FMRP Regulates the Expression of Axonal Transcripts *in vivo*

By

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Akins MR*, **Berk-Rauch HE***, and Fallon JR. Circuit-selective regulation of OMP by FMRP. In preparation.

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RECENT ABSTRACTS

Akins MR, Chang E, **Berk-Rauch HE**, LeBlanc HF, Stackpole EE, Fallon JR. Axonal mRNAs encoding β-catenin and OMP are circuit-selective cargos of Fragile X granules No. 240.12, 2012 Neuroscience Meeting Planner. New Orleans, LA: Society for Neuroscience, 2012.

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Preface

Fragile X syndrome, a leading cause of autism and the most common form of inherited intellectual disability, results from the loss of Fragile X mental retardation protein (FMRP). FMRP is an RNA binding protein with a well-elucidated post-synaptic role in regulation of synaptic plasticity. Emerging evidence also indicates an axonal and presynaptic role for FMRP. Work from our laboratory has identified FMRP as a component of an axonal and presynaptic granule, termed the Fragile X granule (FXG), which also contains its autosomal homologs Fragile X related protein 2 (FXR2P) and Fragile X related protein 1 (FXR1P). The axonal localization of FMRP raises several questions such as: What axonal messages are associated with FMRP? How does FMRP regulate the axonal expression of these messages and proteins? This thesis seeks to address the role of FMRP in regulating axonal mRNAs *in vivo*. I have focused on the circuit between olfactory sensory neurons (OSNs) and the olfactory bulb. Within this system, I have addressed the role of FMRP in regulating axonally localized message for olfactory marker protein (OMP). Work from Michael Akins has shown that the OMP message localizes with a subset of FXGs in the olfactory bulb. I found that FMRP regulates the protein expression of OMP in the axonal domain of OSNs. Interestingly, FMRP does not appear to be required for the localization of OMP mRNA to axons, suggesting that it is not actively involved in the trafficking of this message to this subcellular domain. Taken together, this work provides *in vivo* evidence for FMRP's regulation of an axonal message.

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Introduction

Portions of this chapter (Presynaptic Translation) were previously published in the following article: Akins MR*, Berk-Rauch HE*, Fallon JR. 2009. Presynaptic translation: stepping out of the postsynaptic shadow. Front Neural Circuits. 3:17(Akins et al., 2009). *denotes equal authorship

Background

Neurons are highly polarized cells containing distinct sub-cellular compartments that can function autonomously. This complexity necessitates that the sub-cellular requirements within one domain may differ greatly from the rest of the cell. In humans, distal portions of axons can be located up to a meter from the cell body. Rapid responses to environmental cues are achieved, in part, by local translation. Proteins can be synthesized at the site of action, allowing for rapid alterations in function within distinct sub-cellular domains. This restricted local translation is regulated by several mechanisms orchestrated, in part, by RNA binding proteins such as FMRP.

While the role of local translation postsynaptically is well characterized, our understanding of local translation in the axonal domain is still emerging. Identification of RNA binding proteins, messages and translational machinery within axonal and presynaptic compartments has begun to shape our understanding of local translation in these compartments. Work from our lab has identified FMRP as a component of an axonal structure termed the Fragile X granule (FXG), which also contains fragile X related protein 2 (Christie et al., 2009). FXGs have been identified in several circuits within the brain, including olfactory sensory neurons innervating the olfactory bulb. The presence of the fragile X proteins in FXGs suggests an axonal and presynaptic function for these proteins. Understanding how FMRP acts to regulate axonal proteins may elucidate novel mechanisms regulating presynaptic function. Furthermore, loss of functional FMRP results in fragile X

syndrome the leading inherited cause of autism and intellectual disability (Bhakar et al., 2012). Elucidating the axonal and presynaptic role of FMRP may shed light on the mechanisms underlying this disorder as well as establish potential novel therapeutic targets for fragile X syndrome.

Fragile X Syndrome Results from Loss of the Fragile X Mental Retardation Protein

Fragile X Syndrome

Fragile X syndrome (FXS) results from the loss of functional FMRP, an RNA binding protein. FXS is the most common inherited cause of intellectual disability (ID) and autism, accounting for ~4% of autistic cases (Coffee et al., 2009; Bhakar et al., 2012). Furthermore, the majority of FXS patients exhibit at least one autistic symptom and ~50% can be classified as having an autism spectrum disorder (ASD; Hernandez et al., 2009). Single-gene forms of ASD, such as FXS, provide a unique opportunity to address the molecular mechanisms underlying these disorders. Elucidating the basis of the deficits that arise in FXS may establish shared molecular pathways in various forms of autism.

Genetic Basis of FXS

In the vast majority of known cases, FXS results from transcriptional silencing of the *FMR1* gene, which encodes FMRP. FXS gained its name due to the presence of a thin elongated region of one of the arms of the X chromosome in

afflicted patients (Sutherland, 1983; Lubs et al., 2012). Early work indicated that FXS results from the presence of a dominant mutation on the X-chromosome. Using the fragile arm of the X-chromosome as a marker, pedigree analysis of several families carrying FXS established the mode of transmission of this disorder. Interestingly, of males expressing the fragile X marker approximately 20% appeared to not exhibit FXS symptoms (Sherman et al., 1984, 1985). While the daughters of male carriers are frequently unaffected, their sons (grandson of carrier male) have a high likelihood of exhibiting FXS (Sherman et al., 1984, 1985). The basis of this unusual transmission, termed the Sherman paradox, was elucidated with the identification of a CGG repeat element in the 5`UTR of *FMR1*, the gene that encodes FMRP (Fu et al., 1991; Oberle et al., 1991; Verkerk et al., 1991). Normal patients contain between 20 to 50 CGG's in their 5`UTR, this repeat element can become expanded, to more than 200 repeats in length, resulting in the fragile X phenotype. Carriers of FXS, may exhibit an intermediate number of CGGs, between 55-200 repeats in length, termed a permutation. The repeat element can become expanded in transmission from female carriers, resulting in the observed patterns of heritability (Fu et al., 1991; Oberle et al., 1991). When the CGG repeats become expanded to the full mutation form, it is hypermethylated, a process in which the element and surrounding promoter region undergo abnormal levels of methylation. This hypermethylation results in transcriptional silencing, by blocking transcription factor binding and inducing chromatin remodeling
(reviewed in Penagarikano et al., 2007).

Carriers of the permutation CGG repeat element exhibit a distinct set of physiological deficits from the full mutation form. Premutation males can develop a neurodegenerative disorder called Fragile X related tremor/ataxia syndrome (FXTAS). FXTAS is most commonly observed in older males. The most salient features of FXTAS are development of tremor and gait ataxia, with several other symptoms ranging from physical to cognitive deficits. Female carriers of the permutation form of *FMR1*, exhibit a distinct set of symptoms, termed Fragile X – associated primary ovarian insufficiency FX-POI. FX-POI females exhibit ovarian dysfunction, including infertility, premature onset of menopause, as well as, altered follicle stimulation hormone and anti-mullerian hormone expression (Reviewed in Pirozzi et al., 2011). Interestingly, *FMR1* is transcribed in premutation patients and deficits might arise from a toxic gain-of-function of the expanded repeat element in the mRNA. A hallmark of FXTAS is the presence of intra-nuclear inclusions in the brains of patients (Greco et al., 2002). One possible explanation is that the repeat expansion in *FMR1* mRNA binds and sequesters RNA binding proteins in stress granules. For example, in *Drosophilia*, Pur α is a component of granules formed as a result of the CGG repeat expansion (Jin et al., 2007). Further studies will need to address the exact mechanisms underlying the formation of inclusion in FXTAS and how they result in the neuronal deficits observed in the syndrome.

FXS results from loss of functional FMRP

The majority of identified FXS cases result from transcriptional silencing, due to repeat expansion of the CGG element, as described above. However, several screens have identified other causes of FXS, such as deletions and mutations in both the promoter and introns of *FMR1* (Coffee et al., 2009; Collins et al., 2010). These cases indicate that the symptoms of FXS arise specifically from loss of FMRP function. In particular, identification of cases in which a non-functional form FMRP is translated informs our understanding of the function of FMRP. Work from patient and mouse studies have shown that a point mutation, I304N in the KH2 RNA binding domain of FMRP is sufficient to cause FXS (Boulle et al., 1993; Feng et al., 1997a; Zhang et al., 2009a). Recently a second point mutation, R138Q in the nuclear localization signal of FMRP, as well as three variants in the transcriptional promoter, have been identified in a screen of FXS patients (Collins et al., 2010). These two cases in which a mutated form of FMRP is expressed, highlight the fact that FXS arises specifically from loss of functional FMRP. The cognitive and behavioral symptoms exhibited in FXS suggest that FMRP normally regulates processes underlying learning and memory such as synaptic plasticity. Elucidating how and where FMRP functions will inform the deficits in diseases such as FXS and other autism related disorders.

Fragile X mental retardation protein

FMRP has a well-elucidated role in regulating local translation underlying synaptic plasticity. In response to changes in neurotransmitter release from

presynaptic neurons, synapses can undergo long lasting changes in synaptic strength either strengthening (long-term potentiation) or weakening (long-term depression) the response to subsequent input. One form of long-term depression is mediated by post-synaptic metabotropic glutamate receptors 1/5 (mGLuRs 1/5). In response to signals from the presynaptic neuron, mGluR's initiate a post-synaptic response, including internalization of AMPA receptors, resulting in irreversible long –term depression of the synapse. Importantly, this form of long-term depression requires rapid local protein synthesis at the synaptic site. Upon activation of mGluR5, local translation of mRNA occurs within the synapse, resulting in the sustained loss of AMPA receptors and long-term depression
(Reviewed in Bear et al., 2004). FMRP plays a critical role in this protein synthesis dependent long-term depression (LTD). In the absence of FMRP, regulation of this response is lost, resulting in exaggerated LTD in the hippocampus of *fmr1* knockout mice compared to wild type (Huber et al., 2002). MPEP, an antagonist of mGluR 1/5, has been shown to reverse deficits resulting from loss of FMRP in both mammalian and invertebrate models (Reviewed in Krueger and Bear, 2011). Furthermore, when *fmr1* KO mice are crossed with mGluR5 deficit mice, several of the phenotypes observed in the *fmr1* KO are alleviated, including excessive LTD in the hippocampus (Dölen et al., 2007b). These findings suggest that FMRP may act as a molecular switch in mGLuR5-mediated LTD, regulating translation in response to signaling at the synapse.

FMRP's role in inhibiting protein synthesis appears to underlie its regulation of synaptic processes. Several studies have observed altered protein synthesis in

the *fmr1* knockout mouse. As discussed above, FMRP has a critical role in regulation of mGluR mediated synaptic plasticity, suggesting that it may act as a molecular switch in this process. Several lines of evidence indicate that local translation in response to mGLuR5 signaling is altered in the absence of FMRP. A number of identified messages, such as α-CamKII, PSD-95 and mGluR1/5 exhibit altered polysomal association in the absence of FMRP (Muddashetty et al., 2007). *In vitro*, LTD can be induced by the mGluR agonist, dihydroxyphenylglycine (DHPG). One outcome of DHPG -induced LTD is a translation-dependent, transient increase in the levels of several proteins, including FMRP. Interestingly, in FMRP null hippocampal slices and synaptosomes, de novo protein synthesis in response to DHPG does not occur; suggesting that regulation of mGluR1/5 induced translation is altered (Hou et al., 2006; Muddashetty et al., 2007; Osterweil et al., 2010). In hippocampal slices there is increased basal levels of protein synthesis in the *fmr1* KO compared to wild type (Dölen et al., 2007a; Osterweil et al., 2010). *In vivo*, *fmr1* KO mice exhibit increase basal levels of protein synthesis as measured by incorporation of radioactively labeled leucine (Qin et al., 2005). These findings suggest that FMRP functions as a negative regulator of translation, resulting in constitutively increased protein synthesis in the *fmr1* KO. Furthermore, the altered LTP observed in the *fmr1* KO may be an outcome of dysregulation of protein synthesis.

Critical to understanding FMRP's function in neurons is elucidating the cohort of messages associated with the protein. While multiple wide-scale screens of FMRP associated-messages have been performed, technical limitations of the early efforts made identification of valid targets a challenge. An initial microarray

study of the RNA co-immunoprecipitated with FMRP suggested that it binds as much as 4% of messages within the brain, suggesting a large, but specific, set of messages (Brown et al., 2001). Further analysis of FMRP associated messages, both *in vivo* and *in vitro*, suggested that they that encode proteins falling into several functional classes, including synaptic proteins. These initial studies also suggested that FMRP preferentially bound G-quartet structures in mRNA, via its RGG box motif (Brown et al., 2001; Miyashiro et al., 2003; Darnell et al., 2011). While, these early studies were promising, they were prone to identification of false positive targets due to technical limitations, particularly in RNA-immunoprecipications. Recent work, using HITS-CLIP technique, in which RNA binding proteins are covalently crosslinked to their target messages *in vivo*, has identified a more stringent set of FMRP associated messages (Darnell et al., 2011). Interestingly, although only a subset of the transcriptome was found associated with FMRP, there was not evidence for specific recognition sequences or motifs within these target transcripts. This observation raised the surprising suggestion that there is no FMRP binding sequence in mRNA. The study identified 842 FMRP RNA targets, encompassing several classes of neuronal function. Perhaps not surprisingly, a large number of identified messages encode post-synaptic proteins. Interestingly, FMRP was found to also bind approximately 30% of the presynaptic transcriptome (Darnell et al., 2011).

