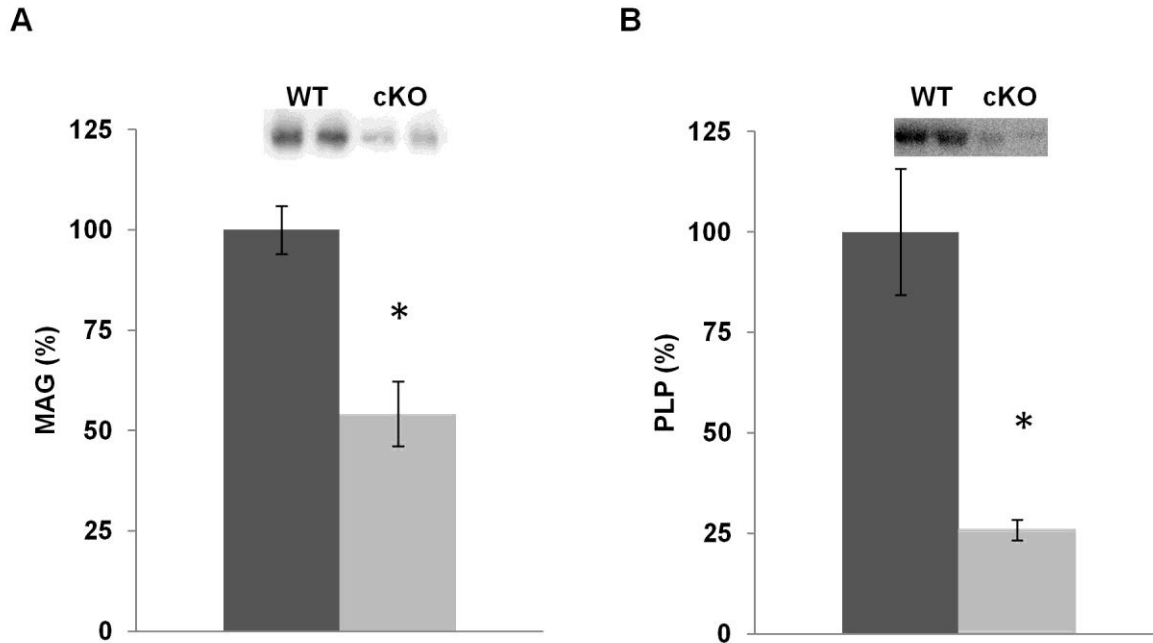


Figure 4-1: MAG and PLP levels are decreased in the CNP TOG cKO. Western blots and densitometric analysis of levels of MAG (A) and PLP (B) in homogenates of cerebral hemispheres of WT (n = 3) and TOG CNP cKO (n=4) at 2 months of age. Values were standardized to β -actin and means normalized to WT. There are significant differences in the levels of MAG and PLP (mean \pm s.e.m.; *t*-test, **p* < 0.01).

OLG development is normal in the TOG heterozygote

Unlike the CNP TOG cKO, TOG is reduced in TOG heterozygote OLGs throughout life. TOG is also reduced in the astrocytes and neurons which signal to OLGs. These reductions in TOG could lead to differences in development of OLGs. To examine this we computed the density of OLG lineage cells in the corpus callosum of the TOG heterozygote at 22 days (D). OLG lineage cells were identified by immunostaining for Olig2 (Fig. 4-2A, B). There was no significant difference in the density of Olig2 cells in the corpus callosum of the TOG heterozygote when compared to wild type (WT) (Fig. 4-2C). We also examined if the quantity of OLG lineage cells in the entire cerebral hemispheres differed in the TOG heterozygote by examining the expression of Olig2. Western blots of Olig2 showed no significant differences in expression between the TOG heterozygote and WT (Fig. 4-2D, E). These results suggest that there is proper number and proper placement of OLG lineage cells in the TOG heterozygote.

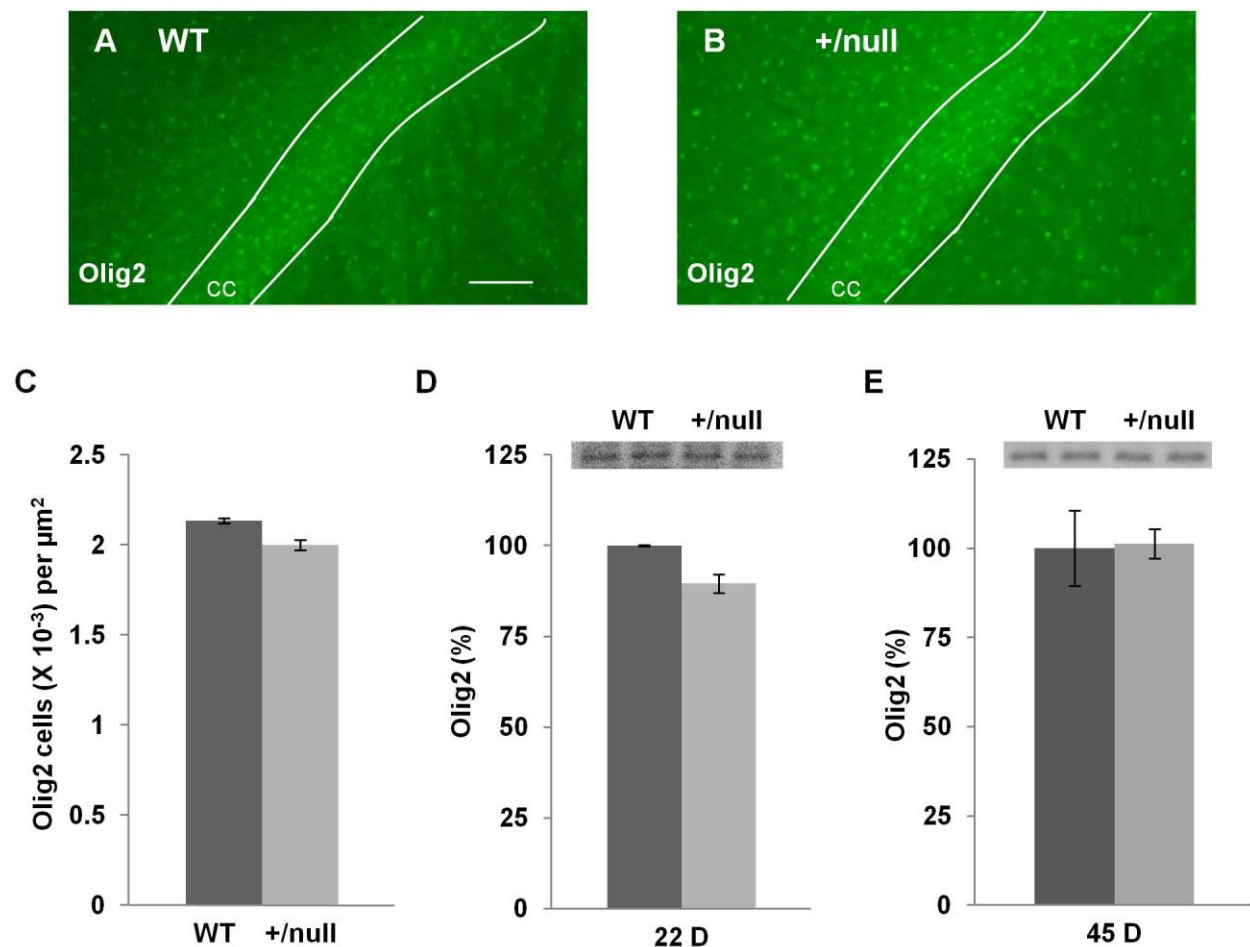


Figure 4-2: The TOG heterozygote displays normal OLG development. Immunostaining for Olig2 in 22 D mouse brains of WT (A) and TOG heterozygote (B). Corpus callosum (CC) is the enclosed region in the image. Scale bar, 100 μm. (C) Average density of Olig2 expressing cells in the corpus callosum of WT (n = 2) and TOG heterozygote (n = 2) at 22 D. There is no significant difference in the density when comparing genotypes (mean ± s.e.m.; *t*-test, *p* > 0.05). (D+E) Detection of Olig2 in Western blots of brain homogenates from WT and TOG heterozygote at 22 D (n = 2 WT, 2 +/null) and 45 D (n = 3 WT, 4 +/null). Densitometric analysis revealed no differences in the expression of Olig2 in the TOG heterozygote when compared to WT at either time point (mean ± s.e.m.; *t*-test, *p* > 0.05). Values were standardized to β-actin and means normalized to WT at each time point.

MBP and other myelin proteins are unchanged in the TOG heterozygote

Results previously obtained in our lab showed that the level of MBP was reduced in the TOG heterozygote at the age of 3 months. To investigate if the decline in MBP was due to decreased expression of MBP during myelination, we examined MBP levels in the cerebral hemispheres during (22 D) and shortly after (45 D) the active period of myelination. MBP levels were found to be unchanged at both time points in the TOG heterozygote (Fig. 4-3A). Levels of other major myelin proteins were also examined at 45 D to see if they were altered as in the TOG CNP cKO. No significant differences were found in the expression of MOG, MAG, PLP, and CNP (Fig. 4-3B-E).

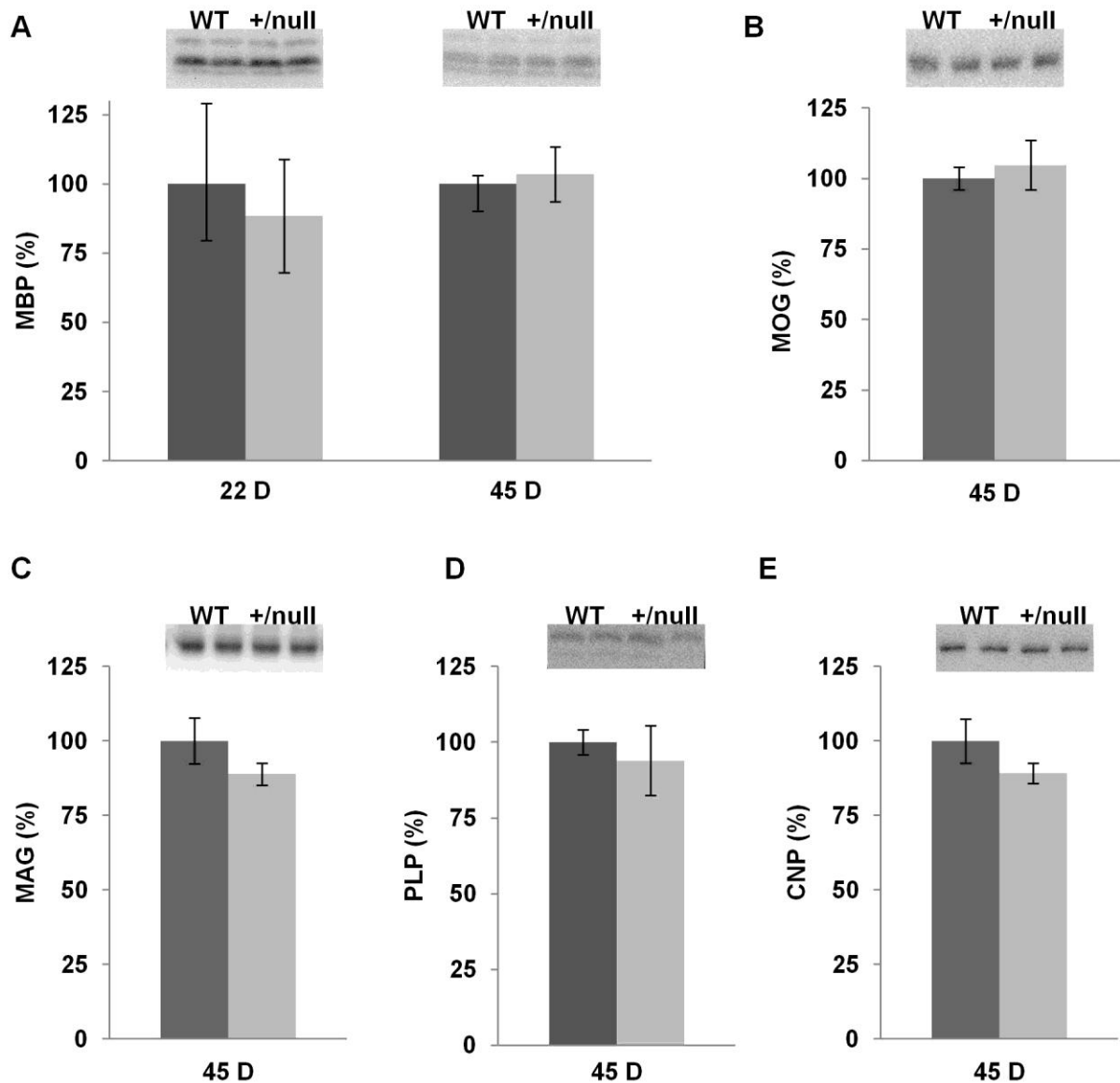


Figure 4-3: Levels of myelin proteins are similar to WT in the TOG heterozygote brain at 22 D and 45 D. Western blots of homogenates of cerebral hemispheres from WT and TOG heterozygote (+/null). (A) MBP at 22 D (n = 2 WT, 2 +/null) and 45 D (n = 3 WT, 3 +/null); (B) MOG at 45 D (n = 3 WT, 4 +/null); (C) MAG at 45 D (n = 2 WT, 3 +/null); (D) PLP at 45 D (n = 3 WT, 4 +/null); (E) CNP at 45 D (n = 3 WT, 4 +/null). 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 TOG heterozygote and WT at any of the time points (mean \pm s.e.m.; *t*-test, $p > 0.05$).