Several structural elements suggest that FMRP may function to regulate mRNA trafficking, translation and stability. FMRP contains tandem KH domains and an RGG box, both of which play a role in RNA binding (Ashley et al., 1993; Siomi et

al., 1993; Darnell et al., 2005; Blackwell et al., 2010). FMRP also contains nuclear import and export sequences allowing for FMRP to shuttle in and out of the nucleus (Ashley et al., 1993; Blackwell et al., 2010). While the majority of FMRP is cytoplasmic, a portion has been observed in the nucleus, suggesting that it may be a component of trafficking mRNPs (Feng et al., 1997b; Kim et al., 2008). In line with a role in mRNP trafficking, FMRP co-localizes with other RNA binding proteins, which have identified roles in mRNA localization (Mazroui et al., 2002; Barbee et al., 2006; Price et al., 2006). Using live cell imaging FMRP was observed as a component of motile granules in PC12 cells, as well as cultured hippocampal and cortical neurons (De Diego Otero et al., 2002; Antar et al., 2005, 2006; Dictenberg et al., 2008). FMRP is localized to the synapse in response to activity, suggesting that it is a component of a trafficking granule that functions during synaptic activity (Antar et al., 2004). Interestingly, the basal expression of several FMRP target mRNAs, at sites of translation, are unaltered in the *Fmr1* KO, suggesting that FMRP may not be required for localization of target messages (Muddashetty et al., 2007). Thus, while FMRP appears to be a component of trafficking mRNPs it is not sufficient for localization of messages within the cell.

The mechanism by which FMRP regulates protein synthesis is not fully understood. Several studies have shown that FMRP is associated with polyribosomes, both in culture and *in vivo* (Khandjian et al., 1995; Corbin et al., 1997; Feng et al., 1997a; Ceman et al., 2003; Stefani et al., 2004). FMRP's association with polyribosomes is RNA dependent, as FMRP is shifted from the polysomal fraction in the presence of RNase A (Stefani et al., 2004). Two pieces of evidence

indicate that the KH domains of FMRP are required for maintaining the association of FMRP with polyribosomes. Firstly, FMRP can be competed out of the polysome fraction by the presence of kissing complex RNA (Darnell et al., 2005). Secondly, a point mutation in the KH2 domain I304N (discussed above) abolishes polyribosome association (Feng et al., 1997a). Thus FMRP's association with ribosomes appears to be mediated by its KH domains, potentially via its interaction with RNA. FMRP's polysomal association appears to be key to its function as a translational repressor.

One emerging explanation for the mechanism underlying FMRP's role in suppressing protein synthesis is its association with stalled polyribosomes. Early studies showed that phosphorylation of FMRP at serine residue 499/500 results in its association with stalled polyribosomes (Ceman et al., 2003). FMRP can be de-phosphorylated at this residue by protein phosphatase 2A (PP2A), suggesting a mechanism by which FMRP-mediated translational suppression may be lifted in response to synaptic activity (Narayanan et al., 2007). Dephosphorylation of serine 500 by PP2A results in increased synthesis of multiple FMRP target messages (Narayanan et al., 2007; Niere et al., 2012). Finally, FMRP's association with stalled polyribosomes has most definitively been shown in a recent HIT-CLIPS study in which FMRP was observed to bind target messages across the coding region and shown to associate with stalled ribosomes in ribosomal run-off assays (Darnell et al., 2011). Thus, it appears that FMRP may act to regulate the state of active translation by stalling ribosome elongation during translation.

Fragile X related proteins 1 and 2

FMRP has two autosomal homologs Fragile X related protein 1 (FXR1P) and Fragile X related protein 2 (FXR2P). All three proteins are highly conserved, sharing approximately seventy percent homology. Both FXR1P and FXR2P bind mRNA and associate with polyribosomes (Bakker et al., 2000; Darnell et al., 2009). FXR1P has been shown to interact with both RISC and AU-rich element containing mRNAs and act as a translational activator of these messages (Vasudevan and Steitz, 2007). FXR1P plays an important function in muscle, where it is required for proper distribution of neuromuscular proteins (Mientjes et al., 2004). The precise function of FXR2P is less well understood however, mice that lack both FMRP and FXR2P show exaggerated long-term depression compared to either knockout alone, suggesting complimentary but distinct functions for each protein (Spencer et al., 2006).

Axonal and Presynaptic Translation

Translation in the Growth Cone

Perhaps the most well-elucidated role of axonal local translation is during growth cone turning and collapse. Translational machinery, mRNA and rRNA have been observed in developing axons and growth cones in both mammalian and *Xenopus* cultures, suggesting that local translation may underlie proper axonal development (Kleiman et al., 1994; Bassell et al., 1998; Campbell and Holt, 2001;

Steward, 2002; Piper et al., 2006). Direct evidence for local translation was first elucidated in *Xenopus* retinal explants where growth cone collapse and turning is dependent on local protein synthesis in the axonal compartment (Campbell and Holt, 2001; Piper and Holt, 2004). The requirement for local translation in the growth cone suggested that specific messages localize to the axonal compartment, where they are translated. β-Actin was one of the first identified proteins whose local synthesis is critical for growth cone function. β-Actin localizes to the distal tip of growth cones in both mammalian and *Xenopus* cultures (Bassell et al., 1998; Zhang et al., 2001a; Leung et al., 2006; Yao et al., 2006). This localization is dependent on association of the RNA binding protein zip-code binding protein (ZBP1) with β-Actin message (Zhang et al., 2001a; Yao et al., 2006). In response to local growth cues β-Actin protein is synthesized within the growth cone. Remarkably, this local synthesis is so tightly regulated that in response to directional netrin-1 signaling, β-Actin is asymmetrically translated such that it only increases in the turning edge of the growth cone (Leung et al., 2006; Yao et al., 2006). Similar requirements for local protein synthesis have been observed during growth cone collapse, during which RhoA is translated intra-axonally in response to Semaphorin 3A (Wu et al., 2005). How local translation in response to growth cues is regulated is not well understood, however a recent study has identified a role for the transmembrane receptor DCC (Deleted in Colorectal Cancer) in this process. DCC forms a complex with translational machinery at the membrane. In response to netrin-1 the translation complex is released from DCC association and local translation can occur (Tcherkezian et al., 2010).

Identification of Axonal Transcripts

Critical to understanding the function of local translation within axons is establishing what messages are localized to this compartment. A number of microarray-based screens have been performed on the axonal transcriptome, in response to axotomy, growth factors and neurotrophins. These studies identified messages encoding several different classes of proteins, such as, cytoskeletal, heat shock and endoplasmic reticulum (ER) proteins, as well as, translational machinery (Willis et al., 2005, 2007; Taylor et al., 2009). Several other studies have utilized the nerve-crush model to further elucidate specific messages that are locally translated in the axonal compartment. Axotomy following injury can result in regeneration of the axon and re-establishment of the synaptic connection with the target cell. In response to injury there is localization of mRNA and translational machinery to the regenerating axon tip (Zhang et al., 2000). Furthermore, there is rapid induction of local translation of a number of messages at the site of regeneration, including vimentin, Importin B, and RANBP1 among others (Zhang et al., 2001a; Hanz et al., 2003; Perlson et al., 2005; Willis et al., 2005; Yudin et al., 2008). A number of axonally synthesized proteins are retrogradely transported to the cell soma, including GAP43, β-Actin, STAT3, Impa1 and SMAD 1/5/8, suggesting a role for local translation in retrograde signaling (Andreassi et al., 2010; Donnelly et al., 2011; Ben-Yaakov et al., 2012; Ji and Jaffrey, 2012). Interestingly, the axons of mature cortical cultures contain a large cohort of messages, suggesting that mRNA is constitutively localized to axons (Taylor et al., 2009).

Translation in Mature Axons

Several recent *in vivo* studies suggest that local translation functions in mature axons. In the mature, intact, mouse brain both mRNA elements and ribosomal proteins have been observed in distal axons, suggesting that local translation occurs after axons have reached their post-synaptic target (Willis et al., 2011; Walker et al., 2012). Recent work in the *Xenopus* retinal ganglion further supports a role for protein synthesis in mature axons. It was observed that the intermediate filament protein Lamin B (LB2) mRNA is localized to retinal ganglion cell axons, where it is locally translated. Interestingly over tadpole development, axons lacking LB2 mRNA reach their target in the optic tectum, but undergo aberrant degeneration, indicating that local translation of LB2 is required for axon maintenance (Yoon et al., 2012). The fragile X proteins are axonally localized in restricted circuits of the intact mouse brain (Christie et al., 2009; Akins et al., 2012). Supporting a circuit selective role for local protein synthesis, SMAD 1/5/8 mRNA localizes maxillary and ophthalmic, but not mandibular, populations of trigeminal axons in embryonic rats, where it may be translated in response to BDNF signaling (Ji and Jaffrey, 2012). These studies suggest that local translation plays a critical role in both the development and maintenance of axons in several model systems. Furthermore, *in vivo* results suggest that local protein synthesis is potentially restricted to certain circuits or populations of neurons.

Presynaptic Translation

Presynaptic translation has been best elucidated in invertebrates. Serotonin-induced long-term facilitation (LTF) does not require the cell body and is blocked by local application of protein synthesis inhibitors in both cultured *Aplysia* neurons (Martin et al., 1997; Sherff and Carew, 1999; Liu et al., 2003) and isolated crayfish preparations (Beaumont et al., 2001). In the crayfish, presynaptic translation was shown by directly injecting the mRNA cap analog m^7GpppG into severed axons or into the postsynaptic muscle. Only the presynaptic injection blocked LTF induction (Beaumont et al., 2001). Similarly, direct injection of a membrane-impermeant protein synthesis inhibitor into the presynaptic, but not the postsynaptic, neuron blocked induction of LTF in the *Aplysia* system (Martin et al., 1997). LTF induction is sensitive to rapamycin
(which targets mTOR; Casadio et al., 1999; Beaumont et al., 2001) and requires MAP kinase, phosphatidylinositol 3-OH kinase, and S6 kinase activities (Beaumont et al., 2001).

The presynaptic localization and translation of the mRNA encoding the neuropeptide sensorin has been well characterized in *Aplysia*. The mRNA for sensorin rapidly localizes to synapses following contact with appropriate postsynaptic targets and is required for synapse formation (Schacher et al., 1999; Lyles et al., 2006). Furthermore, sensorin protein levels are increased in isolated neurites upon serotonin stimulation (Liu et al., 2003), while a reporter under the control of the sensorin 5' and 3' UTRs is translated locally at the presynaptic site in

response to serotonin stimulation (Wang et al., 2009). Of particular interest, this translation only occurs if the neurite has formed a synapse and requires calcium signaling in the postsynaptic neuron (Wang et al., 2009). It should be noted that translational machinery has not yet been identified at the presynaptic apparatus in this system. Nonetheless, these experiments demonstrate that mRNAs are translated at the presynaptic apparatus. Further, these studies show the importance of transsynaptic interactions in the localization and translation of presynaptic messages.

Several lines of evidence support a functionally relevant role for local protein synthesis in vertebrates. BDNF induces synaptic potentiation in *Xenopus* nerve-muscle cultures (Zhang and Poo, 2002). Injection of a membrane-impermeant translation inhibitor into the presynaptic neuron, but not into the postsynaptic muscle cell, inhibited BDNF-induced potentiation (Zhang and Poo, 2002). The specificity of this inhibition was underscored by the finding that NT3-mediated potentiation was not dependent upon protein synthesis. Furthermore, the BDNF-mediated potentiation occurred in the absence of the cell body and could be blocked by bath application of protein synthesis inhibitors (Zhang and Poo, 2002). Presynaptic translation is also implicated in the proper expression of LTD at corticostriatal synapses (Yin et al., 2006) and LTP at hippocampal mossy fiber synapses (Huang and Hsu, 2004). In both cases, plasticity was examined in slice cultures in which the axons had been severed from their somata. Bath application of protein synthesis inhibitors affected plasticity, while direct injection of cap analogs (Huang and Hsu, 2004) or cycloheximide (Yin et al., 2006) into the postsynaptic

neuron did not. While these slice experiments could not rule out a contribution of glial protein synthesis, they point to a role for local translation in mammalian presynaptic plasticity. Finally, in cultured mouse hippocampal neurons presynaptic translation is required for stabilization of newly formed synapses (Sebeo et al., 2009).

Presynaptic RNA Binding Proteins

One way to understand the regulation of presynaptic translation is by assessing the localization and function of RNA binding proteins, a broad class of factors that regulate mRNA processing, subcellular distribution and regulation. Analyses in *Drosophila* have revealed a variety of RNA binding proteins that are critical for presynaptic formation and function. For example, transgenically-expressed Imp localizes to presynaptic sites, and synapses are smaller (but normal in number) in Imp mutants (Boylan et al., 2008). Further, flies null for dFXR (the sole *Drosophila* homolog of mammalian FMRP) exhibit altered neurotransmission, abnormal presynaptic structure, increased synaptic vesicle pools, and deficits in synaptogenesis (Zhang et al., 2001b; Gatto and Broadie, 2008). These animals exhibit an overexuberant axonal arbor – which could reflect abnormal activity-dependent pruning (Zhang et al., 2001b; Pan et al., 2004; Tessier and Broadie, 2011). The RNA binding proteins Pumilio and Nanos, which suppress translation of target transcripts, regulate synapse structure at the neuromuscular junction during development (Menon et al., 2004, 2009). Notably, these studies failed to detect dFXR, Pumilio or Nanos at the presynaptic site using immunohistochemistry (Zhang

et al., 2001b; Menon et al., 2004, 2009). It is not presently clear whether this finding reflects an absence of protein in the presynaptic compartment or technical challenges in visualization.