Mitochondria motility is slightly altered in the TOG heterozygote

TOG is also a microtubule-associated protein and its absence or reduced level could affect microtubule-based transport. In addition to RNA granules, microtubules are necessary for the transport of a variety of components and organelles in the cell. Mitochondrial motility, which was used as an indirect measure of microtubule-based transport of RNA granules, was previously found in our lab to be altered in the CNP TOG cKO. This alteration may contribute to a decrease in MBP expression in OLGs from the CNP TOG cKO, as it could alter the transport of MBP mRNA on microtubules. Measurement of mitochondria motility can easily be assessed by live cell labeling of mitochondria with a fluorescent compound (Mitotracker, Life Technologies) and monitoring them by microscopy. There was no obvious decline in the number of mitochondria found in the processes when comparing the TOG heterozygote and WT, suggesting that mitochondria can properly travel out to the processes in TOG heterozygote OLGs (Fig. 4-4A, B). TOG heterozygote mitochondria displayed a small increase (74.3% in +/-null vs. 71.2% in WT) in frequency of immobility and an increase in displacement in a small subset of frames analyzed (Fig. 4-4C). These results imply there may be a slight change in microtubule dynamics in the TOG heterozygote. However, as MBP expression is unaltered in the TOG heterozygote at 45 D it is highly unlikely that these very slight microtubule changes influence MBP mRNA transport and translation.

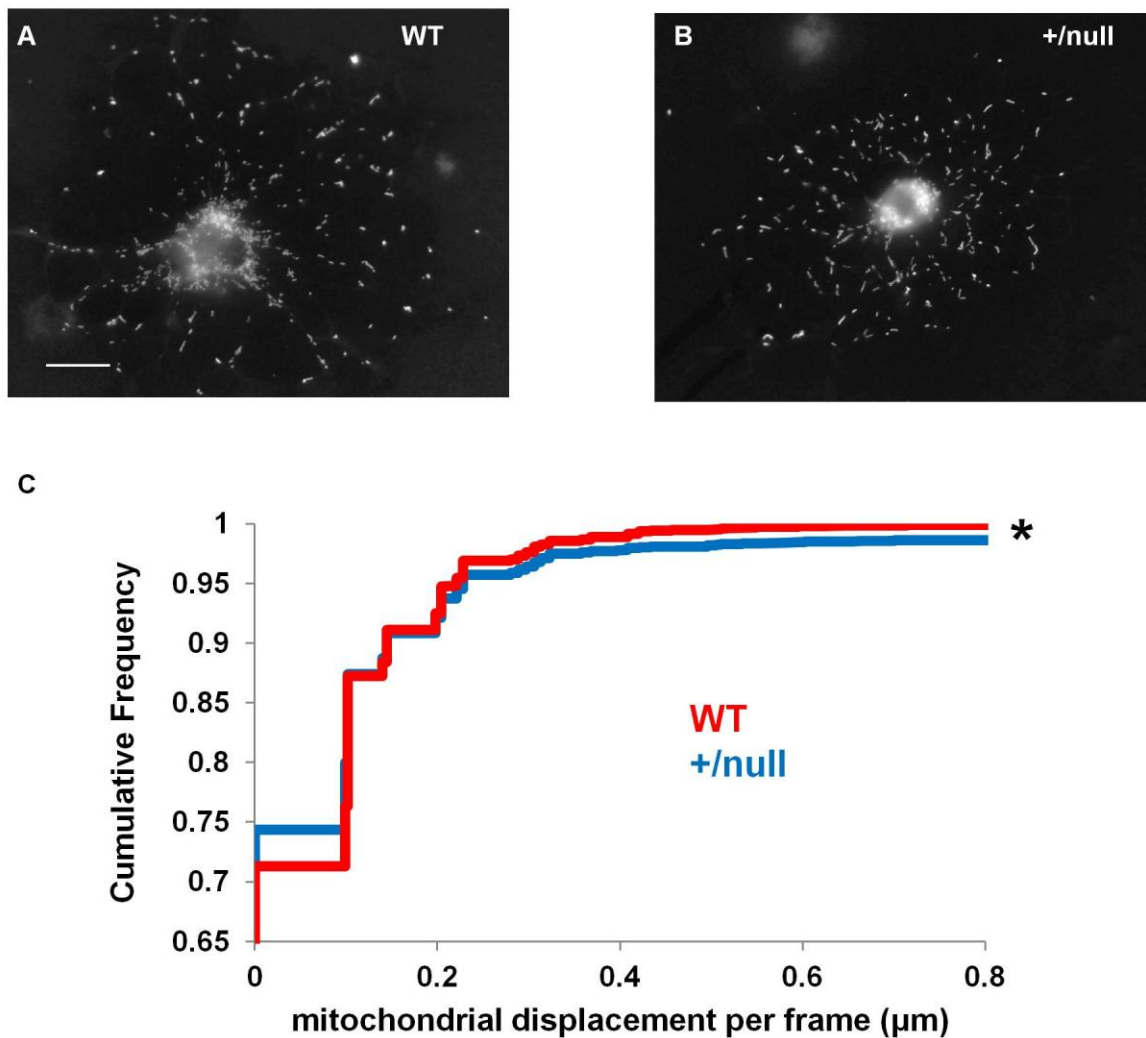


Figure 4-4: Mitochondrial movement is slightly altered in the TOG heterozygote. WT (A) and TOG heterozygote (B) OLGs with mitochondria labeled using Mitotracker. Scale bar, 20 μm . (C) Cumulative frequency plot of mitochondrial displacement per 5 second frame, the distribution of mitochondrial displacement significantly differs in the TOG heterozygote ($n = 11,726$ frames from 455 mitochondria) when compared to control ($n = 13,774$ frames from 400 mitochondria) (* $p < 0.0001$, Kolmogorov-Smirnov test).

Discussion

We found that in addition to the changes in MBP and MOG expression in the CNP TOG cKO, the expression of the myelin proteins PLP and MAG was also altered at 2 months of age. Expression of MBP and other myelin proteins has not been examined at earlier time points in the CNP TOG cKO. However, an intention tremor is present in these mice starting at around two weeks, which strongly implies MBP and myelin levels are very low at those early time points. Examination of the TOG heterozygote revealed that despite changes in MBP at three months, there is no reduction in MBP at 22 D and MBP or other myelin proteins at 45 D. As would be expected with no changes in MBP or myelin proteins, no changes in expression of Olig2 or density of OLGs in the corpus callosum were detected in the TOG heterozygote at the time points examined.

There are several plausible reasons for differences in timing of MBP changes in the CNP TOG cKO and the TOG heterozygote. First, the amount of TOG in the TOG heterozygote OLG may be sufficient to support MBP expression at early time periods but not at later time periods. If the expression of TOG in control OLGs decreases over time then similar decreases in the TOG heterozygote may leave the OLG with insufficient amounts of TOG for MBP expression for myelin maintenance, resulting in decreases in MBP at later time points. Unlike the CNP TOG cKO, TOG is reduced in all cell types in the TOG heterozygote. It may be possible that the amounts of TOG in the OLG are enough for proper MBP expression and myelination, but that dysfunction in neurons and astrocytes may occur at later time points leading to decreases in MBP levels. The effects of reducing TOG in astrocytes has not been examined but conditional knockout of TOG in hippocampal neurons alters dendritic spine density, reduces LTP, and affects behavior (Barbarese et al., 2013). It is quite possible that the decline in TOG in the TOG heterozygote may lead to neuronal changes which then affect MBP expression. The absence of changes in MBP levels in the TOG heterozygote during and soon after the active period of

myelination suggests that MBP synthesis and accumulation may not be altered at all in the TOG heterozygote, but that its degradation may be increased.

Mitochondrial motility is only slightly changed in cultured OLGs of the TOG heterozygote, implying that microtubule-based transport is close to normal. Results of the same assay in CNP TOG cKO showed slower transport. The deficiencies in microtubule-based transport in the CNP TOG cKO may contribute to the more severe and earlier MBP and myelin differences in the CNP TOG cKO when compared to the heterozygote. MBP mRNA travels out to the OLG process on microtubules making them very important for proper localization of MBP mRNA (Ainger et al., 1997). Further investigation into the localization of MBP mRNA in the CNP TOG cKO, such as by *in situ* hybridization, will help determine the role altered transport of MBP mRNA may play in the decreased MBP levels found in the CNP TOG cKO.

The results of our experiments have expanded the knowledge of how OLGs and the expression of MBP and myelin proteins are affected in the CNP TOG cKO and the TOG heterozygote. Based on these results, further experiments should be performed to examine if different mechanisms lead to MBP decreases in the CNP TOG cKO and the TOG heterozygote.

Experimental Methods

Animals

Floxed TOG mice and TOG^{+/*null*} mice were produced at the UCHC Gene Targeting and Transgenic Facility (GTTF). CNP-Cre mice were obtained from Dr. Klaus Nave (Lappe-Siefke et al., 2003). All mice are on a C57/BL6 background.

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-CNP (Millipore mab 326R, 1:5,000), mouse anti-MBP (SMI-94 Covance, 1:3000), rabbit anti-Olig2 (Millipore mab 9610, 1:5,000), 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- β -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).

Immunohistochemistry

Mouse brains were fixed in 4% paraformaldehyde overnight. The following day, brains were cut into 150 μ m thick sections. Brain sections were immunostained by blocking for 2 hours with blocking buffer (10% normal goat serum (NGS) and 0.2% Triton X-100 in PBS). Primary antibody (rabbit anti-Olig2, Millipore mab 9610, 1:350) in blocking buffer was added overnight at 4°C while shaking. The following day sections were washed three times in PBS for five minutes and then the secondary antibody (Molecular Probes, Goat anti-rabbit Alexa 488-A11034,

1:1000) was added for 2 hours in PBS. Sections were then washed three times for five minutes with PBS and mounted onto microscope slides.

Computation of Olig2 density in corpus callosum

Using AxioVision LE software, Olig2 positive cells were counted in enclosed circles, whose area were known, that were drawn in the corpus callosum of 150 μ m mouse brain slices. Corpus callosum was identified by its position in the slice and the alignment of myelinated fibers. Brain sections at similar depth were examined for WT and the TOG heterozygote.

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 per flask were used. 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 min. 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).

Analysis of mitochondrial movement

Mice OLGs cultured in ODM for 3 to 4 days were analyzed. ODM was removed and the culture dish rinsed several times with DMEM-F12 (Lonza, 12-719F). Then, 50 μ M Mitotracker Red CMXRos (Life Technologies) in DMEM-F12 was added to cover the dish and OLGs were

incubated for 25 minutes at 37°C. Following incubation, cells were rinsed several times with DMEM-F12 and then left in DMEM-F12 to be imaged. Live cell imaging was performed by using a Carl Zeiss Axiovert 200M microscope equipped with an incubation chamber. The cells were kept at 37°C while imaging. Cells chosen to be imaged were verified as OLGs by morphology, and highly branched OLGs appearing to be differentiated were used. OLGs were imaged every 5 seconds for up to 5 minutes.

Mitochondria were tracked as described previously (DeVos et al., 2007). The absolute velocity, regardless of direction, was found for each five second interval for each mitochondria. Each mitochondria was analyzed for between 40 and 59 intervals. For each mitochondria, the frequency of stationary events was computed by dividing the number of intervals without any movement by the total number of intervals analyzed. Also, the average absolute velocity of all intervals was found for each mitochondria.

Acknowledgements

I am thankful to Kyle Denton for his tutoring in how to analyze mitochondrial movement and Jennifer Kirkland for her assistance in analyzing mitochondrial movement data.

Chapter 5: Future Directions

Work presented in chapters 2-4 investigated different mechanisms by which MBP expression may be altered. FMRP, a translation inhibitor, was found to have no effect on MBP mRNA translation and MBP expression in rodents, and the fragile X premutation mRNA increased exogenous MBP mRNA translation in rodent OLGs and human fibroblasts. These findings lead to the need for future work to investigate why myelin abnormalities are found in these disorders. Continued investigation of TOG and its role in regulating MBP expression was presented in chapter 4. Though significant amounts of work have been performed in our lab, further work will be necessary to identify the mechanism by which a delayed decline in MBP levels occurs in the TOG heterozygote, how TOG may affect MBP mRNA translation, and how TOG itself is regulated in OLGs.

Oligodendrocyte differentiation

Investigation of myelin protein expression in cultured OLGs can be very informative. Rat oligodendrocyte precursor cells (OPCs) can be obtained in great quantities and survive for over a week after differentiation. In contrast, mouse OPCs are obtained in much lower quantities and usually do not survive much after differentiation. Since most disorders are modeled in mice, this creates a problem for myelin studies. I have unsuccessfully attempted to increase the survival of mouse OLGs by using several different differentiation media and rat OLG conditioned differentiation media. Future work in the field of OLG studies should focus on a protocol that leads to increased survival of mouse OLGs in culture. A protocol which efficiently produces OLGs from human induced pluripotent stem cells (iPSCs) in culture also should be devised. Current protocols yield OPCs for injection into mouse brain, but more work needs to be performed on determining the conditions for prolonged survival of differentiated OLGs in culture

(Wang et al., 2013). The ability to work with cultured OLGs from human iPSCs from diseased and control patients would be very beneficial.

The role of FMRP in OLGs and on myelin protein expression

Investigating the role of FMRP in OLGs

As described in chapter 2, we found FMRP to be expressed in cells throughout the OLG lineage. Prior to our work, the proposed role of FMRP in the OLG was to inhibit MBP mRNA translation. However, we found that not to be the case. Our work leads to the inquiry as to the role FMRP in OLGs. It is possible that FMRP could be involved in MBP mRNA translation inhibition, but not under normal conditions because its role is duplicated by other MBP mRNA translation regulators. FMRP has been found to interact with the 3' UTR of MBP mRNA and there are several regulators of MBP mRNA translation that interact with its 3' UTR. In a situation in which other translational regulators of MBP mRNA are dysfunctional, FMRP may have an increased role in inhibiting MBP mRNA translation. For instance, a reduction in hnRNP E1 activity accompanied by reduced FMRP expression may lead to myelin alterations. To test this, a double knockdown of FMRP and hnRNP E1 in cultured rat OLGs could be generated and MBP mRNA translation compared to that of control and single knockdowns. FMRP may also function in a role that is independent of translational regulation of MBP mRNA or other myelin functions. For instance, FMRP may be involved in metabotropic support to axons or other OLG functions.

Examining the differential effects of FMRP on myelin

Myelin alterations are found in FXS patients. Contrary to what was expected, our examination of the *Fmr1* KO mouse on the C57/BL6 background revealed no changes in any of the major

myelin proteins in the cerebral hemispheres. Our findings suggest that in addition to the absence of FMRP, a genetic or environmental factor, not present in the mice we examined, is necessary to cause myelin alterations. One such factor may be the enriched environmental experiences a human is subjected to as compared to laboratory mice. A future experiment would be to compare the myelination of *Fmr1* KO and control mice in an enriched environment. Rodents in enriched environments have increases in myelin and possibly *Fmr1* KO mice may have a more drastic myelin increase than control (Zhao et al., 2012). Examination of myelin in humans with FXS would also be very useful in determining which additional factors cause myelin changes in FXS, as myelin changes in FXS are not homogenous. Genomic studies of FXS patients with similar myelin phenotypes, based upon MRI, could help decipher any potential genetic factors. In addition, analysis of myelin in FXS patients from similar and different environments could help determining the role environmental factors may play on myelin in FXS.

Investigating the role of myelin in FXTAS

Characterizing MBP and myelin protein expression in the CGG knock-in mouse

Results presented in chapter 3 suggest MBP mRNA translation is increased in the presence of the fragile X premutation. In the peripheral nervous system, hypermyelination in development has been found to lead to demyelination later in life (Adlkofer et al., 1995). In FXTAS, hypermyelination due to increased MBP expression may lead to demyelination and the myelin lesions present in the disorder. Investigation of MBP and other myelin proteins in the knock-in mouse models in which expanded CGG repeats have been incorporated into the 5'UTR of the *FMR1* gene should be performed at various time periods to determine if the presence of the

fragile X premutation can lead to hypermyelination followed by demyelination (Berman et al., 2009).

If myelin changes are not found in the CGG knock-in mouse, it would suggest that additional factors may be necessary for myelin alterations in FXTAS. Different factors which may lead to the white matter phenotype can be investigated in the CGG knock-in mouse. For instance, individuals with fragile X premutation RNA may not remyelinate as well upon insult to myelin. This can be examined by subjecting CGG knock-in mice to demyelination and comparing their ability to remyelinate to controls.

Investigating the role of OLGs in FXTAS pathogenesis

Myelin lesions found in FXTAS may be produced by several different mechanisms. Primary dysfunction in astrocytes and neurons that through secondary effects alters the ability of the OLG to properly myelinate, maintain myelin, or remyelinate is one probable cause of white matter disease in FXTAS. In chapter 2, we show that FMRP is expressed in mature OLGs. This expression makes it possible for the fragile X premutation to directly affect OLGs, which means primary OLG dysfunction may also contribute to the white matter disease present in FXTAS. Further work should be performed to determine which mechanism, or both, cause the myelin phenotype in FXTAS.

Examination of myelination in the CGG knock-in mouse alone will not determine if there is primary dysfunction in OLGs in the presence of the fragile X premutation. It is expected that since OLGs express FMRP, they will have elevated levels of fragile X premutation transcript. Elevated levels of fragile X premutation transcript in the OLG are most likely needed for primary dysfunction of OLGs in FXTAS. Quantitative PCR of premutation transcript levels in OLGs from CGG knock-in mice can be compared to controls. Increased expression of the premutation transcript in CGG knock-in mice would warrant several other experiments to examine if the

presence of the fragile X premutation transcript in OLGs affects OLG function. If myelin differences were found in the mouse model, co-cultures of OLGs derived from the CGG knock-in mouse with wild type neurons can be analyzed to determine if OLGs from the CGG knock-in mouse myelinate differently than control OLGs. In addition, OPCs from the CGG knock-in mice can be added to brain slices or transplanted into the brain of *shiverer* mice and the pattern of myelination compared to that of control OPCs. *Shiverer* mice do not express MBP and addition of exogenous OPCs to brain slices or injection of OPCs into *shiverer* brain leads to the formation of compact myelin (Bin et al., 2012; Najm et al., 2011). Human OPCs derived from iPSCs have also been demonstrated to produce compact myelin and prevent premature death when introduced into *shiverer* brain (Wang et al., 2013). Myelination and survival of *shiverer* mice injected with OPCs derived from FXTAS patients and controls can also be compared. When the protocol has been established for differentiating mature human OLGs from iPSCs in culture, examining MBP and myelin in OLG cultures derived from iPSCs of FXTAS patients should also be informative. If these experiments show primary dysfunction in OLGs to be a cause of myelin differences in the presence of the fragile X premutation, experiments based on Venus-MBP mRNA translation in OLGs with expanded CGG repeats performed in chapter 3 could be expanded upon.

TOG in OLGs

Further analyzing TOG's effects on MBP and other myelin proteins

Results in chapter 4 identify reductions in MBP and other myelin proteins in the CNP TOG cKO. Decline in the other myelin proteins are thought to be a secondary effect to the severe decreases in MBP expression, as expression of these proteins is also reduced in the *shiverer* mouse. The *shiverer* mouse does not express MBP due to a mutation in the MBP gene. To verify that MBP reduction is the cause of the changes in other myelin proteins, transcript levels

of PLP could be examined in the CNP TOG cKO. The *shiverer* mouse has reduced expression of PLP transcript at 18 days and a similar reduction in the CNP TOG cKO would verify that at least some of the changes in PLP level could be attributed to altered levels of MBP (Shiota et al., 1991).

Investigating the delayed decline in MBP levels in the TOG heterozygote

Examination of MBP and other myelin proteins in the brain of the TOG heterozygote, which was presented in chapter 4, revealed no changes in MBP levels at 22 days and no alterations in MBP and other myelin proteins at 45 days. The absence of changes in MBP is surprising as MBP expression has been previously found in our lab to be decreased in the brain of the TOG heterozygote at three months. One important piece of evidence to gather would be if there are changes in other myelin proteins at three months in the TOG heterozygote. This information would be useful in determining if the mechanism leading to decreased MBP levels at three months is specific to MBP or not.

The timing of MBP decline in the TOG heterozygote implies that MBP mRNA translation, at least during the active period of myelination, is not altered. This suggests that an alternative mechanism may lead to the reduction in MBP in the TOG heterozygote at 90 days. One possible mechanism is increased MBP degradation. Future work can be performed to determine if degradation of MBP and other myelin proteins is increased, both in the TOG heterozygote and in the CNP TOG cKO. The TOG heterozygote features reductions of TOG in all cell types. Sufficient amount of TOG may be present in OLGs for the proper expression of MBP at 90 days, but dysfunction in other cell types may be affecting OLG function. This can be investigated by examining MBP and myelin in CNP TOG conditional heterozygotes. These animals are available to be studied in our lab and they have normal expression of TOG in all cells of the CNS except OLGs.

Confirming the role of the A2RE in TOG's regulation of MBP mRNA expression

Though further experiments should still be performed, it is highly probable that TOG specifically regulates the translation of MBP mRNA and not of all mRNAs in the OLG. TOG's binding to hnRNP A2, reduced translation of A2RE containing GFP mRNA but not GFP mRNA in B104 cells in which TOG has been knocked down, and altered translation of A2RE containing Arc mRNA in TOG knockout neurons suggest that the A2RE sequence provides the sensitivity of MBP mRNA to TOG reductions (Barbarese et al., 2013; Francone et al., 2007; Kosturko et al., 2005). To confirm this, expression of microinjected Venus-MBP mRNA without the A2RE sequence could be compared in control OLGs and OLGs with a reduction in TOG. In this instance, we expect the expression of Venus-MBP mRNA without the A2RE sequence to be similar to control when TOG is reduced. Ideally this experiment would be performed in TOG CNP cKO and control. However, microinjection of mouse OLGs has been very difficult when compared to rat OLGs, so this experiment may need to be performed in rat OLGs in which TOG levels have been decreased through shRNA knockdown.

Evaluation of expression of other mRNAs with an A2RE in OLGs can also be performed. One of the transcripts for Myelin-associated Oligodendrocyte Basic Protein (MOBP) contains an A2RE sequence (Ainger et al., 1997). It would be very interesting to compare the translation of the MOBP transcript containing the A2RE in control OLGs to translation in OLGs where TOG is reduced. Those results can then be compared to the results of analyzing translation of the MOBP transcript without an A2RE under the same conditions. It would be expected that translation of the MOBP transcript with the A2RE would be decreased when TOG is reduced in the OLG, but no difference should be found in translation of MOBP without the A2RE when comparing control with TOG reduced OLGs. Discoidin domain receptor 1 (Ddr1) is expressed in myelin and mouse transcripts for Ddr1 contain an A2RE sequence, as well as do 4 of the 5 human transcripts (Roig et al., 2012; Roig et al., 2010). Knockdown of hnRNP A2 in human

OLG cell lines showed decreased expression of Ddr1. It would be interesting to examine levels of Ddr1 in the brain of the TOG heterozygote and the CNP TOG cKO.

Determining the mechanism by which TOG regulates MBP expression

In vitro assays

As previously stated, it is highly likely TOG regulates MBP mRNA translation. However, the exact mechanism by which TOG influences MBP mRNA translation has not been determined. Several experiments can be performed, both *in vitro* and in OLGs, to help identify the mechanism. Analysis of *in vitro* translation of Venus-MBP mRNA in wheat germ lysate in the presence and absence of exogenous TOG would be useful in assessing TOG's role in translation of MBP mRNA. Differences in translation in the presence of exogenous TOG would suggest that TOG may have a role independent of involvement in cell signaling, independent of its role of as a microtubule binding protein, and independent of TOG's ability to affect MBP mRNA localization. One such mechanism that has been proposed is that TOG may promote translation by blocking the binding of the translational inhibitor hnRNP E1 to hnRNP A2 and/or by preventing the binding of another potential translational inhibitor TACC to CPEB on MBP mRNA (Francone et al., 2007). It has also been suggested that TOG binding to hnRNP A2 may prevent hnRNP A2 from inhibiting protein phosphatase 2A (PP2A). PP2A is responsible for dephosphorylating the elongation factor eEF2, which is in its active form when dephosphorylated (Francone et al., 2007). If TOG works through those mechanisms then differences in *in vitro* translation of MBP mRNA may be detected when TOG is present in the wheat germ lysate. This is of course contingent on the presence of those regulators in wheat germ lysate, which would first have to be assessed. If they were not present, assays in which they are added to the wheat germ lysate could be performed to investigate TOG's influence on their regulation of MBP mRNA translation.

Assays in OLGs

Analysis of OLGs in which TOG was knocked down using shRNA, showed no differences in MBP mRNA granule formation and localization (Francone et al., 2007). However, examination of neurons in which TOG was knocked out showed decreased numbers and decreased size of Arc RNA containing granules in dendrites (Barbarese et al., 2013). *In situ* hybridization should be performed in CNP TOG cKO OLGs to reveal if MBP mRNA granule assembly and/or transport are altered in the absence of TOG.

Work on the A2RE containing Arc mRNA in neurons implies that TOG is involved in polysomal translation of A2RE containing mRNAs (Barbarese et al., 2013). However, TOG is thought to associate with MBP mRNA through hnRNP A2, which is believed to be released from binding with MBP mRNA prior to the start of translation. Future work should further investigate TOG's role in polysomal translation of MBP mRNA. A polysome profile examining if TOG associated with polysomes in control OLGs would be helpful in determining if TOG itself associates with translationally active mRNA. In addition, polysome profiling of MBP mRNA in the brain of control and CNP TOG cKO would help determine if indeed TOG promotes polysomal translation in OLGs. If TOG is involved in promoting polysomal translation then a smaller percentage of MBP mRNA should be found associated with polyribosomes in the CNP TOG cKO than in controls.

The mechanism by which TOG influences translation of MBP mRNA in OLGs can further be investigated in several ways. Phosphorylation of hnRNP A2 by fyn kinase is believed to stimulate MBP mRNA translation in OLGs (White et al., 2008). TOG's role in this phosphorylation event could be determined by analyzing the phosphorylation of hnRNP A2 in the corpus callosum of the CNP TOG cKO, the TOG heterozygote, and in rat OLG cultures in which TOG has been knocked down. The mechanisms proposed by Francone et al. (2007),

and discussed in the previous paragraph on *in vitro* assays, could also be analyzed in cultured OLGs with reduced levels of TOG or in homogenate from the corpus callosum of the TOG CNP cKO.

Investigating TOG's regulation in OLGs

TOG has a very important function in MBP expression, yet very little is known about TOG in OLGs in both normal and diseased states. Further analysis of TOG in OLGs should be performed. Examining if TOG levels or the phosphorylation of TOG changes depending on the stage of the OLG would provide insight as to whether TOG expression or phosphorylation of TOG correlate with MBP expression in any way. In addition, little is known on what can alter TOG expression in OLGs. It has been shown that the microRNA miR-155 can decrease TOG expression and that miR-155 is upregulated in multiple sclerosis (Junker et al., 2009).

Investigation of TOG levels in OLGs of individuals affected with multiple sclerosis or mice subjected to experimental autoimmune encephalitis (EAE), a model used for multiple sclerosis, would provide crucial information on TOG regulation in OLGs and its possible role in multiple sclerosis.