Presynaptic FMRP

Several lines of evidence support a role for FMRP at mammalian presynaptic specializations. An elegant demonstration of the importance of FMRP expression in the presynaptic cell was achieved in a system where half the neurons lack FMRP due to X-inactivation. In this system the likelihood of synapse formation was reduced only if the presynaptic neuron lacked FMRP (Hanson and Madison, 2007). Full *fmr1* knockouts exhibited defective baseline synaptic transmission and short-term presynaptic plasticity in the hippocampus and amagydala (Zhang et al., 2009a; Suvrathan et al., 2010; Deng et al., 2011). Furthermore, in the absence of FMRP there is an increase in the size of the readily-releasable pool of synaptic vesicles and the rate of vesicle turn over (Deng et al., 2011; Klemmer et al., 2011). While suggestive, these knockout studies do not distinguish the locus within the presynaptic cell at which FMRP is acting. However, FMRP is localized to axons and growth cones of cultured hippocampal and dorsal root ganglia neurons (Antar et al., 2006; Hengst et al., 2006; Price et al., 2006; Murashov et al., 2007). FMRP is also expressed in hippocampal mossy fibers, which demonstrate both a dysregulation of the FMRP target MAP1B and deficits in pruning of the infrapyramidal bundle in *fmr1* mutant mice (Ivanco and Greenough, 2002; Christie et al., 2009). The presynaptic localization of FMRP suggests that some of its target mRNAs may encode

presynaptic proteins. The expression of several presynaptic proteins associated with the synaptic membrane is altered in the *fmr1* KO (Klemmer et al., 2011). FMRP associates with the presynaptic potassium channel Slack B in a complex that also contains mRNA, indicating that FMRP does interact with presynaptic proteins (Brown et al., 2010). Finally, recent HITS-CLIP screen of FMRP targets identified 30% of the presynaptic proteosome, including Bassoon, neurexin family members, and Piccolo (Darnell et al., 2011).

The Fragile X granule

FMRP is also expressed presynaptically (Feng et al., 1997a; Christie et al., 2009). Recent work from our lab has identified FMRP as a component of a novel presynaptic granule, the FXG (Christie et al., 2009). FXGs also contain the Fragile X-related proteins FXR1P and FXR2P. FXGs localize to a subset of axons and presynaptic sites in several brain regions. Of interest, at least two of these FXG-expressing synapses exhibit long-term presynaptic plasticity (hippocampal mossy fiber and cerebellar fiber terminals; Salin et al., 1996; Nicoll an d Schmitz, 2005). Interestingly, FXGs are enriched in a number of sensory circuits, including those involved in sensorimotor processing (Akins et al., 2012). Of interest for this thesis, within the olfactory bulb FXGs are highly enriched in the circuitry between olfactory sensory neurons and the main olfactory bulb, while they are sparsely observed between vomeronasal sensory neurons (VNS) and the accessory olfactory bulb (Figure 1; Akins et al., 2012).

The Olfactory System and Olfactory Marker Protein

The Olfactory System

One of the regions in which FXG expression is best characterized is within the main olfactory system, which is composed of the olfactory epithelium and olfactory bulb. Olfactory sensory neurons express odorant receptors (ORs), which are G-protein coupled receptors. In response to odorant binding an OR activates G-protein mediated cAMP- transduction, resulting in, among other responses, Ca2+ influx. This transduction occurs in the cell bodies of OSNs, which are located within the olfactory epithelium in the nasal cavity. OSNs project axons through the cribriform plate, a component of the ethmoid bone, into the olfactory bulb where they pass through the olfactory nerve layer and converge within olfactory glomeruli. Within the glomeruli they synapse with olfactory bulb neurons (Mitral, tufted and periglomerular neurons), forming the first circuit of the olfactory system (Reviewed in Nedelec et al., 2005). Experimentally, this anatomical arrangement, in which the cell bodies of OSNs are physically separated from their axonal/presynaptic domains, allows for a straightforward, separate analysis of both the somatic and axonal domain.

Several pieces of evidence indicate that the major source of FXGs within the OB is from OSNs. FXG expression is predominately restricted to the olfactory nerve and glomerular layers, both of which are highly enriched for OSN axons and presynaptic terminals. When rats are treated with methyl bromide, there is wide-spread ablation of OSNs followed by synchronized regeneration and innervation of

the OB. FXG density in the OB initially decreases following OSN ablation and then rises as OSN axons re-innervate the OB. Interestingly FXG expression peaks at approximately 3 weeks post-ablation, which correlates with synapse formation and OSN maturation (Christie et al., 2009). Finally, FMRP localizes in close proximity to the synaptic vesicle pool, indicating presynaptic localization (Christie et al., 2009). Taken together, these data indicates that FMRP is localized to the axonal and presynaptic domains of OSNs.

Olfactory Marker Protein

Olfactory maker protein (OMP) was first identified as a protein that is solely expressed by OSNs in the post-natal mouse. OMP expression is restricted to mature OSNs, whose axons have innervated the OB and reached their glomerular target (Margolis, 1972). OMP is one of the most highly expressed genes in olfactory sensory neurons, comprising approximately 1% of the protein and 0.5 % of the mRNA in olfactory epithelium (Margolis, 1972; Keller and Margolis, 1976). Interestingly, both OMP mRNA and protein are relatively simple in sequence and structure. OMP is a 19kDa protein that is cytosolically localized. NMR and crystal structure studies have shown that OMP is a single domain protein composed of two antiparallel β-sheets that is predominately found in homodimers, forming a β-clam structure, (Smith et al., 2002; Koo et al., 2004).

Several studies indicate that OMP functions in OSN maturation and odorant response. Mice null for OMP exhibit decreased odorant discrimination and threshold sensitivity (Youngentob and Margolis, 1999; Lee et al., 2011). As pups, OMP null

mice have decreased preference for their mother over an unfamiliar female, suggesting a decreased ability to seek their mother's milk, a critical developmental olfactory cue (Lee et al., 2011). Electrophysiologically, OSNs from OMP null mice exhibit reduced response to odorants as compared to wild type. OMP null OSNs exhibit altered responses to changes in calcium dynamics and OMP has been shown to modulate cAMP signaling (Reisert et al., 2007; Kwon et al., 2009). Finally, OMP's role in signal transduction of odorant response appears to contribute to OSN maturation (Lee et al., 2011).

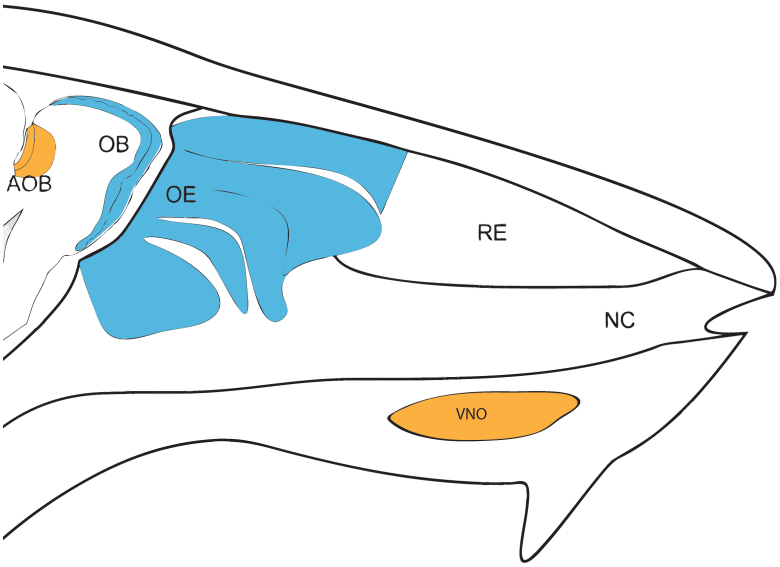
Several lines of evidence suggest that OMP mRNA is translated within the axonal compartment of OSNs. OMP mRNA was initially detected in total RNA preparations from rat olfactory bulbs, at approximately 5-10% the levels found in the olfactory epithelium, suggesting that it was localized to OSN axons (Rogers et al., 1987; Wensley et al., 1995; Strotmann et al., 2004). Using radioactive *in situ* hybridization it was further revealed that OMP mRNA, as well as that of multiple olfactory receptor messages, is localized to olfactory glomeruli within the bulb (Rogers et al., 1987; Ehrlich et al., 1990; Vassar et al., 1994; Wensley et al., 1995). The localization of OMP mRNA to olfactory glomeruli does not formally indicate that it is axonally derived, as it could originate from support cells or post-synaptic neurons. To address this possibility, animals were treated with Triton X-100 or $ZnSO_4$, to abolish the olfactory sensory neuron cell bodies and de-innervate the olfactory bulb. In this system, the OMP mRNA signal in olfactory glomeruli was lost, indicating that the message was localized to olfactory sensory neurons rather than their post-synaptic targets (Ehrlich et al., 1990; Wensley et al., 1995). The

identification of OMP mRNA in axons of OSNs suggests that it is locally translated in this compartment. OMP mRNA has been shown to associate with olfactory bulb polysomes, by polysome fractionation. Interestingly, while the levels of polysome-associated OR messages in the bulb decrease between juvenile and adult mice, equal levels of OMP mRNA are polysome associated at each age point (Dubacq et al., 2009). The association of OMP mRNA with polysomes at all examined age points, suggests that the local translation of OMP within the axonal compartment is not a developmental phenomenon, and it may potentially occur in mature neurons.

Summary

Elucidating the presynaptic role of FMRP will inform our understanding of how it regulates neuronal processes, such as synaptic plasticity, and how these processes go awry in disorders such as FXS. Recent work from our lab has identified FMRP, FXR2P and FXR1P as components of the FXG a uniquely axonal structure, suggesting a novel function for these proteins. Several questions regarding FMRP's role in the axon/presynaptically remain such as: What axonal messages are associated with FMRP? How does FMRP regulate axonal messages? In this thesis I have utilized the olfactory system to determine an *in vivo* role for FMRP in regulation an axonal message, OMP. OMP protein is expressed by somata and axons of both olfactory sensory neurons (which innervate the main olfactory bulb) and vomeronasal sensory neurons (which innervate the accessory olfactory bulb). In contrast, OMP mRNA localizes to axons of olfactory sensory neurons, but not vomeronasal sensory neurons. In olfactory sensory neuron axons, a portion of OMP

messages colocalize with FMRP in FXGs. I have found that FMRP is not required for the axonal localization of OMP mRNA, as axonal OMP mRNA is unchanged in *fmr1* null mice. However, FMRP is required for correct expression of OMP protein in these same axons, as OMP levels are increased in OSN axons in olfactory bulbs of *fmr1* null mice. Interestingly, vomeronasal sensory neurons do not contain FMRP or OMP mRNA in their axons. In these axons, loss of FMRP does not affect the axonal expression of OMP protein. FMRP therefore regulates OMP expression in a circuit-selective manner independent of mRNA trafficking.



(Adapted from Michael Akins)

Figure 1: Schematic of the olfactory system. The cell bodies of olfactory sensory neurons are located in the olfactory epithelium (OE; blue). They project axons through the cribriform plate into the olfactory bulb (OB; blue). OSN axons converge into olfactory glomeruli, where they synapse with olfactory bulb neurons forming the first circuit of the main olfactory bulb. The axonal domains of OSNs in the OB, can be readily separated from their cell bodies in the OE due to the presence of a bone structure called the cribriform plate. A second circuit of the olfactory system contains the vomernasal organ (VNO; orange) whose neurons project axons into accessory olfactory bulb (AOB; orange). FXGs are localized to OSN axons in the OE and OB, but are not detected in VNS axons in the VNO and AOB.

Chapter Two:

Circuit-Selective Regulation of OMP by FMRP

I performed the experiments presented in this chapter. Michael Akins made the initial observation that OMP mRNA co-localizes with FXGs.

ABSTRACT

Fragile X syndrome, a leading cause of autism, results from the loss of the Fragile X mental retardation protein (FMRP). FMRP is an RNA binding protein with a well-elucidated post-synaptic role in regulation of synaptic plasticity. Emerging evidence suggests that FMRP is present and functions in axons. Work from our lab has identified FMRP as a component of an axonal and presynaptic granule, termed the fragile X granule (FXG). FXGs are expressed throughout the brain, in a circuit selective manner (Akins et al., 2012). In the olfactory system, FXGs are expressed in the main olfactory bulb, but not the accessory olfactory bulb (Akins et al., 2012). Here, we have identified a novel FMRP target olfactory marker protein (OMP) that co-localizes with a subset of FXGs within the axonal domains of olfactory sensory neurons. OMP protein is present in both the soma and axonal domains of olfactory sensory neurons and vomernasal sensory neurons. However, the OMP message is localized to the axonal domain of olfactory sensory neurons, but not vomeranasal neurons. The levels of OMP mRNA are not altered in *fmr1* null olfactory sensory neurons, suggesting that it does not directly participate in trafficking of this message. Finally, the level of OMP protein is increased in olfactory sensory neuron axons, but not vomeranasal sensory neuron axons. This finding suggests that there is circuit-selective regulation by FMRP, at the level of protein expression, of a target message.