Reference List

- Adlkofer., K., Martini, R., Aguzzi, A., Zielasek, J., Toyka, K.V., Suter U., 1995. Hypermyelination and demyelinating peripheral neuropathy in Pmp22-deficient mice. *Nat Genet.* 11, 274-80.
- Ahrendsen, J.T., Macklin, W., 2013. Signaling mechanisms regulating myelination in the central nervous system. *Neurosci Bull.* 29, 199-215.
- Ainger, K., Avossa, D., Diana, A.S., Barry, C., Barbarese, E., Carson, J.H., 1997. Transport and localization elements in myelin basic protein mRNA. *J Cell Biol.* 138, 1077-1087.
- Ainger, K., Avossa, D., Morgan, F., Hill, S.J., Barry, C., Barbarese, E., Carson, J.H., 1993. Transport and localization of exogenous myelin basic protein mRNA microinjected into oligodendrocytes. *J Cell Biol.* 123, 431-441.
- Almeida, R.G., Czopka, T., Ffrench-Constant, C., Lyons, D.A., 2011. Individual axons regulate the myelinating potential of single oligodendrocytes in vivo. *Development.* 138, 4443-4450.
- Als, H., Duffy, F.H., McAnulty, G.B., Rivkin, M.J., Vajapeyam, S., Mulkern, R.V., Warfield, S.K., Huppi, P.S., Butler, S.C., Conneman, N., Fischer, C., Eichenwald, E.C., 2004. Early experience alters brain function and structure. *Pediatrics.* 113, 846-857.
- Amur-Umarjee, S., Schonmann, V., Campagnoni, A.T., 1997. Neuronal regulation of myelin basic protein mRNA translocation in oligodendrocytes is mediated by platelet-derived growth factor. *Dev Neurosci.* 19, 143-151.
- Andrade, M.A., Petosa, C., O'Donoghue, S.I., Muller, C.W., Bork, P., 2001. Comparison of ARM and HEAT protein repeats. *J Mol Biol.* 309, 1-18.
- Antar, L.N., Li, C., Zhang, H., Carroll, R.C., Bassell, G.J., 2006. Local functions for FMRP in axon growth cone motility and activity-dependent regulation of filopodia and spine synapses. *Mol Cell Neurosci.* 32, 37-48.
- Ashley, C.T., Jr, Wilkinson, K.D., Reines, D., Warren, S.T., 1993. FMR1 protein: Conserved RNP family domains and selective RNA binding. *Science.* 262, 563-566.
- Aziz, M., Stathopulu, E., Callias, M., Taylor, C., Turk, J., Oostra, B., Willemsen, R., Patton, M., 2003. Clinical features of boys with fragile X premutations and intermediate alleles. *Am J Med Genet B Neuropsychiatr Genet.* 121B, 119-127.
- Balslev, T., Cortez, M.A., Blaser, S.I., Haslam, R.H., 1997. Recurrent seizures in metachromatic leukodystrophy. *Pediatr Neurol.* 17, 150-154.
- Bansal, R., 2002. Fibroblast growth factors and their receptors in oligodendrocyte development: Implications for demyelination and remyelination. *Dev Neurosci.* 24, 35-46.
- Barbarese, E., Ifrim, M.F., Hsieh, L., Guo, C., Tatavarty, V., Maggipinto, M.J., Korza, G., Tutolo, J.W., Giampetruzzi, A., Le, H., Ma, X.M., Levine, E., Bishop, B., Kim, D.O., Kuwada, S., Carson,

- J.H., 2013. Conditional knockout of tumor overexpressed gene in mouse neurons affects RNA granule assembly, granule translation, LTP and short term habituation. *PLoS One*. 8, e69989.
- Barbarese, E., Brumwell, C., Kwon, S., Cui, H., Carson, J.H., 1999. RNA on the road to myelin. *J Neurocytol*. 28, 263-270.
- Barbarese, E., Koppel, D.E., Deutscher, M.P., Smith, C.L., Ainger, K., Morgan, F., Carson, J.H., 1995. Protein translation components are colocalized in granules in oligodendrocytes. *J Cell Sci*. 108 (Pt 8), 2781-2790.
- Barbarese, E., Carson, J.H., Braun, P.E., 1978. Accumulation of the four myelin basic proteins in mouse brain during development. *J Neurochem*. 31, 779-782.
- Bardoni, B., Schenck, A., Mandel, J.L., 1999. A novel RNA-binding nuclear protein that interacts with the fragile X mental retardation (FMR1) protein. *Hum Mol Genet*. 8, 2557-2566.
- Barnea-Goraly, N., Eliez, S., Hedeus, M., Menon, V., White, C.D., Moseley, M., Reiss, A.L., 2003. White matter tract alterations in fragile X syndrome: Preliminary evidence from diffusion tensor imaging. *Am J Med Genet B Neuropsychiatr Genet*. 118B, 81-88.
- Barres, B.A., Raff, M.C., 1993. Proliferation of oligodendrocyte precursor cells depends on electrical activity in axons. *Nature*. 361, 258-260.
- Battistella, G., Niederhauser, J., Fornari, E., Hippolyte, L., Gronchi Perrin, A., Lesca, G., Forzano, F., Hagmann, P., Vingerhoets, F.J., Draganski, B., Maeder, P., Jacquemont, S., 2013. Brain structure in asymptomatic FMR1 premutation carriers at risk for fragile X-associated tremor/ataxia syndrome. *Neurobiol Aging*. 34, 1700-1707.
- Bauer, N.M., Moos, C., van Horssen, J., Witte, M., van der Valk, P., Altenhein, B., Luhmann, H.J., White, R., 2012. Myelin basic protein synthesis is regulated by small non-coding RNA 715. *EMBO Rep*. 13, 827-834.
- Baumann, N., Pham-Dinh, D., 2001. Biology of oligodendrocyte and myelin in the mammalian central nervous system. *Physiol Rev*. 81, 871-927.
- Bear, M.F., Huber, K.M., Warren, S.T., 2004. The mGluR theory of fragile X mental retardation. *Trends Neurosci*. 27, 370-377.
- Bengtsson, S.L., Nagy, Z., Skare, S., Forsman, L., Forssberg, H., Ullen, F., 2005. Extensive piano practicing has regionally specific effects on white matter development. *Nat Neurosci*. 8, 1148-1150.
- Bengtsson, S.L., Nagy, Z., Skare, S., Forsman, L., Forssberg, H., Ullen, F., 2005. Extensive piano practicing has regionally specific effects on white matter development. *Nat Neurosci*. 8, 1148-1150.
- Berman, R.F., Willemsen, R., 2009. Mouse models of fragile X-associated tremor ataxia. *J Investig Med*. 57, 837-41.

Bhakar, A.L., Dolen, G., Bear, M.F., 2012. The pathophysiology of fragile X (and what it teaches us about synapses). *Annu Rev Neurosci.* 35, 417-443.

Bin, J.M., Leong, S.Y., Bull, S.J., Antel, J.P., Kennedy, T.E., 2012. Oligodendrocyte precursor cell transplantation into organotypic cerebellar shiverer slices: A model to study myelination and myelin maintenance. *PLoS One.* 7, e41237.

Boggs, J.M., 2006a. Myelin basic protein: A multifunctional protein. *Cell Mol Life Sci.* 63, 1945-1961.

Boggs, J.M., Rangaraj, G., Gao, W., Heng, Y.M., 2006b. Effect of phosphorylation of myelin basic protein by MAPK on its interactions with actin and actin binding to a lipid membrane in vitro. *Biochemistry.* 45, 391-401.

Brackett, D.M., Qing, F., Amieux, P.S., Sellers, D.L., Horner, P.J., Morris, D.R., 2013. FMR1 transcript isoforms: Association with polyribosomes; regional and developmental expression in mouse brain. *PLoS One.* 8, e58296.

Bradl, M., Lassmann, H., 2010. Oligodendrocytes: Biology and pathology. *Acta Neuropathol.* 119, 37-53.

Brady, S.T., Witt, A.S., Kirkpatrick, L.L., de Waegh, S.M., Readhead, C., Tu, P.H., Lee, V.M., 1999. Formation of compact myelin is required for maturation of the axonal cytoskeleton. *J Neurosci.* 19, 7278-7288.

Bregman, B.S., Kunkel-Bagden, E., Schnell, L., Dai, H.N., Gao, D., Schwab, M.E., 1995. Recovery from spinal cord injury mediated by antibodies to neurite growth inhibitors. *Nature.* 378, 498-501.

Brennan, F.X., Albeck, D.S., Paylor, R., 2006. Fmr1 knockout mice are impaired in a leverpress escape/avoidance task. *Genes Brain Behav.* 5, 467-471.

Brinkmann, B.G., Agarwal, A., Sereda, M.W., Garratt, A.N., Muller, T., Wende, H., Stassart, R.M., Nawaz, S., Humml, C., Velanac, V., Radyushkin, K., Goebbels, S., Fischer, T.M., Franklin, R.J., Lai, C., Ehrenreich, H., Birchmeier, C., Schwab, M.H., Nave, K.A., 2008. Neuregulin-1/ErbB signaling serves distinct functions in myelination of the peripheral and central nervous system. *Neuron.* 59, 581-595.

Brouhard, G.J., Stear, J.H., Noetzel, T.L., Al-Bassam, J., Kinoshita, K., Harrison, S.C., Howard, J., Hyman, A.A., 2008. XMAP215 is a processive microtubule polymerase. *Cell.* 132, 79-88.

Brumwell, C., Antolik, C., Carson, J.H., Barbarese, E., 2002. Intracellular trafficking of hnRNP A2 in oligodendrocytes. *Exp Cell Res.* 279, 310-320.

Cammer, W., Kahn, S., Zimmerman, T., 1984. Biochemical abnormalities in spinal cord myelin and CNS homogenates in heterozygotes affected by the shiverer mutation. *J Neurochem.* 42, 1372-1378.

Campagnoni, A.T., Macklin, W.B., 1988. Cellular and molecular aspects of myelin protein gene expression. *Mol Neurobiol.* Spring 2, 41-89.

Campagnoni, A.T., Skoff, R.P., 2001. The pathobiology of myelin mutants reveal novel biological functions of the MBP and PLP genes. *Brain Pathol.* 11, 74-91.

Campagnoni, A.T., Verdi, J.M., Verity, A.N., Amur-Umarjee, S., Byravan, S., 1991. Posttranscriptional regulation of myelin protein gene expression. *Ann N Y Acad Sci.* 633, 178-188.

Cao, N., Yao, Z.X., 2013. Oligodendrocyte N-methyl-D-aspartate receptor signaling: Insights into its functions. *Mol Neurobiol.* 47, 845-856.

Carson, J.H., Cui, H., Barbarese, E., 2001. The balance of power in RNA trafficking. *Curr Opin Neurobiol.* 11, 558-563.

Carson, J.H., Worboys, K., Ainger, K., Barbarese, E., 1997. Translocation of myelin basic protein mRNA in oligodendrocytes requires microtubules and kinesin. *Cell Motil Cytoskeleton.* 38, 318-328.

Cassimeris, L., Morabito, J., 2004. TOGp, the human homolog of XMAP215/Dis1, is required for centrosome integrity, spindle pole organization, and bipolar spindle assembly. *Mol Biol Cell.* 15, 1580-1590.

Ceman, S., O'Donnell, W.T., Reed, M., Patton, S., Pohl, J., Warren, S.T., 2003. Phosphorylation influences the translation state of FMRP-associated polyribosomes. *Hum Mol Genet.* 12, 3295-3305.

Charles, P., Hernandez, M.P., Stankoff, B., Aigrot, M.S., Colin, C., Rougon, G., Zalc, B., Lubetzki, C., 2000. Negative regulation of central nervous system myelination by polysialylated-neural cell adhesion molecule. *Proc Natl Acad Sci U S A.* 97, 7585-7590.

Charrasse, S., Schroeder, M., Gauthier-Rouviere, C., Ango, F., Cassimeris, L., Gard, D.L., Larroque, C., 1998. The TOGp protein is a new human microtubule-associated protein homologous to the xenopus XMAP215. *J Cell Sci.* 111 (Pt 10), 1371-1383.

Chen, Y., Wu, H., Wang, S., Koito, H., Li, J., Ye, F., Hoang, J., Escobar, S.S., Gow, A., Arnett, H.A., Trapp, B.D., Karandikar, N.J., Hsieh, J., Lu, Q.R., 2009. The oligodendrocyte-specific G protein-coupled receptor GPR17 is a cell-intrinsic timer of myelination. *Nat Neurosci.* 12, 1398-1406.

Chomiak, T., Hu, B., 2009. What is the optimal value of the g-ratio for myelinated fibers in the rat CNS? A theoretical approach. *PLoS One.* 4, e7754.

Coffee, B., Keith, K., Albizua, I., Malone, T., Mowrey, J., Sherman, S.L., Warren, S.T., 2009. Incidence of fragile X syndrome by newborn screening for methylated FMR1 DNA. *Am J Hum Genet.* 85, 503-514.