Introduction

Fragile X Syndrome (FXS), the leading inherited cause of autism and intellectual disability, results from loss of functional Fragile X mental retardation protein (FMRP). Symptoms of FXS include, but are not limited to: sensory hypersensitivity, hyperactivity, and mental impairment (Bhakar et al., 2012). The majority of FXS patients exhibit at least one autistic feature, suggesting that elucidation of the molecular basis underlying FXS may shed light on shared mechanisms underlying other forms of autism (Hernandez et al., 2009). In the majority of identified cases a CGG repeat element in the promoter of *FMR1*, which encodes FMRP, becomes expanded resulting in transcriptional silencing of the gene (Reviewed in Oostra and Willemsen, 2009). However, FXS can arise various other mutations and deletions in *FMR1*, indicating that the primary symptoms of FXS are a result of loss of FMRP function (De Boulle et al., 1993; Feng et al., 1997a; Coffee et al., 2009; Zhang et al., 2009b; Collins et al., 2010). FMRP is an RNA binding protein, which contains two KH domains and an RGG box domain (Ashley et al., 1993; Siomi et al., 1993). Several lines of evidence indicate that FMRP acts to regulate protein synthesis. FMRP associates with polyribosomes in an RNA dependent manner (Khandjian et al., 1995; Corbin et al., 1997; Feng et al., 1997a; Ceman et al., 2003; Stefani et al., 2004). In the *fmr1* knockout mouse there are altered basal levels of protein synthesis, suggesting that the protein negatively regulates translation (Qin et al., 2005; Dölen et al., 2007a; Osterweil et al., 2010). Finally, FMRP has been shown to associate with a large cohort of messages in the brain, including those

encoding both post- and pre-synaptic proteins as well as several other classes of neuronal function (Darnell et al., 2011).

FMRP has a well-elucidated role in regulation of protein synthesis dependent synaptic plasticity. The mechanisms underlying FMRP's role in this process have been best elucidated postsynaptically. However, recent work has indicated that FMRP is present and functions presynaptically. In mice that are mosaic for FMRP, due to X-inactivation, synapse formation is reduced when FMRP is lost from the presynaptic, but not the postsynaptic neuron (Hanson and Madison, 2007). Full *fmr1* knockout mice exhibit defective baseline synaptic transmission and short-term presynaptic plasticity, which maybe due, in part, to alterations in the size of the readily-releasable pool of synaptic vesicles and rate of vesicle turn over (Zhang et al., 2009b; Suvrathan et al., 2010; Deng et al., 2011; Klemmer et al., 2011). FMRP localizes to axons and growth cones of cultured neurons and to hippocampal mossy fibers *in vivo* (Ivanco and Greenough, 2002; Antar et al., 2006; Hengst et al., 2006; Price et al., 2006; Murashov et al., 2007; Christie et al., 2009). These findings suggest that FMRP may play a critical role in the axonal and presynaptic compartment.

Work from our lab has identified FMRP as a component of the Fragile X granule (FXG) an axonal structure, which also contains FMRP's homologs Fragile X related protein 1 (FXR1P) and Fragile X related protein 2(FXR2P). FXR2P is an essential component of FXGs, while FMRP is present in the majority and FXR1P is a subset (Christie et al., 2009). Interestingly, while the Fragile X proteins appear to be

expressed ubiquitously in neurons, FXG expression is limited to restricted circuits in the brain (Christie et al., 2009; Akins et al., 2012). Of interest, expression of FXGs was observed in both sensory and motor processing circuits (Akins et al., 2012). Within the olfactory bulb, FXGs are observed in the axons of olfactory sensory neurons innervating the glomeruli of the main olfactory bulb, but were rarely detected in the axons of vomernasal sensory neurons (VSNs) in the accessory olfactory bulb (Akins et al., 2012).

The identification of axonal and circuit selective expression raises several questions. What messages are regulated by FMRP in the axonal compartment? How does FMRP regulate the expression of axonal target messages? And is FMRP's regulation of certain targets limited to restricted circuits? To begin to address these questions we focused on the olfactory system, which is an enticing model for understanding the axonal role of FMRP for several reasons. As discussed above, FMRP is axonally expressed in a circuit selective manner in the olfactory bulb. Furthermore, it is well established that multiple messages are localized to the axonal domains of olfactory sensory neurons, including those encoding olfactory marker protein (OMP) and several odorant receptors (Rogers et al., 1987; Ehrlich et al., 1990; Vassar et al., 1994; Wensley et al., 1995).

We began by asking whether FMRP might play a role in regulation of OMP localization and expression. OMP is a cytosolic protein that is solely expressed in mature OSNs, where it is highly expressed throughout the neuron (Margolis, 1972). Mice null for OMP exhibit reduced odorant discrimination, response and threshold

sensitivity, as well as, altered maturation of OSNs (Youngentob and Margolis, 1999; Lee et al., 2011). Functionally, OMP plays a role in regulating Ca2+ dynamics via regulation of cAMP signaling in response to odorant receptor transduction (Kwon et al., 2009). As discussed above, OMP mRNA localizes to the axons of OSNs, where it associates with polyribosomes, suggesting that it is locally translated (Dubacq et al., 2009).

The axonal localization of OMP mRNA suggested that it might be a novel axonal target of FMRP. To address this possibility we took advantage of the anatomy of the olfactory system, which allows for straightforward analysis of the axonal compartment of OSNs in comparison to their cell bodies. We sought to address the association of OMP mRNA with FXGs, as well as, the role of FMRP in regulation of the axonal expression of OMP mRNA and protein. We observed that OMP mRNA is associated with a subset of FXGs in OSN axons. Interestingly, we observe that *fmr1* knockout mice exhibit no detectable alteration in the axonal expression of OMP mRNA, suggesting that FMRP is not required for its localization. Finally, we observe increased OMP in the *fmr1* knockout, suggesting that FMRP acts to negatively regulate its expression. This increase is circuit-specific, as it appears to only occur the main olfactory bulb and not the accessory bulb.

Materials and methods

Immunoblotting. P23 wild type and *fmr1* knockout mice were sacrificed and the olfactory bulb and olfactory epithelium were isolated. Tissue from three animals was pooled for each genotype. Tissue was extracted in RIPA lysis buffer (25mM

Tris-HCl pH8.0, 150mM NaCl, 0.1%SDS, 1% NP-40, 5% sodium deoxycholate, Roche complete proteinase-inhibitor cocktail, sodium orthovanidate and AEBSF). Protein levels were determined by BCA assay (Pierce). Samples were run on 15% acrylamide gels and transferred to PVDF membrane. Blots were probed with rabbit anti-OMP (1:200; Abcam) and mouse anti-Beta-Actin (1:10,000; Sigma) at 4°C overnight. Blots were incubated in the following secondaires for one hour at room temperature: HRP conjugated anti-mouse and anti-rabbit (1:2000; from Invitrogen). Blots were developed with ECL (GE healthcare) and imaged on ECL hyperfilm (GE healthcare). Similar results were observed in three separate experiments. Nine animals were pooled into three sets of three animals for each genotype.

Immunofluorescence in Fresh Frozen Tissue. Three WT and three *fmr1* KO littermate mice were sacrificed at P23. Brains were removed, embedded in OCT and snap frozen in an ethanol and dry ice slurry. Brains were sectioned on a Leica cryostat in 20μm coronal sections and slide mounted. Slides were stored at -20°C, and brought to room temperature at the time of staining. Sections were fixed on the slide with 4% paraformaldehyde for 15 minutes at room temperature. Antigen retrieval was performed at 95°C in 0.01M sodium citrate pH6.0 for ten minutes and sections were blocked with blocking reagent (Roche) for one hour at room temperature. The following primary antibodies were applied at room temperature over night: Goat anti-OMP 1:500 (Wako), Rabbit anti-GAP43 1:1000 (Novus) and mouse IgG1 anti-vglut2 1:1000 (neuromab). The following secondaries were used for one hour at room temperature: Alexa flour 555anti-goat, Alexa flour 647 anti-

rabbit and Alexa flour 488 anti-mouse IgG1 (all secondaries antibodies acquired from invitrogen). Slides were mounted in NPG and sealed with coverslip glass.

Quantification of OMP immunofluorescence. Images were collected at 20x on a Nikon E800 microscope for quantification. Three wild type and three *fmr1* KO littermates were used for this analysis, for each animal, six main olfactory bulb and six accessory olfactory bulb images were collected. In each image, the intensity of OMP signal across an area of the glomerulus was measured. Background signal was determined by measuring the intensity in a region lacking OMP signal and subtracted from the OMP signal in each image. Statistics were determined by two-way anova, with a $p = 0.0031$.

Immunofluorescence in Perfusion Fixed Tissue. P23 animals were anesthetized by isolflurane inhalation. They were then perfused with 100mM HEPES, pH 7.4, 150 mM sodium chloride, 0.5% sodium nitrite and 1U/ml heprin. They were then fixed with 4% paraformaldehyde in 100mM phosphate buffered saline. Brains were removed and immersed in 4%paraformaldhyde overnight. The brains were then washed in PBS and cyro-protected in 30% sucrose in PBS overnight. They were then embedded in OCT and frozen at -80°C. Brains were sectioned on a Leica cyrostat in 20μm sections. The following primary antibodies were applied at room temperature over night: Goat anti-OMP 1:1000 (Wako), Rabbit anti-GAP43 1:1000 (Novus) and mouse IgG1 anti-vglut2 1:1000 (NeuroMab). The following secondaries were used for one hour at room temperature: Alexa flour 555anti-goat, Alexa flour 647 anti-rabbit and Alexa flour 488 anti-mouse IgG1 (1:1000; all secondaries antibodies

acquired from invitrogen). Slides were mounted in NPG. Images for presentation were collected on a Ziess LSM 510 scanning confocal microscope at 63x.

Quantitative RT-PCR. Littermate wild type and *fmr1* knockout mice were sacrificed at P23 and the olfactory bulbs and epithelium were separately dissected out. Quantification was performed on three separate experiments. In each experiment, multiple animals were pooled within each genotype to minimize inter-animal variation. RNA was extracted using Trizol reagent (invitrogen), DNase treated (turbo Dnase, Ambion) and cleaned with RNAeasy mini clean up kit (qiagen). 200ng of RNA were used to reverse transcribe cDNA. QPCR using primers against OMP, GAPDH and N-Cadherin (qiagen) was performed. Total relative levels of OMP were determined by ΔΔCt.

***In situ* Hybridization**. Three WT and three *fmr1* KO littermate mice were sacrificed at P23. Brains were removed, embedded in OCT and snap frozen in an ethanol and dry ice slurry. Brains were sectioned on a Lycia cryostat in 20μm coronal sections and slide mounted. Slides were stored at -20°C, and brought to room temperature at the time of staining. Sections were fixed on the slide with 4% paraformaldehyde for 10 minutes at room temperature and washed three times in PBS. Antigen retrieval was performed at 95°C in 0.01M sodium citrate pH6.0 for ten minutes. Slides were then treated with 0.2M HCl for ten minutes, 1% Triton X-100 in PBS for two minutes, PBS for one minute twice and 2X saline sodium citrate (SSC) in 10% formamide for ten minutes. Slides were incubated in hybridization solution [10% Dextran Sulfate, 1 mg/mL E. coli tRNA (Roche), 2 mM Vanadyl Ribonucleosides

(NEB), 200 μg/mL BSA (Roche), 2X SSC (Ambion), 10% Deionized Formamide (Ambion)]. *In situ* oligonucleotide mix against the OMP message (1:100) and primary antibody against FXR2P (A42, 1:500) overnight at 37°c in a humid chamber. Stellaris *In situ* hybrization probes are a mixture of 48 unique oligonucleotides against OMP purchased from Biosearch. The following day slides were washed twice in 2XSSC in 10% formamide for thirty minutes at 37°c, once in 2XSSC and once in 1% triton-X 100. Alexa flour 488 anti-mouse IgG1 applied in blocking solution for one hour at room temperature. Slides were mounted in NPG and imaged the same day to avoid loss of signal on a Ziess LSM 510 scanning confocal microscope at 63x.

Immunoprecipitation. Wild type mice were sacrificed and their olfactory epithelium dissected out. Tissue was homogenized in polysome lysis buffer as described in Darnell et al. 2011(Darnell, 2011). Lysate was precleared with protein A beads (dynabeads, invitrogen) for one hour at 4°C. Immunoprecpitation was performed with the following antibodies pre-bound to protein A beads: rabbit anti-FMRP (abcam 1772), rabbit anti-FXR2P, mouse anti-rRNA (Y10b, specifically binds 5s and 5.8s rRNA), mouse IgG and rabbit IgG. Samples were incubated with beads for two hours at 4°C. Half of each sample was run on 10% polyacrylimide gels and transferred to nitrocellulose. Blots were probed for FMRP (2F5 1:400) or FXR2P (BD55 1:5,000; B and D transduction siganling). The other half of each sample was extracted in Trizol reagent (Invitrogen) for RNA. RNA amounts were quantified with Quanti-ribogreen (Invitrogen) and reverse transcribed to cDNA. The presence of OMP mRNA in each sample was determined by PCR analysis.

Results

Circuit-selective expression of FXGs and OMP mRNA

As we were interested in identifying messages associated with FXGs we focused on the olfactory system, where several messages have been observed in the axons of OSNs. FXGs are detected in the glomerular layer of the main olfactory bulb, but not the accessory bulb (Previously published in, Akins et al., 2012; Fig 1. a, b). One possible explanation for the lack of FXGs in the accessory olfactory bulb is that VNO neurons do not express FMRP and/or FXR2P, however both Fragile X proteins are readily detected in the VNO (Fig. 1e,f). We next asked whether OMP mRNA is expressed in the axons innervating both the main and accessory olfactory bulbs. In agreement with several previously published studies, OMP mRNA is present in OSN axons in the main olfactory bulb. OMP mRNA was observed localized to distinct puncta throughout the main olfactory bulb glomerular layer and olfactory nerve layer (Fig1. c). In contrast, OMP mRNA was rarely observed in the accessory olfactory bulb, suggesting that it is not localized to the axons of those neurons (Fig. 1d). These findings indicate that OMP mRNA is specifically localized to the same circuitry as FXGs.

OMP mRNA Localizes to a subset of FXGs in the mouse olfactory bulb

The similar expression pattern of FXGs and OMP mRNA in the olfactory bulb, suggested that there might be an association between the two structures. Co-localization was assessed by *in situ* hybridization for OMP mRNA in conjunction with immunohistochemistry for FXR2P, as a marker for FXGs. In both the

glomerular layer and olfactory nerve layer OMP mRNA was observed in a subset of FXGs (Fig. 2a,b, c). However, large populations of both OMP granules and FXGs were detected that did not co-localize with each other (Fig. 2a, b, c). We next asked whether FMRP is required for the localization of OMP mRNA to FXGs, by performing *in situ* hybridization in the *FMR1* KO olfactory bulb. It was observed that in the absence of FMRP the association of OMP mRNA with FXGs persists (Fig. 2d, e, f). This finding indicates that FMRP is not required for the localization of at least one target message to FXGs. Interestingly, the majority of FXGs do not contain detectable OMP mRNA in either the wild type or *fmr1* KO olfactory bulb suggesting a limited or transient association.

FMRP and FXR2P Associate with the OMP Message

The co-localization of OMP mRNA with FXGS in the olfactory bulb, suggest that it may be a target message of the Fragile X proteins. To test this possibility, we asked whether FMRP and FXR2P associate with OMP mRNA in the olfactory epithelium, which contains the cell bodies of OSNs. The association between FMRP and FXR2P were assessed by RNA-immunoprecipitation from WT olfactory epithelium (Figure 3). Following immunoprecipitation, both FMRP and FXR2P were readily detected in the immunoprecipitate of specific antibodies against each protein. Conversely, they were not observed in negative control immunoprecipitates (Figure 3a, b). The mRNA cohort immunoprecipitated with each protein was isolated and analyzed for the presence of the OMP message. OMP mRNA was found to associate with FMRP and FXR2P, as well as the positive control

immunprecipitation for ribosomal rRNA (Figure 3c). These results suggest that OMP mRNA is associated with both FMRP and FXR2P, suggesting that the Fragile X proteins may regulate OMP by direct interaction with the message.

FMRP is not required for axonal localization of OMP mRNA

One explanation for the association between OMP mRNA and FXGs is that the FMRP play a role in the trafficking and/or localization of the message. To test this possibility we asked whether the expression of OMP mRNA is altered in the *fmr1* knockout as compared to wild type. The localization of OMP mRNA in the olfactory bulb of *fmr1* KO mice compared to wild was assessed by *in situ* hybridization. OMP mRNA granules were detected in the glomerular and olfactory nerve layer of *fmr1* KO mice and there were no obvious differences as compared to wild type (Fig. 3a,b). We observed high levels of heterogeneity in the distribution of OMP mRNA within the olfactory bulb in both wild type and *fmr1* KO mice. Therefore, to address whether FMRP regulates the total amount of axonal OMP mRNA, we performed qPCR on olfactory bulb, as well as olfactory epithelium, from both genotypes (Fig. 3c). No difference between *fmr1* KO and wild type in the total amount of OMP mRNA was observed, when assessed in either the olfactory bulb or the olfactory epithelium. These results indicate that FMRP is not required for the axonal localization of OMP mRNA.

FMRP regulates the expression of OMP

We next sought to determine whether FMRP regulates the expression of OMP. FMRP has been shown to regulate the protein expression of target messages in

the dendrites and soma of multiple neuronal populations. To assess OMP protein levels in the axonal domain of OSNs we again took advantage of the anatomy of the olfactory system and isolated the olfactory bulb and olfactory epithelium from wild type and *fmr1* KO mice for western blot analysis. In the absence of FMRP there was an increase of OMP in the olfactory bulb and olfactory epithelium (Fig. 4a). This observation suggests that FMRP acts to negatively regulate the expression of OMP.

As discussed above, both OMP mRNA and FXGs are expressed in restricted circuits of the olfactory bulb, both localizing to OSN axons innervating the main olfactory bulb and absent from the VSN axons in the accessory olfactory bulb. This observation suggests that FMRP may act to regulate OMP expression in specific circuits. To address this possibility, we performed immunohistochemistry in the olfactory bulb of wild type and *fmr1* KO mice. In conjunction with the results of the Western blot analysis, an increase in the levels of OMP in the glomeruli of the main olfactory bulb was observed (Fig. 4b,c, f; $P = 0.0031$). Interestingly, in the accessory olfactory bulb no difference in the expression of OMP was detected (Fig. 4d,e, f). The same analysis for vGluT2 a synaptic protein that is highly enriched in main olfactory bulb and accessory bulb glomeruli, was performed. No detectable difference in the vGluT2 levels between the *fmr1* knockout and wild type in either olfactory bulb region was observed (data not shown). This finding suggests that OMP expression in the axonal domain is regulated by FMRP in a circuit-selective manner.

Discussion

Summary

As we begin to elucidate the role of FMRP in axonal and presynaptic function, it will be critical to identify proteins whose expression it regulates in these compartments. Here, we have identified a novel association between FXGs, which contain FMRP as well as FXR2P, and OMP mRNA in the olfactory bulb. FMRP is not required for the axonal localization of OMP mRNA, nor its association with FXGs. This suggests that FMRP is not required for the localization of at least one target message to the axon. Interestingly, both FXGs and OMP mRNA exhibit circuit specific expression in the olfactory bulb. In both cases, expression is limited to the axons of OSNs innervating the main olfactory glomeruli and rare in VNO neuron axons in the accessory olfactory bulb. This selective expression is not reflective of differences in the cell bodies, as both classes of neurons expression OMP and the Fragile X proteins. We next asked whether FMRP regulates the protein expression of OMP. In the *fmr1* knockout olfactory system, we observed an increase in the levels of OMP. This increase suggests that FMRP may normally regulate the expression of OMP protein. Given the circuit selective expression of OMP mRNA and FXGs, we next asked if FMRP's regulation of OMP was confined to OSN axons. We found that the increase in OMP in the absence of FMRP was restricted to OSNs axons, in comparison to VNO neuron axons. One possible explanation for this finding is that OMP is local translated in an FMRP dependent manner in OSN axons. Future work will seek to address this possibility.

FMRP is not required for axonal localization of potential target messages

We found that FMRP does not appear to be required for the axonal expression of OMP mRNA. This observation raise's the interesting possibility that the localization of axonal messages is regulated by a separate class of RNA binding proteins than those that regulated the expression of the proteins which they encode. Furthermore, it suggests that FMRP is not directly responsible for the localization of certain target messages. FMRP co-localizes with trafficking mRNPs in cultured neurons and can trans-locate within the synapse in response to activity, suggesting that FMRP is a component of trafficking granules (De Diego Otero et al., 2002; Antar et al., 2004, 2005, 2006; Dictenberg et al., 2008). However, it has been noted that postsynaptically, the basal levels of several FMRP targets are unaltered in the *fmr1* knockout (Steward et al., 1998; Muddashetty et al., 2007). One possible interpretation is that FMRP is a passive component of trafficking mRNPs, the presence of which is not necessary for localization of the message. Our findings support this model, wherein FMRP regulates target messages at the level of protein expression.

FMRP negatively regulates OMP expression within OSN axons

In this study, we observed an increase in the levels of OMP in the *fmr1* knockout mouse olfactory system, indicating that FMRP is critical for regulating the levels of this protein. One possible explanation for this observation is that FMRP regulates the translation of OMP mRNA. As discussed above, several lines of evidence suggest that in the soma-dendritic compartment of neurons, FMRP

regulates translation, specifically acting to suppress protein synthesis. Whether FMRP plays a similar role in axons is less well understood. The localization of OMP mRNA to the axons of OSNs suggests that it maybe locally translated in this compartment. Furthermore, this message's association with FXGs and altered expression in the *fmr1* knockout suggests that FMRP regulates this process. Future studies will seek to directly address FMRP-dependent local translation of OMP in axons of OSNs.

OMP is regulated by FMRP in selective circuits within the olfactory bulb

Previous work from our lab established that FXG expression is restricted to specific circuits in the brain (Christie et al., 2009; Akins et al., 2012). This suggests that, presynaptically, the Fragile X proteins function in only a subset of circuits. The presence of FXGs in these circuits further suggests that these axons are sites of local translation. The anatomy of the olfactory system allowed us a unique ability to assess the affect of FMRP on the expression of target messages in two circuits, OSNs to the main olfactory bulb and VNO neurons to the accessory olfactory bulb. We observed that in the *fmr1* knockout, OMP expression is specifically dysregulated in one of these circuits, OSNs innervating the main olfactory bulb. This finding indicates that FMRP does not exhibit a global function throughout the nervous system. FXGs are expressed in several circuits, which underlie symptoms of FXS, such as those involved in sensory and motor processing (Akins et al., 2012). Understanding how FMRP function is modulated within individual circuits will inform our understanding of its normal function and how it goes awry in FXS.

Circuit-Selective FXG Expression

While the Fragile X proteins are expressed in essentially all neuronal cell bodies, FXGs are expressed within resticted circuits throughout the brain (Christie, 2009; Akins 2012). This limited expression, suggests that there maybe a distinct axonal and presynaptic function for the Fragile X proteins in FXG-containing circuits. Here I have addressed the differences between two circuits, the main olfactory system, which contains FXGs and the accessory olfactory system, which does not (Akins, 2012). I have found that the axonal expression of OMP is regulated in the main olfactory by FMRP, while it appears to be independent of FMRP in the accessory olfactory system. This suggests a specific role for FMRP in those axons that contain FXGs. It is not currently clear why certain axons contain FXGs. However, elucidating the distinctions between circuits that contain FXGs and those that do not may shed light on this question and the function of FXGs in general.

A critical component of olfactory development is ongoing exposure to novel odorants and the ability to detect and discrimination between odors. The main olfactory system is relatively immature at birth and it continues to increase during the first postnatal month as animals are exposed to a range of novel odor experiences. In particular, odorant exposure plays an important role in the maturation of OSNs (Lee et al, 2011). Individual OSNs response to odorant exposure via the G-protein coupled odorant receptors, which activate intracellular signaling, a process in which OMP plays a role (Youngentob and Margolis, 1999; Reisert et al., 2007; Kwon et al., 2009; Lee et al., 2011). This odorant response ultimately results

in synaptic activity between OSNs and their postsynaptic targets in the olfactory bulb. The ability to response to and modulate the system for ongoing exposure to novel odors, suggests that the circuitry of the main olfactory system maybe particularly plastic. Thus, one enticing possibility is that the presence of FXGs and associated messages represent a system by which the axonal compartment of OSNs can be quickly modulated during periods of rapid plasticity.

Similarly to OSNs the cell bodies of VSNs are located in the nasal cavity where they are exposed to inhaled substances. VSNs express vomeranasal receptors that detect pheromones, chemical compounds that induce social responses between animals. Upon detection of pheromones, vomeranasal receptors are activated resulting in signaling by VSNs to their postsynaptic targets in the accessory olfactory bulb. One potentially important difference between the main and accessory olfactory systems is in the form of the behavioural response they evoke. For example, the ability to response to specific odorants mediated by the main olfactory system is a result of adaptive behavior, in which previous exposures informs future responses. This is in contrast to the detection of pheromones by the accessory olfactory system, which elicits an innate behavioral response. Importantly, it is possibility, that the more adaptive nature of the main olfactory system requires higher levels of plasticity in the neuronal circuitry, than in the accessory olfactory system. If OSNs are a component of a more plasticity system than VSNs, it is likely that the local requirements within each circuit differ. Thus, resulting in a requirement for localization of RNA and proteins involved in protein synthesis to

the axons of OSNs, but not VSNs. This difference may explain, in part the selective expression of FXGs in OSN, but not VSN axons.