- Cohen, S., Masyn, K., Adams, J., Hessel, D., Rivera, S., Tassone, F., Brunberg, J., DeCarli, C., Zhang, L., Cogswell, J., Loesch, D., Leehey, M., Grigsby, J., Hagerman, P.J., Hagerman, R., 2006. Molecular and imaging correlates of the fragile X-associated tremor/ataxia syndrome. *Neurology*. 67, 1426-1431.
- Collins, S.C., Bray, S.M., Suhl, J.A., Cutler, D.J., Coffee, B., Zwick, M.E., Warren, S.T., 2010. Identification of novel FMR1 variants by massively parallel sequencing in developmentally delayed males. *Am J Med Genet A*. 152A, 2512-2520.
- Colman, D.R., Kreibich, G., Frey, A.B., Sabatini, D.D., 1982. Synthesis and incorporation of myelin polypeptides into CNS myelin. *J Cell Biol*. 95, 598-608.
- Cornish, K.M., Kogan, C.S., Li, L., Turk, J., Jacquemont, S., Hagerman, R.J., 2009. Lifespan changes in working memory in fragile X premutation males. *Brain Cogn*. 69, 551-558.
- Cruz-Martin, A., Crespo, M., Portera-Cailliau, C., 2010. Delayed stabilization of dendritic spines in fragile X mice. *J Neurosci*. 30, 7793-7803.
- Cui, Q.L., Fogle, E., Almazan, G., 2006. Muscarinic acetylcholine receptors mediate oligodendrocyte progenitor survival through src-like tyrosine kinases and PI3K/Akt pathways. *Neurochem Int*. 48, 383-393.
- Darnell, J.C., Van Driesche, S.J., Zhang, C., Hung, K.Y., Mele, A., Fraser, C.E., Stone, E.F., Chen, C., Fak, J.J., Chi, S.W., Licatalosi, D.D., Richter, J.D., Darnell, R.B., 2011. FMRP stalls ribosomal translocation on mRNAs linked to synaptic function and autism. *Cell*. 146, 247-261.
- Dawe GS, Schachner M, Patterson M, Zhi-Cheng X., 2006. Ensheathing the Node of Ranvier? *Neuron glia biology*. 2, 149.
- de Vries, H., de Jonge, J.C., Schrage, C., van der Haar, M.E., Hoekstra, D., 1997. Differential and cell development-dependent localization of myelin mRNAs in oligodendrocytes. *J Neurosci Res*. 47, 479-488.
- Delassalle, A., Zalc, B., Lachapelle, F., Raoul, M., Collier, P., Jacque, C., 1981. Regional distribution of myelin basic protein in the central nervous system of quaking, jimpy, and normal mice during development and aging. *J Neurosci Res*. 6, 303-313.
- Demerens, C., Stankoff, B., Logak, M., Anglade, P., Allinquant, B., Couraud, F., Zalc, B., Lubetzki, C., 1996. Induction of myelination in the central nervous system by electrical activity. *Proc Natl Acad Sci U S A*. 93, 9887-9892.
- Devys, D., Lutz, Y., Rouyer, N., Bellocq, J.P., Mandel, J.L., 1993. The FMR-1 protein is cytoplasmic, most abundant in neurons and appears normal in carriers of a fragile X premutation. *Nat Genet*. 4, 335-340.
- D'Hooge, R., Nagels, G., Franck, F., Bakker, C.E., Reyniers, E., Storm, K., Kooy, R.F., Oostra, B.A., Willems, P.J., De Deyn, P.P., 1997. Mildly impaired water maze performance in male *Fmr1* knockout mice. *Neuroscience*. 76, 367-376.

- Dickson, K.S., Bilger, A., Ballantyne, S., Wickens, M.P., 1999. The cleavage and polyadenylation specificity factor in *xenopus laevis* oocytes is a cytoplasmic factor involved in regulated polyadenylation. *Mol Cell Biol.* 19, 5707-5717.
- Du, L., Richter, J.D., 2005. Activity-dependent polyadenylation in neurons. *Rna.* 11, 1340-1347.
- Dutch-Belgian fragile X consortium. 1994. *Fmr1* knockout mice: A model to study fragile X mental retardation. *Cell.* 78, 23-33.
- Dyer, C.A., Philibotte, T.M., Billings-Gagliardi, S., Wolf, M.K., 1995. Cytoskeleton in myelin-basic-protein-deficient shiverer oligodendrocytes. *Dev Neurosci.* 17, 53-62.
- Dyer, C.A., Philibotte, T.M., Wolf, M.K., Billings-Gagliardi, S., 1994. Myelin basic protein mediates extracellular signals that regulate microtubule stability in oligodendrocyte membrane sheets. *J Neurosci Res.* 39, 97-107.
- Ellegood, J., Pacey, L.K., Hampson, D.R., Lerch, J.P., Henkelman, R.M., 2010. Anatomical phenotyping in a mouse model of fragile X syndrome with magnetic resonance imaging. *Neuroimage.* 53, 1023-1029.
- Feng, Y., Absher, D., Eberhart, D.E., Brown, V., Malter, H.E., Warren, S.T., 1997. FMRP associates with polyribosomes as an mRNP, and the I304N mutation of severe fragile X syndrome abolishes this association. *Mol Cell.* 1, 109-118.
- Feng, Y., Gutekunst, C.A., Eberhart, D.E., Yi, H., Warren, S.T., Hersch, S.M., 1997. Fragile X mental retardation protein: Nucleocytoplasmic shuttling and association with somatodendritic ribosomes. *J Neurosci.* 17, 1539-1547.
- Fields, R.D., 2008. White matter in learning, cognition and psychiatric disorders. *Trends Neurosci.* 31, 361-370.
- Fitzner, D., Schneider, A., Kippert, A., Mobius, W., Willig, K.I., Hell, S.W., Bunt, G., Gaus, K., Simons, M., 2006. Myelin basic protein-dependent plasma membrane reorganization in the formation of myelin. *Embo j.* 25, 5037-5048.
- Francone, V.P., Maggipinto, M.J., Kosturko, L.D., Barbarese, E., 2007. The microtubule-associated protein tumor overexpressed gene/cytoskeleton-associated protein 5 is necessary for myelin basic protein expression in oligodendrocytes. *J Neurosci.* 27, 7654-7662.
- Funfschilling, U., Supplie, L.M., Mahad, D., Boretius, S., Saab, A.S., Edgar, J., Brinkmann, B.G., Kassmann, C.M., Tzvetanova, I.D., Mobius, W., Diaz, F., Meijer, D., Suter, U., Hamprecht, B., Sereda, M.W., Moraes, C.T., Frahm, J., Goebbels, S., Nave, K.A., 2012. Glycolytic oligodendrocytes maintain myelin and long-term axonal integrity. *Nature.* 485, 517-521.
- Gao, Y., Tatavarty, V., Korza, G., Levin, M.K., Carson, J.H., 2008. Multiplexed dendritic targeting of alpha calcium calmodulin-dependent protein kinase II, neurogranin, and activity-regulated cytoskeleton-associated protein RNAs by the A2 pathway. *Mol Biol Cell.* 19, 2311-2327.

- Garcia-Arocena, D., Hagerman, P.J., 2010. Advances in understanding the molecular basis of FXTAS. *Hum Mol Genet.* 19, R83-9.
- Garcia-Arocena, D., Yang, J.E., Brouwer, J.R., Tassone, F., Iwahashi, C., Berry-Kravis, E.M., Goetz, C.G., Sumis, A.M., Zhou, L., Nguyen, D.V., Campos, L., Howell, E., Ludwig, A., Greco, C., Willemsen, R., Hagerman, R.J., Hagerman, P.J., 2010. Fibroblast phenotype in male carriers of FMR1 premutation alleles. *Hum Mol Genet.* 19, 299-312.
- Gard, D.L., Becker, B.E., Josh Romney, S., 2004. MAPping the eukaryotic tree of life: Structure, function, and evolution of the MAP215/Dis1 family of microtubule-associated proteins. *Int Rev Cytol.* 239, 179-272.
- Gary, D.S., Malone, M., Capestany, P., Houdayer, T., McDonald, J.W., 2012. Electrical stimulation promotes the survival of oligodendrocytes in mixed cortical cultures. *J Neurosci Res.* 90, 72-83.
- Gay, C.T., Hardies, L.J., Rauch, R.A., Lancaster, J.L., Plaetke, R., DuPont, B.R., Cody, J.D., Cornell, J.E., Herndon, R.C., Ghidoni, P.D., Schiff, J.M., Kaye, C.I., Leach, R.J., Fox, P.T., 1997. Magnetic resonance imaging demonstrates incomplete myelination in 18q- syndrome: Evidence for myelin basic protein haploinsufficiency. *Am J Med Genet.* 74, 422-431.
- Geiser, M., Cebe, R., Drewello, D., Schmitz, R., 2001. Integration of PCR fragments at any specific site within cloning vectors without the use of restriction enzymes and DNA ligase. *Biotechniques.* 31, 88-90, 92.
- Gergely, F., Draviam, V.M., Raff, J.W., 2003. The ch-TOG/XMAP215 protein is essential for spindle pole organization in human somatic cells. *Genes Dev.* 17, 336-341.
- Giampetruzzi, A., Carson, J.H., Barbarese, E., 2013. FMRP and myelin protein expression in oligodendrocytes. *Mol Cell Neurosci.* 56, 333-341.
- Givogri, M.I., Bongarzone, E.R., Schonmann, V., Campagnoni, A.T., 2001. Expression and regulation of golli products of myelin basic protein gene during in vitro development of oligodendrocytes. *J Neurosci Res.* 66, 679-690.
- Greco, C.M., Berman, R.F., Martin, R.M., Tassone, F., Schwartz, P.H., Chang, A., Trapp, B.D., Iwahashi, C., Brunberg, J., Grigsby, J., Hessel, D., Becker, E.J., Papazian, J., Leehey, M.A., Hagerman, R.J., Hagerman, P.J., 2006. Neuropathology of fragile X-associated tremor/ataxia syndrome (FXTAS). *Brain.* 129, 243-255.
- Greco, C.M., Hagerman, R.J., Tassone, F., Chudley, A.E., Del Bigio, M.R., Jacquemont, S., Leehey, M., Hagerman, P.J., 2002. Neuronal intranuclear inclusions in a new cerebellar tremor/ataxia syndrome among fragile X carriers. *Brain.* 125, 1760-1771.
- Greenough, W.T., Klintsova, A.Y., Irwin, S.A., Galvez, R., Bates, K.E., Weiler, I.J., 2001. Synaptic regulation of protein synthesis and the fragile X protein. *Proc Natl Acad Sci U S A.* 98, 7101-7106.

Griffiths, I., Klugmann, M., Anderson, T., Yool, D., Thomson, C., Schwab, M.H., Schneider, A., Zimmermann, F., McCulloch, M., Nadon, N., Nave, K.A., 1998. Axonal swellings and degeneration in mice lacking the major proteolipid of myelin. *Science*. 280, 1610-1613.

Grinthal, A., Adamovic, I., Weiner, B., Karplus, M., Kleckner, N., 2010. PR65, the HEAT-repeat scaffold of phosphatase PP2A, is an elastic connector that links force and catalysis. *Proc Natl Acad Sci U S A*. 107, 2467-2472.

Gross, C., Yao, X., Pong, D.L., Jeromin, A., Bassell, G.J., 2011. Fragile X mental retardation protein regulates protein expression and mRNA translation of the potassium channel Kv4.2. *J Neurosci*. 31, 5693-5698.

Gyllenstein, L., Malmfors, T., 1963. Myelination of the optic nerve and its dependence on visual function--a quantitative investigation in mice. *J Embryol Exp Morphol*. 11, 255-266.

Haas, B.W., Barnea-Goraly, N., Lightbody, A.A., Patnaik, S.S., Hoeft, F., Hazlett, H., Piven, J., Reiss, A.L., 2009. Early white-matter abnormalities of the ventral frontostriatal pathway in fragile X syndrome. *Dev Med Child Neurol*. 51, 593-599.

Hagemeyer, N., Goebbels, S., Papiol, S., Kastner, A., Hofer, S., Begemann, M., Gerwig, U.C., Boretius, S., Wieser, G.L., Ronnenberg, A., Gurvich, A., Heckers, S.H., Frahm, J., Nave, K.A., Ehrenreich, H., 2012. A myelin gene causative of a catatonia-depression syndrome upon aging. *EMBO Mol Med*. 4, 528-539.

Hagerman, P.J., Hagerman, R.J., 2004. The fragile-X premutation: A maturing perspective. *Am J Hum Genet*. 74, 805-816.

Hallahan, B.P., Craig, M.C., Toal, F., Daly, E.M., Moore, C.J., Ambikapathy, A., Robertson, D., Murphy, K.C., Murphy, D.G., 2011. In vivo brain anatomy of adult males with fragile X syndrome: An MRI study. *Neuroimage*. 54, 16-24.

Han, S.P., Friend, L.R., Carson, J.H., Korza, G., Barbarese, E., Maggipinto, M., Hatfield, J.T., Rothnagel, J.A., Smith, R., 2010. Differential subcellular distributions and trafficking functions of hnRNP A2/B1 spliceoforms. *Traffic*. 11, 886-898.

Handa, V., Goldwater, D., Stiles, D., Cam, M., Poy, G., Kumari, D., Usdin, K., 2005. Long CGG-repeat tracts are toxic to human cells: Implications for carriers of fragile X premutation alleles. *FEBS Lett*. 579, 2702-2708.

Harauz, G., Ishiyama, N., Hill, C.M., Bates, I.R., Libich, D.S., Fares, C., 2004. Myelin basic protein-diverse conformational states of an intrinsically unstructured protein and its roles in myelin assembly and multiple sclerosis. *Micron*. 35, 503-542.

Harris, S.W., Hessler, D., Goodlin-Jones, B., Ferranti, J., Bacalman, S., Barbato, I., Tassone, F., Hagerman, P.J., Herman, H., Hagerman, R.J., 2008. Autism profiles of males with fragile X syndrome. *Am J Ment Retard*. 113, 427-438.

Hashem, V., Galloway, J.N., Mori, M., Willemsen, R., Oostra, B.A., Paylor, R., Nelson, D.L., 2009. Ectopic expression of CGG containing mRNA is neurotoxic in mammals. *Hum Mol Genet.* 18, 2443-2451.

Hildebrand, C., Remahl, S., Persson, H., Bjartmar, C., 1993. Myelinated nerve fibres in the CNS. *Prog Neurobiol.* 40, 319-384.

Hill, C.M., Harauz, G., 2005. Charge effects modulate actin assembly by classic myelin basic protein isoforms. *Biochem Biophys Res Commun.* 329, 362-369.

Hinds, H.L., Ashley, C.T., Sutcliffe, J.S., Nelson, D.L., Warren, S.T., Housman, D.E., Schalling, M., 1993. Tissue specific expression of FMR-1 provides evidence for a functional role in fragile X syndrome. *Nat Genet.* 3, 36-43.

Hodgman, R., Tay, J., Mendez, R., Richter, J.D., 2001. CPEB phosphorylation and cytoplasmic polyadenylation are catalyzed by the kinase IAK1/Eg2 in maturing mouse oocytes. *Development.* 128, 2815-2822.