Implications for Fragile X Syndrome

As discussed above loss of functional FMRP results in Fragile X syndrome, one of the leading inherited causes of autism and intellectual disability. Thus, understanding the normal function of FMRP will inform our understanding of the molecular mechanisms underlying Fragile X syndrome and related disorders. The majority of studies have focused on the postsynaptic deficits in Fragile X syndrome and how they maybe treated. However, the axonal localization of FMRP, as well as, presynaptic deficits in the *fmr1* null mouse, suggest that it may play an important role in these cellular compartments (Christie et al., 2009; Hanson and Madison, 2007; Zhang et al. 2009;Suvrathan et al., 2010; Deng et al., 2011; Klimmer et al. 2011). Furthermore, these findings suggest that some of the symptoms observed in Fragile X syndrome may result from axonal and/or presynaptic deficits. Interestingly, while FMRP is expressed in the soma of essentially every neuron, axonal FMRP, as a component of FXGs, are localized to specific circuits through out the brain. Importantly, a number of the FXG containing circuits underlie several of the processes that are altered in Fragile X syndrome. Thus, understanding the function of FXGs, and axonal FMRP, will be critical to addressing the deficits observed in this disorder.

The focus of my thesis has been on elucidating the role of FMRP in regulating axonal transcripts in the olfactory system. The olfactory system provides an

excellent system for addressing the function of FXGs as its anatomy allows for a straightforward isolation of axonal and cellular compartments. Fragile X patients exhibit symptoms in sensory processing, including deficits in olfaction (Rogers et al., 2003). Thus, these findings will directly inform FMRP's role in olfactory processing. Furthermore, elucidating the role of FMRP in regulating axonal messages in the olfactory system will inform our understanding of its function in other FXG containing circuits. In turn, these findings may have important implications for understanding the deficits in Fragile X syndrome, as well as informing avenues for treatment of the disorder.

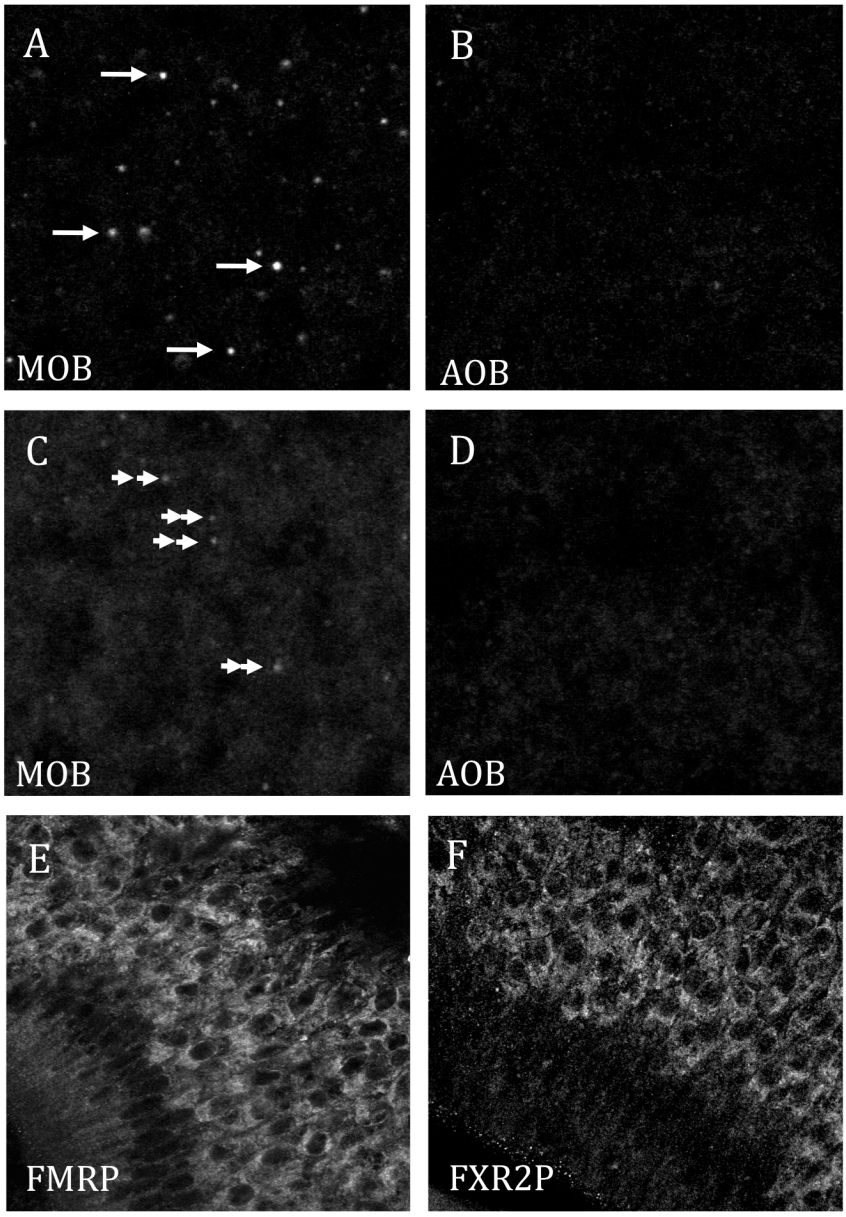


Figure 1. Circuit-selective expression of FXGs and OMP mRNA. FXGs, identified by the marker FXR2P, were highly expressed in main olfactory bulb glomerulus (A, arrows) and not detectable in the accessory olfactory bulb (B). Similarly, OMP mRNA is observed in discrete granules within the main olfactory glomeruli (C, double arrow heads) and rarely detect in the accessory olfactory bulb (D). While FXGs are not detectable in VNS axons, both FMRP (E) and FXR2P (F) are expressed in the cell bodies. N= three animals per genotype.

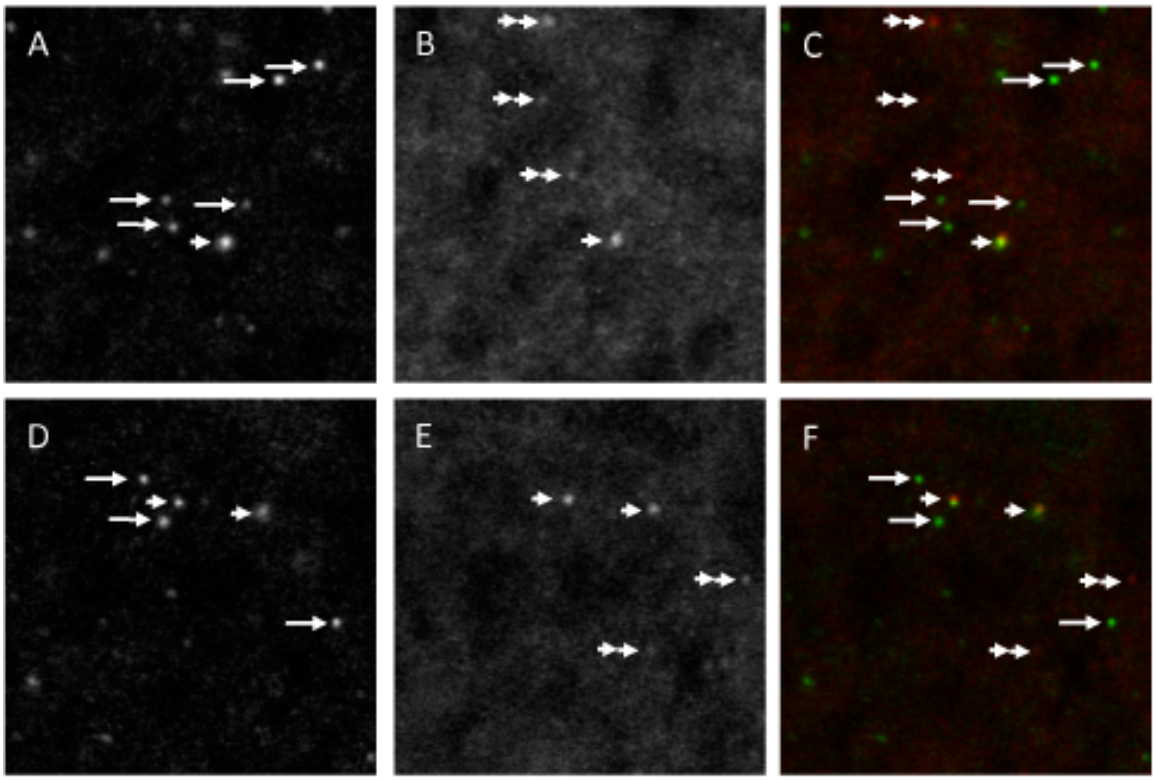


Figure 2. OMP is localized to a subset FXGs in the olfactory bulb. The association between OMP mRNA and FXGs was assess by *in situ* hybrization for the message and immunohistochemistry for FXR2P, a marker for FXGs. (arrows denote FXGS, double arrow heads OM mRNA and co-localiztion single arrow heads). In wild type mice OMP mRNA co-localizes with a subset of FXGs (A, B, C). Similarly to wild type, in the *fmr1* knockout, OMP mRNA remained associated with FXGs (D, E, F). n= three animals per genotype.

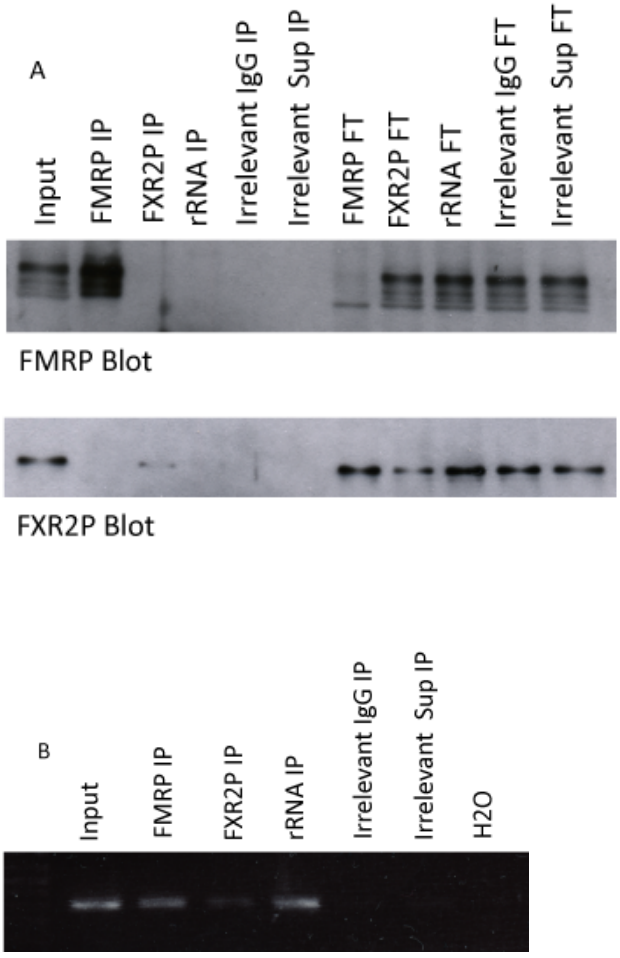


Figure 2: FMRP associates with OMP mRNA in the olfactory epithelium. To determine if FMRP and FXR2P associates with OMP mRNA, olfactory epithelium was isolated. The lysate was split between the following six conditions: Input, Immunoprecipitation for FMRP, FXR2P, rRNA, irrelevant rabbit IgG, and irrelevant mouse supernatant. The immunoprecipitation for FMRP and FXR2P pulled down both proteins (A, B). Furthermore, OMP mRNA was detected in the immunoprecipitation for FMRP and FXR2P, as well as the positive control rRNA (C). OMP mRNA was not detected in either of the control immunoprecipitations (C).

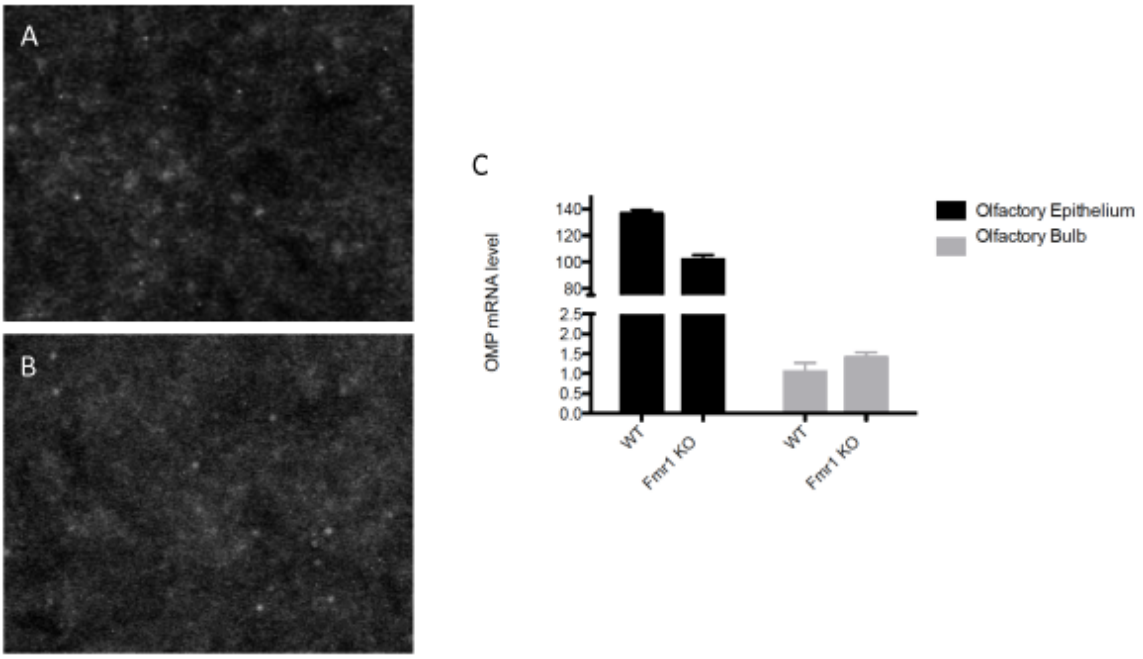


Figure 4. FMRP is not required for the axonal localization of the OMP message. Similar expression of the OMP message was observed in wild type and *fmr1* KO olfactory bulbs (A, B). qPCR on the olfactory epithelium and bulb of wild type and *fmr1* knockout mice was used to further assess FMRP role in over-all OMP mRNA abundance. Relative levels of OMP were measured in three independent experiments. The levels of OMP mRNA in the olfactory epithelium and bulb were not significantly different between WT and *fmr1* KO mice. (C).

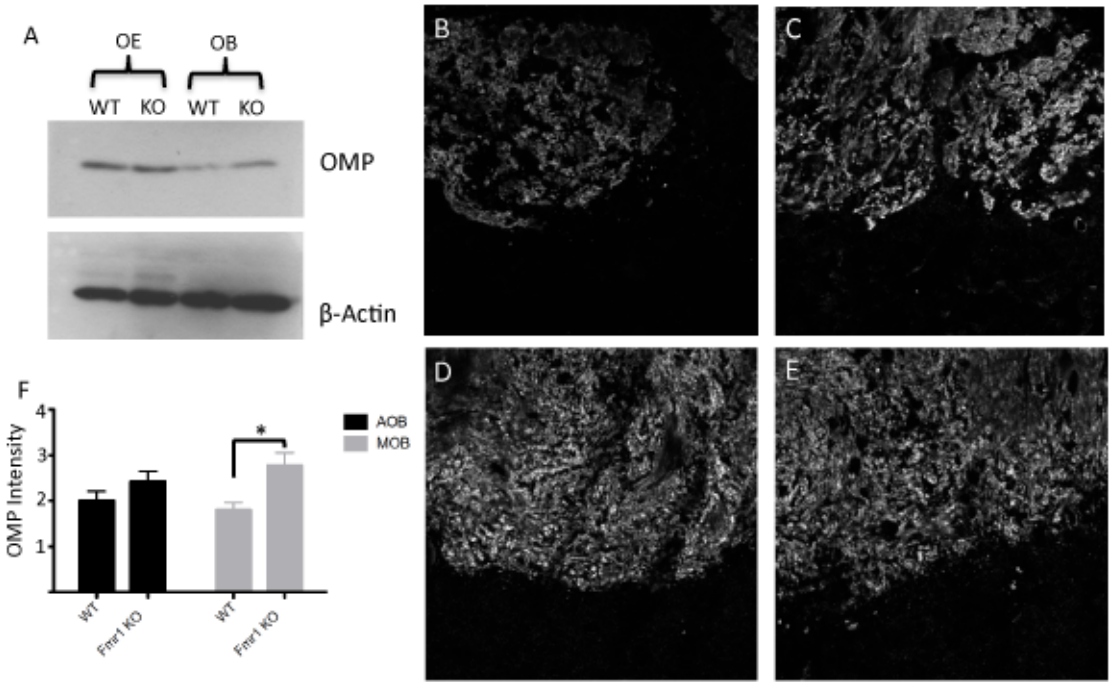


Figure 5: Circuit-selective regulation of OMP by FMRP. The total levels of OMP in wild type and *fmr1* knockout olfactory epithelium and olfactory blub, were assessed by western blot. OMP levels were increased in the *fmr1* knockout olfactory epithelium and bulb compared to wild type. β-Actin was used as a loading control. (A), similar results were observed in three separate experiments. OMP expression was further assessed by immunofluorescence in the main olfactory bulb (B, C) and accessory olfactory bulb (D, E) of wild and *fmr1* knockout mice (three animals per genotype were analyzed). The intensity of OMP signal in each of these regions was quantified. OMP expression was increased in the main olfactory bulb (MOB, $P \leq 0.0031$), but not the accessory olfactory bulb (AOB) of *fmr1* knockout mice.

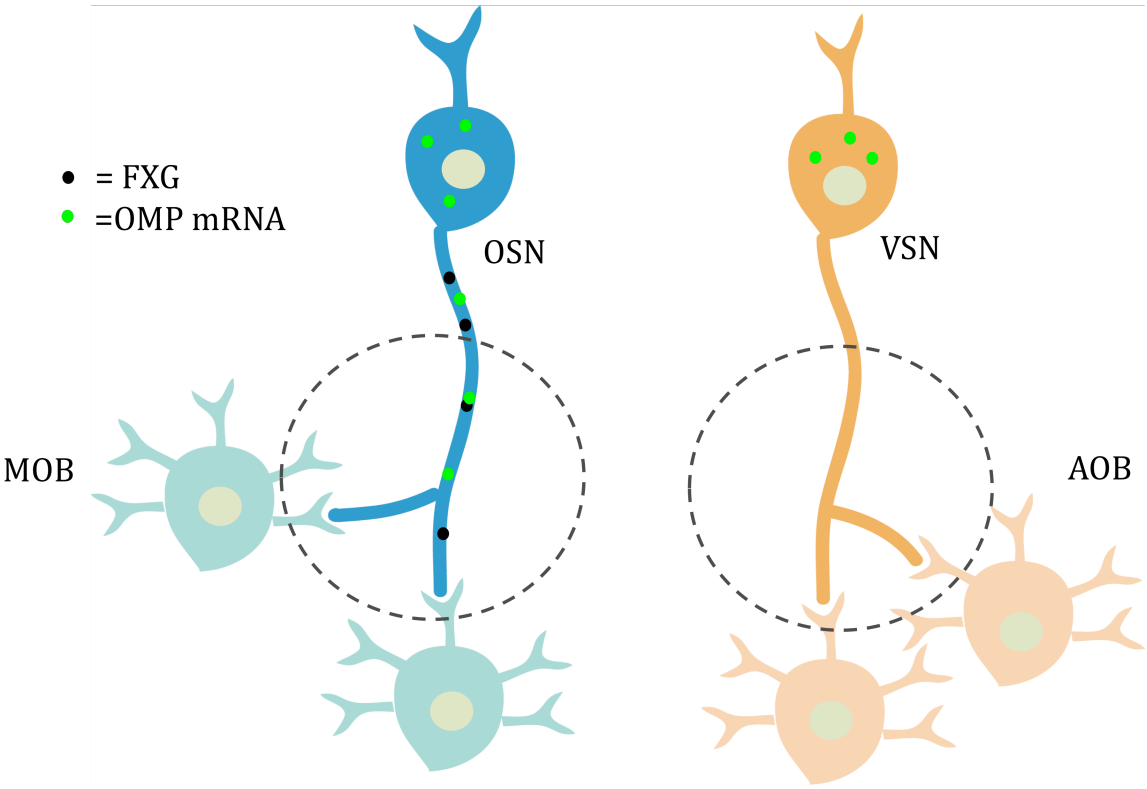


Figure 6: Circuit-Selective Expression of FXGS and OMP mRNA in the olfactory System. The cell bodies of ofactory sensory neurons (OSNs) are located in the nasal cavity where they detect odorants. They project axons into the main olfactory bulb (MOB), where they converge into glomeruli and synapse with their postsynaptic targets, comprising the main olfactory system (in blue). Vomeranasal sensory neurons (VSNs) are located in the vomeranasal organ, where they detect pheromones. They project axons into the accessory olfactory bulb (AOB; orange). FXGs (black) are highly expressed throughout the axons of OSNs, but rarely detected in VSN axons. Similarly, while OMP mRNA is expressed in the cell bodies of OSNs and VSNs, it is only detected in the axonal compartments of OSNs.

Chapter Three:

Discussion

Summary

This work addresses the role of FMRP in regulating the axonal expression of target messages and proteins. Evidence of an axonal role for FMRP is rapidly emerging. Determining the proteins whose axonal expression is regulated by FMRP is critical to understanding its role in this cellular compartment. As previously established, OMP mRNA is localized to the axons of OSNs. In this work we have established that OMP mRNA associates with a subset of FXGs in the main olfactory bulb. We found that OMP mRNA is localized to axons in the absence of FMRP and that the total levels of the message are unaltered. This finding suggests that FMRP is not required for the localization and expression levels of at least one target message. I next asked whether FMRP regulates the axonal expression of the protein levels of OMP. I found that in the absence of FMRP the levels of OMP are increased compared to wild type. We observed that while OMP mRNA is localized to OSN axons in the olfactory bulb, it is not detectable in VNO neuron axons innervating the accessory olfactory bulb. This circuit selective expression is similar to the pattern observed for FXGs. I found that FMRP dependent alterations in OMP expression were also restricted to the circuit between OSNs and the main olfactory bulb glomeruli. These results suggest that the protein levels of OMP are negatively regulated by FMRP in selective circuits.

Future Directions

Does FMRP directly associate with OMP mRNA?

One critical question that I have not answered in this study is whether FMRP directly associates with OMP mRNA. Our observation that OMP mRNA co-localizes with FXGs, suggests an association with the fragile X proteins. However, whether there is a direct association between FMRP and the OMP message has not been determined. In a preliminary set of experiments, I performed RNA immunoprecipitations to assess the association between the fragile X proteins, FMRP and FXR2P, and OMP mRNA. I detected OMP mRNA in the immunoprecipitation lysates of FMRP and FXR2P, suggesting that OMP mRNA associates with FMRP and FXR2P. While these results are promising, there are several technical limitations to this approach, which can lead to identification of false-positive targets of RNA binding proteins. Thus, we intend to use HITS-CLIP analysis, a more stringent approach to determine the association between FMRP and OMP mRNA. We anticipate that OMP mRNA will associate with FMRP in the olfactory epithelium. However, it is possible that FMRP indirectly regulates OMP expression. We have observed co-localization between FXGs and OMP mRNA in the axons of OSNs, however FMRP is not required for the localization of this message. One possibility is that FXR2P or another RNA binding protein is directly associated with OMP mRNA, and responsible for its localization.

What is the role of FXR2P in OMP regulation?

We observed that OMP mRNA associates with a subset of FXGs in the main olfactory bulb. Besides FMRP, FXGs in the olfactory bulb also contain FXR2P. In the *fxr2* knockout expression of FXGs is abolished, indicating that FXR2P is required for their expression (Christie et al., 2009). The differential roles of FMRP and FXR2P in FXG expression suggest that they are not functionally redundant. To test this possibility, we will assess the expression of both OMP mRNA and protein in the *fxr2* knockout. Preliminary results from a post-doc in the lab, Michael Akins, shows that OMP mRNA is localized the olfactory bulb in the *fxr2* KO by *in situ* hybridization. Thus, neither Fragile X protein appears to be necessary for the axonal localization of this message. However, we are unable to determine the total amount of mRNA in the bulb by *in situ* hybrization. Thus, future experiments will use qPCR to determine whether the amount of axonal OMP message is altered in the *fxr2* KO. I have shown in this thesis that FMRP is not required for axonal localization of OMP mRNA. If FXR2P is similarly not required for localization of this message, it will strongly indicate that FXGs are not primarily transport granules. It is possible that Determining FXR2P, contribution to regulation of OMP will provide insight into the functional purpose of FXGs.

The data that I have presented in this thesis indicates that FMRP regulates the expression of OMP in specific circuits of the olfactory system. Furthermore, my data supports the hypothesis that in the *fmr1* knockout mouse, OMP is increase in the axons of OSNs. One of the challenges of this study has been the limitation of the

available reagents for analysis of protein levels of OMP. However, I have recently determined western blot conditions, in which the native form of OMP, in its homodimer form can be readily detected (Fig.2). This protocol can be used to produce quantifiable western blots for OMP in both the *fmr1* and *fxr2* knockouts.

Does FMRP regulate the local translation of OMP in the axonal compartment?

The data presented in this thesis, as well as other studies, suggest FMRP regulates local translation in axons and presynaptically, however to date, there is little evidence to directly support this hypothesis. The identification of OMP as an axonal target of FMRP regulation, suggests an excellent model for assessing local translation in this compartment. One challenge in addressing this question is identification of cues, which induce local translation of OMP. However, we can take advantage of the anatomy of the olfactory bulb to readily separate the axonal domains of OSNs within the olfactory bulb, from their cell bodies, to screen for treatments that induce local translation of OMP. Simultaneously, we can address whether FMRP regulates local translation of OMP in response to any identified cues, by testing the *fmr1* KO mouse olfactory bulbs in conjunction with wild type. It is possible that FMRP does not regulate the local translation of OMP, but rather the global expression, we will address this possibility, by analysis of OMP expression in both the axonal and cell body compartments of OSNs.