Hoeft, F., Walter, E., Lightbody, A.A., Hazlett, H.C., Chang, C., Piven, J., Reiss, A.L., 2011. Neuroanatomical differences in toddler boys with fragile x syndrome and idiopathic autism. *Arch Gen Psychiatry.* 68, 295-305.

Hoeft, F., Carter, J.C., Lightbody, A.A., Cody Hazlett, H., Piven, J., Reiss, A.L., 2010. Region-specific alterations in brain development in one- to three-year-old boys with fragile X syndrome. *Proc Natl Acad Sci U S A.* 107, 9335-9339.

Hoek, K.S., Kidd, G.J., Carson, J.H., Smith, R., 1998. hnRNP A2 selectively binds the cytoplasmic transport sequence of myelin basic protein mRNA. *Biochemistry.* 37, 7021-7029.

Hood, F.E., Williams, S.J., Burgess, S.G., Richards, M.W., Roth, D., Straube, A., Pfuhl, M., Bayliss, R., Royle, S.J., 2013. Coordination of adjacent domains mediates TACC3-ch-TOG-clathrin assembly and mitotic spindle binding. *J Cell Biol.* 202, 463-478.

Huber, K.M., Gallagher, S.M., Warren, S.T., Bear, M.F., 2002. Altered synaptic plasticity in a mouse model of fragile X mental retardation. *Proc Natl Acad Sci U S A.* 99, 7746-7750.

Hunsaker, M.R., Wenzel, H.J., Willemsen, R., Berman, R.F., 2009. Progressive spatial processing deficits in a mouse model of the fragile X premutation. *Behav Neurosci.* 123, 1315-1324.

Inagawa, K., Watanabe, S., Tsukada, Y., Mikoshiba, K., 1988. The role of myelination in learning performance observed in two strains of myelin-deficient mutant mice (shiverer and mld). *Behav Neural Biol.* 50, 184-192.

Irwin, S.A., Patel, B., Idupulapati, M., Harris, J.B., Crisostomo, R.A., Larsen, B.P., Kooy, F., Willems, P.J., Cras, P., Kozlowski, P.B., Swain, R.A., Weiler, I.J., Greenough, W.T., 2001. Abnormal dendritic spine characteristics in the temporal and visual cortices of patients with fragile-X syndrome: A quantitative examination. *Am J Med Genet.* 98, 161-167.

Ishibashi, T., Dakin, K.A., Stevens, B., Lee, P.R., Kozlov, S.V., Stewart, C.L., Fields, R.D., 2006. Astrocytes promote myelination in response to electrical impulses. *Neuron*. 49, 823-832.

Iwahashi, C.K., Yasui, D.H., An, H.J., Greco, C.M., Tassone, F., Nannen, K., Babineau, B., Lebrilla, C.B., Hagerman, R.J., Hagerman, P.J., 2006. Protein composition of the intranuclear inclusions of FXTAS. *Brain*. 129, 256-271.

Jacobs, S., Doering, L.C., 2010. Astrocytes prevent abnormal neuronal development in the fragile x mouse. *J Neurosci*. 30, 4508-4514.

Jacobs, S., Nathwani, M., Doering, L.C., 2010. Fragile X astrocytes induce developmental delays in dendrite maturation and synaptic protein expression. *BMC Neurosci*. 11, 132-2202-11-132.

Jakovcevski, I., Filipovic, R., Mo, Z., Rakic, S., Zecevic, N., 2009. Oligodendrocyte development and the onset of myelination in the human fetal brain. *Front Neuroanat*. 3, 5.

Jin, P., Duan, R., Qurashi, A., Qin, Y., Tian, D., Rosser, T.C., Liu, H., Feng, Y., Warren, S.T., 2007. Pur alpha binds to rCGG repeats and modulates repeat-mediated neurodegeneration in a drosophila model of fragile X tremor/ataxia syndrome. *Neuron*. 55, 556-564.

Jin, P., Zarnescu, D.C., Zhang, F., Pearson, C.E., Lucchesi, J.C., Moses, K., Warren, S.T., 2003. RNA-mediated neurodegeneration caused by the fragile X premutation rCGG repeats in drosophila. *Neuron*. 39, 739-747.

Junker, A., Krumbholz, M., Eisele, S., Mohan, H., Augstein, F., Bittner, R., Lassmann, H., Wekerle, H., Hohlfeld, R., Meinl, E., 2009. MicroRNA profiling of multiple sclerosis lesions identifies modulators of the regulatory protein CD47. *Brain*. 132, 3342-3352.

Kamholz J., de Ferra F., Puckett C., Lazzarini R., 1986. Identification of three forms of human myelin basic protein by cDNA cloning. *Proc Natl Acad Sci U S A*. 83,4962-6.

Kanfer, J., Parenty, M., Goujet-Zalc, C., Monge, M., Bernier, L., Campagnoni, A.T., Dautigny, A., Zalc, B., 1989. Developmental expression of myelin proteolipid, basic protein, and 2',3'-cyclic nucleotide 3'-phosphodiesterase transcripts in different rat brain regions. *J Mol Neurosci*. 1, 39-46.

Keegan, B.M., Noseworthy, J.H., 2002. Multiple sclerosis. *Annu Rev Med*. 53, 285-302.

Khateb, S., Weisman-Shomer, P., Hershcó-Shani, I., Ludwig, A.L., Fry, M., 2007. The tetraplex (CGG)_n destabilizing proteins hnRNP A2 and CBF-A enhance the in vivo translation of fragile X premutation mRNA. *Nucleic Acids Res*. 35, 5775-5788.

Kimura, M., Sato, M., Akatsuka, A., Saito, S., Ando, K., Yokoyama, M., Katsuki, M., 1998. Overexpression of a minor component of myelin basic protein isoform (17.2 kDa) can restore myelinogenesis in transgenic shiverer mice. *Brain Res*. 785, 245-252.

Kirschner, D.A., Ganser, A.L., 1980. Compact myelin exists in the absence of basic protein in the shiverer mutant mouse. *Nature*. 283, 207-210.

- Kolodny, E.H., 1993. Dysmyelinating and demyelinating conditions in infancy. *Curr Opin Neurol Neurosurg.* 6, 379-386.
- Kosturko, L.D., Maggipinto, M.J., Korza, G., Lee, J.W., Carson, J.H., Barbarese, E., 2006. Heterogeneous nuclear ribonucleoprotein (hnRNP) E1 binds to hnRNP A2 and inhibits translation of A2 response element mRNAs. *Mol Biol Cell.* 17, 3521-3533.
- Kosturko, L.D., Maggipinto, M.J., D'Sa, C., Carson, J.H., Barbarese, E., 2005. The microtubule-associated protein tumor overexpressed gene binds to the RNA trafficking protein heterogeneous nuclear ribonucleoprotein A2. *Mol Biol Cell.* 16, 1938-1947.
- Kumar, S., Mattan, N.S., de Vellis, J., 2006. Canavan disease: A white matter disorder. *Ment Retard Dev Disabil Res Rev.* 12, 157-165.
- Lachiewicz, A.M., Dawson, D.V., 1994. Do young boys with fragile X syndrome have macroorchidism? *Pediatrics.* 93, 992-995.
- Laggerbauer, B., Ostareck, D., Keidel, E.M., Ostareck-Lederer, A., Fischer, U., 2001. Evidence that fragile X mental retardation protein is a negative regulator of translation. *Hum Mol Genet.* 10, 329-338.
- Lappe-Siefke, C., Goebbels, S., Gravel, M., Nicksch, E., Lee, J., Braun, P.E., Griffiths, I.R., Nave, K.A., 2003. Disruption of *Cnp1* uncouples oligodendroglial functions in axonal support and myelination. *Nat Genet.* 33, 366-374.
- Laursen, L.S., Chan, C.W., French-Constant, C., 2011. Translation of myelin basic protein mRNA in oligodendrocytes is regulated by integrin activation and hnRNP-K. *J Cell Biol.* 192, 797-811.
- Laursen, L.S., Chan, C.W., French-Constant, C., 2009. An integrin-cadherin complex regulates CNS myelination by differential fyn phosphorylation. *J Neurosci.* 29, 9174-9185.
- Lee, H.Y., Ge, W.P., Huang, W., He, Y., Wang, G.X., Rowson-Baldwin, A., Smith, S.J., Jan, Y.N., Jan, L.Y., 2011. Bidirectional regulation of dendritic voltage-gated potassium channels by the fragile X mental retardation protein. *Neuron.* 72, 630-642.
- Lee, M.J., Gergely, F., Jeffers, K., Peak-Chew, S.Y., Raff, J.W., 2001. Msp/XP215 interacts with the centrosomal protein D-TACC to regulate microtubule behaviour. *Nat Cell Biol.* 3, 643-649.
- Lee, S., Leach, M.K., Redmond, S.A., Chong, S.Y., Mellon, S.H., Tuck, S.J., Feng, Z.Q., Corey, J.M., Chan, J.R., 2012. A culture system to study oligodendrocyte myelination processes using engineered nanofibers. *Nat Methods.* 9, 917-922.
- Leehey, M.A., Berry-Kravis, E., Goetz, C.G., Zhang, L., Hall, D.A., Li, L., Rice, C.D., Lara, R., Cogswell, J., Reynolds, A., Gane, L., Jacquemont, S., Tassone, F., Grigsby, J., Hagerman, R.J., Hagerman, P.J., 2008. FMR1 CGG repeat length predicts motor dysfunction in premutation carriers. *Neurology.* 70, 1397-1402.

- Li, L.B., Bonini, N.M., 2010. Roles of trinucleotide-repeat RNA in neurological disease and degeneration. *Trends Neurosci.* 33, 292-298.
- Li, Z., Zhang, Y., Ku, L., Wilkinson, K.D., Warren, S.T., Feng, Y., 2001. The fragile X mental retardation protein inhibits translation via interacting with mRNA. *Nucleic Acids Res.* 29, 2276-2283.
- Liu, X., Bates, R., Yin, D.M., Shen, C., Wang, F., Su, N., Kirov, S.A., Luo, Y., Wang, J.Z., Xiong, W.C., Mei, L., 2011. Specific regulation of NRG1 isoform expression by neuronal activity. *J Neurosci.* 31, 8491-8501.
- Lossner, C., Meier, J., Warnken, U., Rogers, M.A., Lichter, P., Pscherer, A., Schnolzer, M., 2011. Quantitative proteomics identify novel miR-155 target proteins. *PLoS One.* 6, e22146.
- Lu, P.H., Lee, G.J., Tishler, T.A., Meghpara, M., Thompson, P.M., Bartzokis, G., 2013. Myelin breakdown mediates age-related slowing in cognitive processing speed in healthy elderly men. *Brain Cogn.* 81, 131-138.
- Lu, R., Wang, H., Liang, Z., Ku, L., O'donnell, W.T., Li, W., Warren, S.T., Feng, Y., 2004. The fragile X protein controls microtubule-associated protein 1B translation and microtubule stability in brain neuron development. *Proc Natl Acad Sci U S A.* 101, 15201-15206.
- Luyt, K., Varadi, A., Durant, C.F., Molnar, E., 2006. Oligodendroglial metabotropic glutamate receptors are developmentally regulated and involved in the prevention of apoptosis. *J Neurochem.* 99, 641-656.
- Makinodan, M., Rosen, K.M., Ito, S., Corfas, G., 2012. A critical period for social experience-dependent oligodendrocyte maturation and myelination. *Science.* 337, 1357-1360.
- Martin, M., Hiltner, T.D., Wood, J.C., Fraser, S.E., Jacobs, R.E., Readhead, C., 2006. Myelin deficiencies visualized in vivo: Visually evoked potentials and T2-weighted magnetic resonance images of shiverer mutant and wild-type mice. *J Neurosci Res.* 84, 1716-1726.
- Mathisen, P.M., Johnson, J.M., Kawczak, J.A., 1997. Stabilization of myelin mRNAs as measured in a brain slice system. *J Neurosci Res.* 50, 1030-1039.
- McCarthy, K.D., de Vellis, J., 1980. Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissue. *J Cell Biol.* 85, 890-902.
- McKerracher, L., David, S., Jackson, D.L., Kottis, V., Dunn, R.J., Braun, P.E., 1994. Identification of myelin-associated glycoprotein as a major myelin-derived inhibitor of neurite growth. *Neuron.* 13, 805-811.
- Mendez, R., Hake, L.E., Andresson, T., Littlepage, L.E., Ruderman, J.V., Richter, J.D., 2000. Phosphorylation of CPE binding factor by Eg2 regulates translation of c-mos mRNA. *Nature.* 404, 302-307.
- Meyer-Franke, A., Shen, S., Barres, B.A., 1999. Astrocytes induce oligodendrocyte processes to align with and adhere to axons. *Mol Cell Neurosci.* 14, 385-397.