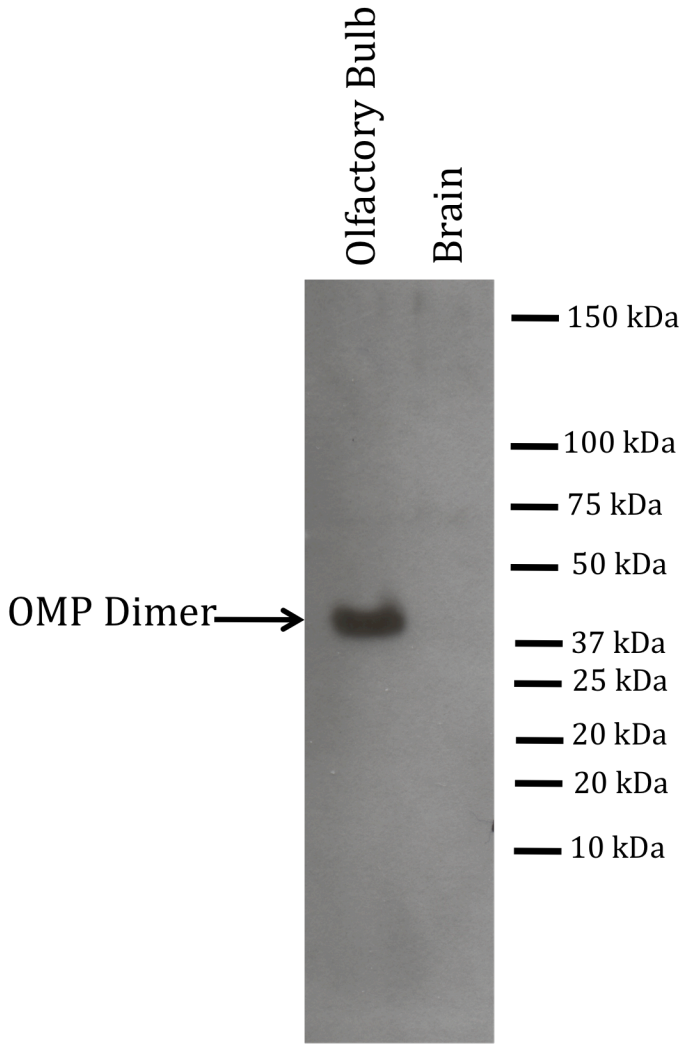


Figure Two: Detection of OMP by Native-PAGE. Olfactory bulbs and whole brains were extracted in RIPA buffer. 10ug of protein, for each sample, was run on reducing, non-denaturing gradient (5%-15% acrylamide) and transferred to PVDF membrane. Blots were probed with goat anti-OMP at 1:1000 (wako chemicals). A strong band was detected in the OB sample at ~38 kDa, which is the expected size of an OMP dimer. Conversely, no band was detectable in brain lysate, confirming the specificity of the antibody for OMP. This indicates the specificity of the OMP antibody.

Chapter Four:

A Role For FMRP in Neuroligin-Neurexin Mediated Presynaptic Assembly

Summary

Loss of the fragile X mental retardation protein (FMRP) results in fragile X syndrome (FXS), the most common single gene cause of autism. FMRP is an RNA binding protein encoded by the *FMR*1 gene that regulates synaptic plasticity by modulating local protein synthesis. The function of FMRP in the postsynaptic compartment is well established. However, recent work suggests that FMRP also functions presynaptically. Here I sought to determine whether FMRP in the presynaptic neuron contributes to differentiation of the presynaptic apparatus, focusing on presynaptic differentiation mediated by neuroligin-neurexin signaling.

Neuroligins and neurexins are synaptic adhesion molecules that are expressed post- and pre-synaptically respectively. They bind across the synaptic cleft and act to regulate synaptic function. There are four members of the neuroligin family, neuroligin-1, neuroligin-2, neuroligin-3 and neuroligin-4 and three members of the neurexin family neurexin-1, neurexin-2 and neurexin-3. Both neuroligins and neurexins are subject to alternative splicing which acts to regulate their adhesive properties and synaptic function. Furthermore, each neurexin family member contains an alternative promoter, resulting in production of a α- and β-isoform for each neurexin (Reviewed in Sudhof, 2008). Using primary neurons, which are co-cultured with heterologous cells transfected with either neuroligin or neurexins, it was observed that the synaptic adhesion molecules could induce assembly of

synaptic machinery at contact sites (Scheiffele et al., 2000). While these initial studies suggested a role for neuroligins and neurexins in synapse formation, *in vivo* studies indicate a more complex role in regulation of synapse function. Mice lacking neuroligins -1, -2, and -3 do not exhibit altered synapse number but rather deficits in synapse transmission (Varoqueaux et al., 2006). Similarly, in mice in which the α-neurexins have been knocked out there is no change in synapse number but rather altered synaptic transmission and organization of presynaptic Ca2+ channels (Missler et al., 2003). Taken together these studies indicate a complex function for the neuroligin-neurexin pathway at the synapse.

Despite evidence indicating that neuroligins and neurexins role in organization of synaptic machinery, the molecular mechanisms underlying this induction are unknown. I, therefore, investigated whether FMRP may contribute to the regulation of this pathway. The neuroligin-neurexin pathway was an enticing possible target of FMRP for several reasons. Both neuroligins and neurexins have been implicated in autism spectrum disorders. Thus, any interactions between FMRP, neuroligins and neurexins might elucidate a common mechanism underlying autism spectrum disorders. Furthermore, postsynaptically, over-expression of neuroligin-1 can ameliorate some of the deficits observed in the *fmr1* knockout mice(Dahlhaus and El-Husseini, 2009). Finally, recent HITS-CLIP data has shown that several members of the neuroligin and neurexin families are mRNA targets of FMRP (Darnell et al., 2011). Together these studies suggest that FMRP might play a role in neuroligin-neurexin mediated synapse function. To begin to assess whether there is an interaction between FMRP and the neuroligin-neurexin pathway, I have

adapted the heterologous co-culture assay described above for olfactory sensory neurons (OSNs), a neuronal population that expresses presynaptic FMRP (Christie et al., 2009). I have assessed the expression and role in synapse induction of members of the neuroligin and neurexin family member in wild and *fmr1* KO olfactory systems. I have found preliminary evidence that FMRP functionally interacts with this pathway in the presynaptic neuron.

Materials and Methods

Co-culture assay COS7 cells were seeded onto Permanox chamberslides coated with 20µg/ml poly-d-lysine (Sigma) and 20µg/ml laminin (Invitrogen). Twenty-fours later they were transfected with pNICE-HA-NL1 or GFP using FugeneHD (Ambion). After three hours, wild type and *fmr1* knockout mice from P1-4 were sacrificed and the olfactory epithelium removed in Hanks balanced salt solution. Olfactory sensory neurons were dissociated with Papain for one hour to fully dissociate neurons. OSNs were seeded onto transfected COS7 cells. Cultures were fixed forty-eight hours later with 4% Paraformaldehyde and 4% sucrose in phosphate buffer saline (PBS) for fifteen minutes. Cultures were then stained for Mouse IgG1 anti-SV2 (1:400, developmental hybridoma studies), Rat anti-NCAM (1:1000, XXX) and Chicken anti-HA (1:5,000, Millipore).

Quantification of SV2 Induction. Images were collected on a Nikon E800 microscope at 60x objective. The experiment was performed twice, for a total of 50 images per condition. The intensity of SV2 signal was measured crossing a neuroligin-1 expressing COS7 cell or an untransfected or transfected with GFP

alone. In experiments in which GFP was included, the SV2 intensity was measured over the area of NCAM that was over the area of the transfected COS7 cell (E.g. the signal in an axon crossing a transfected cell.). For both of these data sets an induction ratio was created of the SV2 intensity in axons crossing neuroligin-1 transfected COS7 cells versus crossing GFP transfected COS7 cells for each genotype.

Quantification of Baseline SV2 Levels WT and *FMR1* KO OSNs were cultured, processed and imaged as described above. SV2 intensity was measured per micron of axon length for individual axons crossing the substrate-coated coverslip. A total of 29 axons were measured for each genotype.

Results

To begin to assess the interaction between FMRP and the neuroligin-neurexin pathway I focused on the olfactory system, as FMRP is expressed in OSNs (Christie et al., 2009). I first asked whether neuroligin-1 is expressed at the post-synaptic targets of OSNs. Using immunohistochemistry, I assessed Neuroligin-1 expression in the developing olfactory bulb, and found that it was highly expressed in the olfactory glomerulus, which contain the synapses between OSNs and their post-synaptic target (Fig. 1). This suggests that neuroligin-1 is localized to, and may function at synapses in olfactory glomeruli.

To assess the contribution of presynaptic FMRP in neuroligin-neurexin mediated synapse formation, I adapted a heterologous cell culture protocol from Scheiffele et al. (Scheiffele et al., 2000). OSNs from either wild type of FMRP null mice were cultured with neuroligin-1 transfected COS7 cells. Cultures were fixed

and stained with the presynaptic marker SV2, to assess clustering of presynaptic machinery at contact sites between OSN axons and neuroligin-1. At contact sites between wild type OSN axons and neuroligin-1 there appeared to be clustering of SV2, suggesting possible presynaptic assembly. Conversely, at contact sites between FMRP null OSN axons and neuroligin-1 there appeared to be very little clustering of SV2 suggesting a possible defect in response to neuroligin in the absence of FMRP (Fig. 2A, B and C). One possible explanation for this observation was that there are elevated baseline levels of SV2, and other presynaptic machinery, in the absence of FMRP. To assess this possibility, I measured the baseline levels of SV2 intensity in WT and *FMR1* KO OSN axons (Fig. 3), and observed that the baseline levels of SV2 are unaltered in the absence of FMRP. These results suggest that FMRP acts to regulate the presynaptic response of OSNs to neuroligin.

Conclusions

Initial results from this project suggested a role for FMRP in the presynaptic neuron in regulation of synaptic assembly in response to neuroligin-1. However, further experiments are needed to further establish this interaction. Furthermore, preliminary results suggest that FMRP may act to selectively regulate expression of some members of the neurexin family. Given recent evident that neuroligins and neurexins are FMRP targets it is quite possible that there expression/ function is regulated by FMRP. In particular, it will be interesting to see in future studies whether presynaptic FMRP regulates expression and assembly of presynaptic machinery in response to neuroligins and neurexins, or other synaptic regulators.

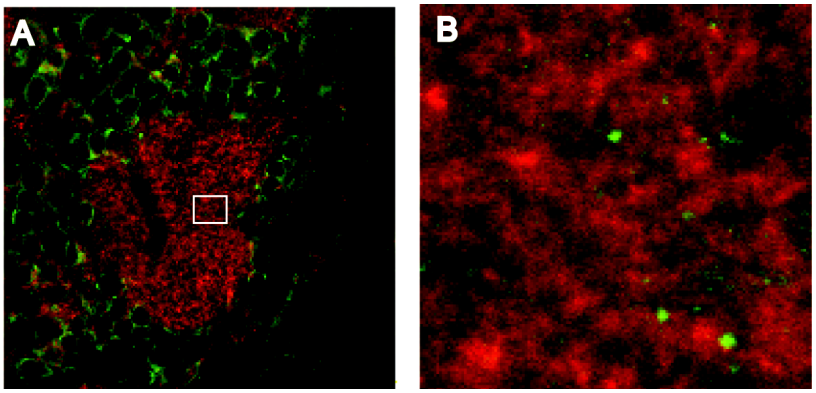


Figure 1. Neuroligin expression in wild type and *fmr1* knockout olfactory system. Neuroligin-1 (red) is highly expressed in the olfactory glomerulus of the developing mouse, which contains the synapses of OSNs (A).

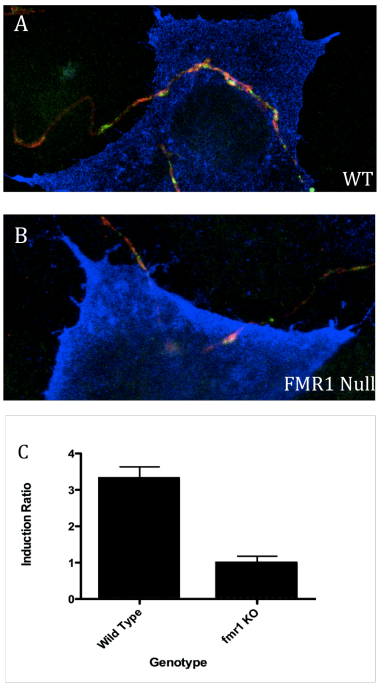


Figure 2. Neuroligin-induced presynaptic differentiation requires FMRP. OSNs were co-cultured with COS7 cells expressing neuroligin-1. Axons (NCAM, red) from neurons of both genotypes contact neuroligin-expressing COS cells (blue). Presynaptic clusters (SV2, green) were enriched upon neuroligin contact in axons of wild (A) but not *fmr1* null (B) neurons. SV2 area in axons crossing neuroligin-expressing cells was normalized to that of axons crossing GFP-expressing cells to generate the induction ratio (C).

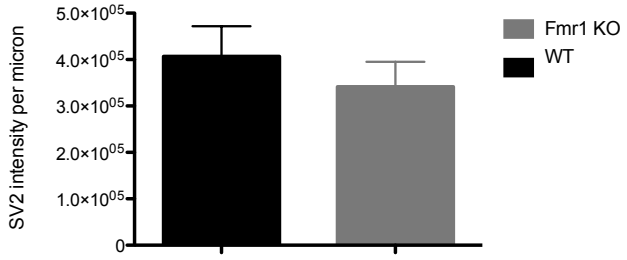


Figure 3. Baseline levels of SV2 in FMRP null axons is unaltered compared to WT. SV2 intensity per micron was measured in either WT or *fmr1* KO OSN axons. There is no significant different between the two genotypes in baseline SV2 levels.

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