- Mi, S., Miller, R.H., Lee, X., Scott, M.L., Shulag-Morskaya, S., Shao, Z., Chang, J., Thill, G., Levesque, M., Zhang, M., Hession, C., Sah, D., Trapp, B., He, Z., Jung, V., McCoy, J.M., Pepinsky, R.B., 2005. LINGO-1 negatively regulates myelination by oligodendrocytes. *Nat Neurosci.* 8, 745-751.
- Mignot, C., Boespflug-Tanguy, O., Gelot, A., Dautigny, A., Pham-Dinh, D., Rodriguez, D., 2004. Alexander disease: Putative mechanisms of an astrocytic encephalopathy. *Cell Mol Life Sci.* 61, 369-385.
- Miller, D.J., Duka, T., Stimpson, C.D., Schapiro, S.J., Baze, W.B., McArthur, M.J., Fobbs, A.J., Sousa, A.M., Sestan, N., Wildman, D.E., Lipovich, L., Kuzawa, C.W., Hof, P.R., Sherwood, C.C., 2012. Prolonged myelination in human neocortical evolution. *Proc Natl Acad Sci U S A.* 109, 16480-16485.
- Mimault, C., Giraud, G., Courtois, V., Cailloux, F., Boire, J.Y., Dastugue, B., Boespflug-Tanguy, O., 1999. Proteolipoprotein gene analysis in 82 patients with sporadic pelizaeus-merzbacher disease: Duplications, the major cause of the disease, originate more frequently in male germ cells, but point mutations do not. the clinical european network on brain dysmyelinating disease. *Am J Hum Genet.* 65, 360-369.
- Mineur, Y.S., Sluyter, F., de Wit, S., Oostra, B.A., Crusio, W.E., 2002. Behavioral and neuroanatomical characterization of the *Fmr1* knockout mouse. *Hippocampus.* 12, 39-46.
- Modesti, N.M., Barra, H.S., 1986. The interaction of myelin basic protein with tubulin and the inhibition of tubulin carboxypeptidase activity. *Biochem Biophys Res Commun.* 136, 482-489.
- Monzo, K., Papoulas, O., Cantin, G.T., Wang, Y., Yates, J.R., 3rd, Sisson, J.C., 2006. Fragile X mental retardation protein controls trailer hitch expression and cleavage furrow formation in drosophila embryos. *Proc Natl Acad Sci U S A.* 103, 18160-18165.
- Moore, C.S., Milner, R., Nishiyama, A., Frausto, R.F., Serwanski, D.R., Pagarigan, R.R., Whitton, J.L., Miller, R.H., Crocker, S.J., 2011. Astrocytic tissue inhibitor of metalloproteinase-1 (TIMP-1) promotes oligodendrocyte differentiation and enhances CNS myelination. *J Neurosci.* 31, 6247-6254.
- Morgan, F., Barbarese, E., Carson, J.H., 1992. Visualizing cells in three dimensions using confocal microscopy, image reconstruction and isosurface rendering: Application to glial cells in mouse central nervous system. *Scanning Microsc.* 6, 345-56; discussion 356-7.
- Mouland, A.J., Xu, H., Cui, H., Krueger, W., Munro, T.P., Prasol, M., Mercier, J., Rekosh, D., Smith, R., Barbarese, E., Cohen, E.A., Carson, J.H., 2001. RNA trafficking signals in human immunodeficiency virus type 1. *Mol Cell Biol.* 21, 2133-2143.
- Muddashetty, R.S., Kelic, S., Gross, C., Xu, M., Bassell, G.J., 2007. Dysregulated metabotropic glutamate receptor-dependent translation of AMPA receptor and postsynaptic density-95 mRNAs at synapses in a mouse model of fragile X syndrome. *J Neurosci.* 27, 5338-5348.
- Munro, T.P., Magee, R.J., Kidd, G.J., Carson, J.H., Barbarese, E., Smith, L.M., Smith, R., 1999. Mutational analysis of a heterogeneous nuclear ribonucleoprotein A2 response element for RNA trafficking. *J Biol Chem.* 274, 34389-34395.

- Nagai, T., Ibata, K., Park, E.S., Kubota, M., Mikoshiba, K., Miyawaki, A., 2002. A variant of yellow fluorescent protein with fast and efficient maturation for cell-biological applications. *Nat Biotechnol.* 20, 87-90.
- Najm, F.J., Zaremba, A., Caprariello, A.V., Nayak, S., Freundt, E.C., Scacheri, P.C., Miller, R.H., Tesar, P.J., 2011. Rapid and robust generation of functional oligodendrocyte progenitor cells from epiblast stem cells. *Nat Methods.* 8, 957-962.
- Napoli, E., Ross-Inta, C., Wong, S., Omanska-Klusek, A., Barrow, C., Iwahashi, C., Garcia-Arocena, D., Sakaguchi, D., Berry-Kravis, E., Hagerman, R., Hagerman, P.J., Giulivi, C., 2011. Altered zinc transport disrupts mitochondrial protein processing/import in fragile X-associated tremor/ataxia syndrome. *Hum Mol Genet.* 20, 3079-3092.
- Napoli, I., Mercaldo, V., Boyl, P.P., Eleuteri, B., Zalfa, F., De Rubeis, S., Di Marino, D., Mohr, E., Massimi, M., Falconi, M., Witke, W., Costa-Mattioli, M., Sonenberg, N., Achsel, T., Bagni, C., 2008. The fragile X syndrome protein represses activity-dependent translation through CYFIP1, a new 4E-BP. *Cell.* 134, 1042-1054.
- Narayanan, U., Nalavadi, V., Nakamoto, M., Pallas, D.C., Ceman, S., Bassell, G.J., Warren, S.T., 2007. FMRP phosphorylation reveals an immediate-early signaling pathway triggered by group I mGluR and mediated by PP2A. *J Neurosci.* 27, 14349-14357.
- Nave, K.A., 2010. Myelination and support of axonal integrity by glia. *Nature.* 468, 244-252.
- Neuwald, A.F., Hirano, T., 2000. HEAT repeats associated with condensins, cohesins, and other complexes involved in chromosome-related functions. *Genome Res.* 10, 1445-1452.
- O'Connor, L.T., Goetz, B.D., Kwiecien, J.M., Delaney, K.H., Fletch, A.L., Duncan, I.D., 1999. Insertion of a retrotransposon in mbp disrupts mRNA splicing and myelination in a new mutant rat. *J Neurosci.* 19, 3404-3413.
- O'Meara, R.W., Ryan, S.D., Colognato, H., Kothary, R., 2011. Derivation of enriched oligodendrocyte cultures and oligodendrocyte/neuron myelinating co-cultures from post-natal murine tissues. *J Vis Exp.* (54). pii: 3324. doi, 10.3791/3324.
- Oostra, B.A., Willemsen, R., 2009. FMR1: A gene with three faces. *Biochim Biophys Acta.* 1790, 467-477.
- Orr, H.T., Zoghbi, H.Y., 2007. Trinucleotide repeat disorders. *Annu Rev Neurosci.* 30, 575-621.
- Pacey, L.K., Xuan, I.C., Guan, S., Sussman, D., Henkelman, R.M., Chen, Y., Thomsen, C., Hampson, D.R., 2013. Delayed myelination in a mouse model of fragile X syndrome. *Hum Mol Genet.* 22, 3920-3930.
- Pacey, L.K., Doering, L.C., 2007. Developmental expression of FMRP in the astrocyte lineage: Implications for fragile X syndrome. *Glia.* 55, 1601-1609.
- Pedraza, L., 1997. Nuclear transport of myelin basic protein. *J Neurosci Res.* 50, 258-264.

- Peier, A.M., McIlwain, K.L., Kenneson, A., Warren, S.T., Paylor, R., Nelson, D.L., 2000. (Over)correction of FMR1 deficiency with YAC transgenics: Behavioral and physical features. *Hum Mol Genet.* 9, 1145-1159.
- Peprah, E., He, W., Allen, E., Oliver, T., Boyne, A., Sherman, S.L., 2010. Examination of FMR1 transcript and protein levels among 74 premutation carriers. *J Hum Genet.* 55, 66-68.
- Pieretti, M., Zhang, F.P., Fu, Y.H., Warren, S.T., Oostra, B.A., Caskey, C.T., Nelson, D.L., 1991. Absence of expression of the FMR-1 gene in fragile X syndrome. *Cell.* 66, 817-822.
- Pribyl, T.M., Campagnoni, C.W., Kampf, K., Kashima, T., Handley, V.W., McMahon, J., Campagnoni, A.T., 1993. The human myelin basic protein gene is included within a 179-kilobase transcription unit: Expression in the immune and central nervous systems. *Proc Natl Acad Sci U S A.* 90, 10695-10699.
- Qin, M., Entezam, A., Usdin, K., Huang, T., Liu, Z.H., Hoffman, G.E., Smith, C.B., 2011. A mouse model of the fragile X premutation: Effects on behavior, dendrite morphology, and regional rates of cerebral protein synthesis. *Neurobiol Dis.* 42, 85-98.
- Quarles, R. H., Macklin, W. B., Morell, P., 2006. Myelin formation, structure and biochemistry. Siegel, G., Albers, R.W., Brady, S., Albers, W., editors. London: Elsevier Academic Press; Basic neurochemistry 7th edition. 51-72.
- Readhead, C., Hood, L., 1990. The dysmyelinating mouse mutations shiverer (shi) and myelin deficient (shimld). *Behav Genet.* 20, 213-234.
- Relucio, J., Tzvetanova, I.D., Ao, W., Lindquist, S., Colognato, H., 2009. Laminin alters fyn regulatory mechanisms and promotes oligodendrocyte development. *J Neurosci.* 29, 11794-11806.
- Reynolds, R., Wilkin, G.P., 1988. Development of macroglial cells in rat cerebellum. II. an in situ immunohistochemical study of oligodendroglial lineage from precursor to mature myelinating cell. *Development.* 102, 409-425.
- Rich, R.L., Myszka, D.G., 2007. Higher-throughput, label-free, real-time molecular interaction analysis. *Anal Biochem.* 361, 1-6.
- Roig, B., Moyano, S., Martorell, L., Costas, J., Vilella, E., 2012. The discoidin domain receptor 1 gene has a functional A2RE sequence. *J Neurochem.* 120, 408-418.
- Roig, B., Franco-Pons, N., Martorell, L., Tomas, J., Vogel, W.F., Vilella, E., 2010. Expression of the tyrosine kinase discoidin domain receptor 1 (DDR1) in human central nervous system myelin. *Brain Res.* 1336, 22-29.
- Rosenberg, S.S., Kelland, E.E., Tokar, E., De la Torre, A.R., Chan, J.R., 2008. The geometric and spatial constraints of the microenvironment induce oligodendrocyte differentiation. *Proc Natl Acad Sci U S A.* 105, 14662-14667.

Ross-Inta, C., Omanska-Klusek, A., Wong, S., Barrow, C., Garcia-Arocena, D., Iwahashi, C., Berry-Kravis, E., Hagerman, R.J., Hagerman, P.J., Giulivi, C., 2010. Evidence of mitochondrial dysfunction in fragile X-associated tremor/ataxia syndrome. *Biochem J.* 429, 545-552.

Roth, G.A., Gonzalez, M.D., Monferran, C.G., De Santis, M.L., Cumar, F.A., 1993. Myelin basic protein domains involved in the interaction with actin. *Neurochem Int.* 23, 459-465.

Sabri, M.I., Bone, A.H., Davison, A.N., 1974. Turnover of myelin and other structural proteins in the developing rat brain. *Biochem J.* 142, 499-507.

Sanchez, I., Hassinger, L., Paskevich, P.A., Shine, H.D., Nixon, R.A., 1996. Oligodendroglia regulate the regional expansion of axon caliber and local accumulation of neurofilaments during development independently of myelin formation. *J Neurosci.* 16, 5095-5105.

Santoro, M.R., Bray, S.M., Warren, S.T., 2012. Molecular mechanisms of fragile X syndrome: A twenty-year perspective. *Annu Rev Pathol.* 7, 219-245.

Schaeffer, C., Bardoni, B., Mandel, J.L., Ehresmann, B., Ehresmann, C., Moine, H., 2001. The fragile X mental retardation protein binds specifically to its mRNA via a purine quartet motif. *Embo j.* 20, 4803-4813.

Schapiro, M.B., Murphy, D.G., Hagerman, R.J., Azari, N.P., Alexander, G.E., Miezieski, C.M., Hinton, V.J., Horwitz, B., Haxby, J.V., Kumar, A., 1995. Adult fragile X syndrome: Neuropsychology, brain anatomy, and metabolism. *Am J Med Genet.* 60, 480-493.

Schwab, M.E., Thoenen, H., 1985. Dissociated neurons regenerate into sciatic but not optic nerve explants in culture irrespective of neurotrophic factors. *J Neurosci.* 5, 2415-2423.

Seiwa, C., Kojima-Aikawa, K., Matsumoto, I., Asou, H., 2002. CNS myelinogenesis in vitro: Myelin basic protein deficient shiverer oligodendrocytes. *J Neurosci Res.* 69, 305-317.

Sellier, C., Rau, F., Liu, Y., Tassone, F., Hukema, R.K., Gattoni, R., Schneider, A., Richard, S., Willemsen, R., Elliott, D.J., Hagerman, P.J., Charlet-Berguerand, N., 2010. Sam68 sequestration and partial loss of function are associated with splicing alterations in FXTAS patients. *Embo j.* 29, 1248-1261.

Shan, J., Moran-Jones, K., Munro, T.P., Kidd, G.J., Winzor, D.J., Hoek, K.S., Smith, R., 2000. Binding of an RNA trafficking response element to heterogeneous nuclear ribonucleoproteins A1 and A2. *J Biol Chem.* 275, 38286-38295.

Sheedlo, H.J., Siegel, G.J., 1986. Myelin-associated glycoprotein (MAG) in the CNS of adult shiverer (Shi/Shi) mice. *J Neurosci Res.* 16, 629-641.

Shine, H.D., Readhead, C., Popko, B., Hood, L., Sidman, R.L., 1992. Morphometric analysis of normal, mutant, and transgenic CNS: Correlation of myelin basic protein expression to myelinogenesis. *J Neurochem.* 58, 342-349.

Shiota, C., Ikenaka, K., Mikoshiba, K., 1991. Developmental expression of myelin protein genes in dysmyelinating mutant mice: Analysis by nuclear run-off transcription assay, in situ hybridization, and immunohistochemistry. *J Neurochem.* 56, 818-826.

Smith, C.M., Cooksey, E., Duncan, I.D., 2013. Myelin loss does not lead to axonal degeneration in a long-lived model of chronic demyelination. *J Neurosci.* 33, 2718-2727.

Smith, G.S., Paez, P.M., Spreuer, V., Campagnoni, C.W., Boggs, J.M., Campagnoni, A.T., Harauz, G., 2011. Classical 18.5-and 21.5-kDa isoforms of myelin basic protein inhibit calcium influx into oligodendroglial cells, in contrast to golli isoforms. *J Neurosci Res.* 89, 467-480.

Smith, L.E., Barker, E.T., Seltzer, M.M., Abbeduto, L., Greenberg, J.S., 2012. Behavioral phenotype of fragile X syndrome in adolescence and adulthood. *Am J Intellect Dev Disabil.* 117, 1-17.

Sofola, O.A., Jin, P., Qin, Y., Duan, R., Liu, H., de Haro, M., Nelson, D.L., Botas, J., 2007. RNA-binding proteins hnRNP A2/B1 and CUGBP1 suppress fragile X CGG premutation repeat-induced neurodegeneration in a drosophila model of FXTAS. *Neuron.* 55, 565-571.

Sorg, B.A., Smith, M.M., Campagnoni, A.T., 1987. Developmental expression of the myelin proteolipid protein and basic protein mRNAs in normal and dysmyelinating mutant mice. *J Neurochem.* 49, 1146-1154.

Spencer, C.M., Alekseyenko, O., Hamilton, S.M., Thomas, A.M., Serysheva, E., Yuva-Paylor, L.A., Paylor, R., 2011. Modifying behavioral phenotypes in *Fmr1*KO mice: Genetic background differences reveal autistic-like responses. *Autism Res.* 4, 40-56.

Sprinkle, T.J., 1989. 2',3'-cyclic nucleotide 3'-phosphodiesterase, an oligodendrocyte-schwann cell and myelin-associated enzyme of the nervous system. *Crit Rev Neurobiol.* 4, 235-301.

Stefani, G., Fraser, C.E., Darnell, J.C., Darnell, R.B., 2004. Fragile X mental retardation protein is associated with translating polyribosomes in neuronal cells. *J Neurosci.* 24, 7272-7276.

Stevens, B., Porta, S., Haak, L.L., Gallo, V., Fields, R.D., 2002. Adenosine: A neuron-glia transmitter promoting myelination in the CNS in response to action potentials. *Neuron.* 36, 855-868.

Strait, K.A., Carlson, D.J., Schwartz, H.L., Oppenheimer, J.H., 1997. Transient stimulation of myelin basic protein gene expression in differentiating cultured oligodendrocytes: A model for 3,5,3'-triiodothyronine-induced brain development. *Endocrinology.* 138, 635-641.

Swarr, D.T., Bloom, D., Lewis, R.A., Elenberg, E., Friedman, E.M., Glotzbach, C., Wissman, S.D., Shaffer, L.G., Potocki, L., 2010. Potocki-shaffer syndrome: Comprehensive clinical assessment, review of the literature, and proposals for medical management. *Am J Med Genet A.* 152A, 565-572.

Takikita, S., Fukuda, T., Mohri, I., Yagi, T., Suzuki, K., 2004. Perturbed myelination process of premyelinating oligodendrocyte in niemann-pick type C mouse. *J Neuropathol Exp Neurol.* 63, 660-673.

Takikita, S., Fukuda, T., Mohri, I., Yagi, T., Suzuki, K., 2004. Perturbed myelination process of premyelinating oligodendrocyte in niemann-pick type C mouse. *J Neuropathol Exp Neurol.* 63, 660-673.

Tassone, F., Adams, J., Berry-Kravis, E.M., Cohen, S.S., Brusco, A., Leehey, M.A., Li, L., Hagerman, R.J., Hagerman, P.J., 2007. CGG repeat length correlates with age of onset of motor signs of the fragile X-associated tremor/ataxia syndrome (FXTAS). *Am J Med Genet B Neuropsychiatr Genet.* 144B, 566-569.

Tassone, F., Hagerman, R.J., Taylor, A.K., Mills, J.B., Harris, S.W., Gane, L.W., Hagerman, P.J., 2000. Clinical involvement and protein expression in individuals with the FMR1 premutation. *Am J Med Genet.* 91, 144-152.

Tatavarty, V., Ifrim, M.F., Levin, M., Korza, G., Barbarese, E., Yu, J., Carson, J.H., 2012. Single-molecule imaging of translational output from individual RNA granules in neurons. *Mol Biol Cell.* 23, 918-929.

Tauber, H., Waehneltd, T.V., Neuheff, V., 1980. Myelination in rabbit optic nerves is accelerated by artificial eye opening. *Neurosci Lett.* 16, 235-238.

Teicher, M.H., Dumont, N.L., Ito, Y., Vaituzis, C., Giedd, J.N., Andersen, S.L., 2004. Childhood neglect is associated with reduced corpus callosum area. *Biol Psychiatry.* 56, 80-85.

Temple, S., Raff, M.C., 1986. Clonal analysis of oligodendrocyte development in culture: Evidence for a developmental clock that counts cell divisions. *Cell.* 44, 773-779.

Todd, P.K., Oh, S.Y., Krans, A., He, F., Sellier, C., Frazer, M., Renoux, A.J., Chen, K.C., Scaglione, K.M., Basrur, V., Elenitoba-Johnson, K., Vonsattel, J.P., Louis, E.D., Sutton, M.A., Taylor, J.P., Mills, R.E., Charlet-Berguerand, N., Paulson, H.L., 2013. CGG repeat-associated translation mediates neurodegeneration in fragile X tremor ataxia syndrome. *Neuron.* 78, 440-455.

Todd, P.K., Oh, S.Y., Krans, A., Pandey, U.B., Di Prospero, N.A., Min, K.T., Taylor, J.P., Paulson, H.L., 2010. Histone deacetylases suppress CGG repeat-induced neurodegeneration via transcriptional silencing in models of fragile X tremor ataxia syndrome. *PLoS Genet.* 6, e1001240.

Tomoo, K., Matsushita, Y., Fujisaki, H., Abiko, F., Shen, X., Taniguchi, T., Miyagawa, H., Kitamura, K., Miura, K., Ishida, T., 2005. Structural basis for mRNA Cap-Binding regulation of eukaryotic initiation factor 4E by 4E-binding protein, studied by spectroscopic, X-ray crystal structural, and molecular dynamics simulation methods. *Biochim Biophys Acta.* 2, 191-208.

Trapp, B.D., Moench, T., Pulley, M., Barbosa, E., Tennekoon, G., Griffin, J., 1987. Spatial segregation of mRNA encoding myelin-specific proteins. *Proc Natl Acad Sci U S A.* 84, 7773-7777.

Tsiouris, J.A., Brown, W.T., 2004. Neuropsychiatric symptoms of fragile X syndrome: Pathophysiology and pharmacotherapy. *CNS Drugs.* 18, 687-703.

- Uchida, N., Hoshino, S., Imataka, H., Sonenberg, N., Katada, T., 2002. A novel role of the mammalian GSPT/eRF3 associating with poly(A)-binding protein in Cap/Poly(A)-dependent translation. *J Biol Chem.* 277, 50286-50292.
- Van Dam, D., Errijgers, V., Kooy, R.F., Willemsen, R., Mientjes, E., Oostra, B.A., De Deyn, P.P., 2005. Cognitive decline, neuromotor and behavioural disturbances in a mouse model for fragile-X-associated tremor/ataxia syndrome (FXTAS). *Behav Brain Res.* 162, 233-239.
- van Dijk, E.J., Breteler, M.M., Schmidt, R., Berger, K., Nilsson, L.G., Oudkerk, M., Pajak, A., Sans, S., de Ridder, M., Dufouil, C., Fuhrer, R., Giampaoli, S., Launer, L.J., Hofman, A., CASCADE Consortium, 2004. The association between blood pressure, hypertension, and cerebral white matter lesions: Cardiovascular determinants of dementia study. *Hypertension.* 44, 625-630.
- Vasquez, R.J., Gard, D.L., Cassimeris, L., 1999. Phosphorylation by CDK1 regulates XMAP215 function in vitro. *Cell Motil Cytoskeleton.* 43, 310-321.
- Verity, A.N., Campagnoni, A.T., 1988. Regional expression of myelin protein genes in the developing mouse brain: In situ hybridization studies. *J Neurosci Res.* 21, 238-248.
- Vogel, U.S., Thompson, R.J., 1988. Molecular structure, localization, and possible functions of the myelin-associated enzyme 2',3'-cyclic nucleotide 3'-phosphodiesterase. *J Neurochem.* 50, 1667-1677.
- Volk, L.J., Pfeiffer, B.E., Gibson, J.R., Huber, K.M., 2007. Multiple gq-coupled receptors converge on a common protein synthesis-dependent long-term depression that is affected in fragile X syndrome mental retardation. *J Neurosci.* 27, 11624-11634.
- Wake, H., Lee, P.R., Fields, R.D., 2011. Control of local protein synthesis and initial events in myelination by action potentials. *Science.* 333, 1647-1651.
- Wang, H., Ku, L., Osterhout, D.J., Li, W., Ahmadian, A., Liang, Z., Feng, Y., 2004. Developmentally-programmed FMRP expression in oligodendrocytes: A potential role of FMRP in regulating translation in oligodendroglia progenitors. *Hum Mol Genet.* 13, 79-89.
- Wang, S., Bates, J., Li, X., Schanz, S., Chandler-Militello, D., Levine, C., Maherali, N., Studer, L., Hochedlinger, K., Windrem, M., Goldman, S.A., 2013. Human iPSC-derived oligodendrocyte progenitor cells can myelinate and rescue a mouse model of congenital hypomyelination. *Cell Stem Cell.* 12, 252-264.
- Wang, S., Sdrulla, A.D., diSibio, G., Bush, G., Nofziger, D., Hicks, C., Weinmaster, G., Barres, B.A., 1998. Notch receptor activation inhibits oligodendrocyte differentiation. *Neuron.* 21, 63-75.
- Wang, X., Zhao, Y., Zhang, X., Badie, H., Zhou, Y., Mu, Y., Loo, L.S., Cai, L., Thompson, R.C., Yang, B., Chen, Y., Johnson, P.F., Wu, C., Bu, G., Mobley, W.C., Zhang, D., Gage, F.H., Ranscht, B., Zhang, Y.W., Lipton, S.A., Hong, W., Xu, H., 2013. Loss of sorting nexin 27 contributes to excitatory synaptic dysfunction by modulating glutamate receptor recycling in down's syndrome. *Nat Med.* 19, 473-480.

Weiler, I.J., Irwin, S.A., Klintsova, A.Y., Spencer, C.M., Brazelton, A.D., Miyashiro, K., Comery, T.A., Patel, B., Eberwine, J., Greenough, W.T., 1997. Fragile X mental retardation protein is translated near synapses in response to neurotransmitter activation. *Proc Natl Acad Sci U S A*. 94, 5395-5400.

Westenbroek, R.E., Bausch, S.B., Lin, R.C., Franck, J.E., Noebels, J.L., Catterall, W.A., 1998. Upregulation of L-type Ca²⁺ channels in reactive astrocytes after brain injury, hypomyelination, and ischemia. *J Neurosci*. 18, 2321-2334.

White, R., Gonsior, C., Bauer, N.M., Kramer-Albers, E.M., Luhmann, H.J., Trotter, J., 2012. Heterogeneous nuclear ribonucleoprotein (hnRNP) F is a novel component of oligodendroglial RNA transport granules contributing to regulation of myelin basic protein (MBP) synthesis. *J Biol Chem*. 287, 1742-1754.

White, R., Gonsior, C., Kramer-Albers, E.M., Stohr, N., Huttelmaier, S., Trotter, J., 2008. Activation of oligodendroglial fyn kinase enhances translation of mRNAs transported in hnRNP A2-dependent RNA granules. *J Cell Biol*. 181, 579-586.

Widlund, P.O., Stear, J.H., Pozniakovsky, A., Zanic, M., Reber, S., Brouhard, G.J., Hyman, A.A., Howard, J., 2011. XMAP215 polymerase activity is built by combining multiple tubulin-binding TOG domains and a basic lattice-binding region. *Proc Natl Acad Sci U S A*. 108, 2741-2746.

Willemsen, R., Hoogeveen-Westerveld, M., Reis, S., Holstege, J., Severijnen, L.A., Nieuwenhuizen, I.M., Schrier, M., van Unen, L., Tassone, F., Hoogeveen, A.T., Hagerman, P.J., Mientjes, E.J., Oostra, B.A., 2003. The FMR1 CGG repeat mouse displays ubiquitin-positive intranuclear neuronal inclusions; implications for the cerebellar tremor/ataxia syndrome. *Hum Mol Genet*. 12, 949-959.

Yakovlev, P.I., Lecours, A.R., 1966. Regional Development of the Brain in Early Life. Minkovski, A., editor. Oxford, UK: Blackwell; The myelogenic cycles of regional maturation in the brain. 3-70.

Yin, X., Baek, R.C., Kirschner, D.A., Peterson, A., Fujii, Y., Nave, K.A., Macklin, W.B., Trapp, B.D., 2006. Evolution of a neuroprotective function of central nervous system myelin. *J Cell Biol*. 172, 469-478.

Zalfa, F., Giorgi, M., Primerano, B., Moro, A., Di Penta, A., Reis, S., Oostra, B., Bagni, C., 2003. The fragile X syndrome protein FMRP associates with BC1 RNA and regulates the translation of specific mRNAs at synapses. *Cell*. 112, 317-327.

Zeller, N.K., Behar, T.N., Dubois-Dalcq, M.E., Lazzarini, R.A., 1985. The timely expression of myelin basic protein gene in cultured rat brain oligodendrocytes is independent of continuous neuronal influences. *J Neurosci*. 5, 2955-2962.

Zhao YY, Shi XY, Qiu X, Lu W, Yang S, Li C, Chen L, Zhang L, Cheng GH, Tang Y. 2012. Enriched environment increases the myelinated nerve fibers of aged rat corpus callosum. *Anat Rec*. 295, 999-1005.

Zhu, D., Xu, G., Ghandhi, S., Hubbard, K., 2002. Modulation of the expression of p16INK4a and p14ARF by hnRNP A1 and A2 RNA binding proteins: Implications for cellular senescence. *J Cell Physiol.* 193, 19-25.