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The Role of FMRP in the Regulation of PI3-Kinase Signaling in the Fragile X Mouse Model

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A thesis submitted to the Faculty of Emory College of Arts and Sciences
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Abstract

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By So Yim

A fundamental yet unexplained phenotype of animal models for the mental retardation disease fragile X syndrome (FXS) is exaggerated signaling through group 1 metabotropic glutamate receptors (gp1 mGluRs) and dysregulated protein synthesis. FXS is caused by the inherited loss of Fragile X Mental Retardation Protein (FMRP), an mRNA binding protein that can inhibit the translation of select target mRNAs and control protein synthesis dependent synaptic plasticity. Preliminary data from the Bassell lab indicates that one specific downstream pathway of gp1 mGluRs, the PI3K signaling pathway, is regulated by FMRP and is overly active in FXS. We thus hypothesized that FMRP directly regulates PI3K activity, leading to exaggerated PI3K signaling in the absence of FMRP. To assess PI3K signaling, we first quantified Akt levels in dendrites and at synaptic membranes from wild type and FMRP deficient mice by immunocytochemistry of hippocampal neurons and western blot analyses of biochemical purifications of synaptic membrane fractions. Akt is translocated to the plasma membrane via its interaction with phosphoinositide-3-phosphates (PI3P), the product of PI3K activity. We observed an increase of Akt protein in dendrites of cultured *Fmr1* KO neurons, as well as upregulated membrane levels of Akt in *Fmr1* KO cortical synaptic fractions, indicating enhanced PI3K enzymatic activity in the absence of FMRP. Furthermore, our study suggests that FMRP directly regulates the protein expression of the PI3K enhancer PIKE-L. Our results show that PIKE-L protein levels are increased in synaptic fractions of *Fmr1* KO mice, and that PIKE-L mRNA associates with FMRP in cortical lysates. My

study has significant implications for the FXS field because it (1) points to novel therapeutic strategies for FXS by targeting excess PI3K activity, and (2) validates the PI3K activator PIKE as an important FMRP target playing a role for dysregulated gp1 mGluR signaling in FXS.

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Introduction

Fragile X Syndrome (FXS) is an inherited neurodevelopmental disorder and the most common genetic cause of mental retardation and autism. Its name is derived from the "fragile" appearance of the affected X chromosome, in which a section of the chromosome seems to be almost detached at the site of a gene mutation. Approximately 1 in 4000 males are born carrying the full mutation, amounting to 37,000 males in the United States. The prevalence of females with the full mutation is slightly lower at an estimated 1 in 4000 – 6000 (Beckett *et al*., 2005). While the majority of males with the mutation display symptoms of FXS, around half of these females appear unaffected, thus reflecting the sex-linked nature of this disease. Some of the prominent physical features characterized in FXS are an elongated face, prominent ears, flat feet, and macroorchidism, or enlarged testicles (Figure 1; Butler *et al*., 1992). Commonly observed behavioral and cognitive symptoms include mental retardation, motor and speech delay, epilepsy, and various autism spectrum disorders, such as ADHD (Reiss and Freund, 1992; Rogers *et al*., 2001). The expression of these symptoms ranges by the individual, particularly within females who in general are less severely affected due to their additional X chromosome (Freund and Reiss, 2005). Nonetheless, this wide scope of physical and mental impairments contributed to this disorder makes FXS an important area of research.

FXS is caused by the expansion of a CGG trinucleotide repeat in the 5' UTR of the *FMR1* gene (Kremer *et al*. 1991; Ashley *et al*. 1993). The average number of repeats in unaffected individuals is around 5-55. Between 55 – 200 repeats denotes a

premutation, in which no or few symptoms may be displayed, but there is an increased susceptibility of a full mutation within the offspring of female carriers (Fu *et al*., 1991). A full mutation results when there are more than 200 CGG repeats. At this range, hypermethylation of the CpG island embedded within the *FMR1* promoter occurs, leading to the silencing of the fragile X mental retardation protein, or FMRP (McConkie-Rosell *et al*., 1993; Stoger *et al.,* 1997). FMRP is an mRNA binding protein that plays a role in regulating translation and protein synthesis in response to the activation of group 1 mGluRs (Bassell and Warren, 2008). It has been shown to be ubiquitously expressed throughout cells in the body, and within neurons it is localized mainly in the cytoplasm (Eberhart *et al,* 1996). Its function in the translational control of various mRNAs is indicated within its domain structure (Figure 2), where several RNA-binding motifs: two KH domains (KH1 and KH2), an RGG box, and an N-terminal domain (Siomi *et al*., 1993; Zalfa *et al*., 2005), have been identified. Furthermore, a nuclear localization sequence and a conserved nuclear export signal has been observed (Eberhart *et al,* 1996), indicating a nucleo-cytoplasmic shuttling of FMRP (Feng *et al*., 1997; Tamanini *et al*., 1999). It has been noted early on that a point mutation of the KH2 domain resulted in FXS despite normal CGG repeat lengths and an unmethylated CpG island (De Boulle *et al*., 1993). Later evidence in vitro showed that the KH2 domain binds to synthetic RNAs which harbor a tertiary structure called the FMRP "kissing complex," a structure which was suggested to provide a necessary platform for the association of polyribosomes with FMRP and specific "kissing complex" RNAs (Darnell *et al.,* 2005). The mutation of the KH2 domain resulted in the formation of abnormal FMRP-containing mRNP particles, and prevented FMRP from associating with translating polyribosomes (Feng *et al*., 1997).

However, specific mRNA targets that bind to the KH2 domain have yet to be identified. In contrast, several mRNA targets have been reported to bind the RGG box of FMRP via a so-called G-quartet within their 3'UTR (Darnell *et al*., 2001), and FMRP was shown to regulate translation, dendritic localization and stability of these mRNAs (Bassell and Warren, 2008). The molecular mechanisms by which FMRP regulates the translation of target mRNAs are controversial. Besides the above mentioned possible role of FMRP within polysomes, several other reports have suggested that FMRP plays a role during the initiation step of translation. A recent study for example, identified CYFIP1 as FMRP interacting protein, which associates with the initiation factor eIF4E (Napoli *et al*., 2008). CYFIP1 was proposed to function as a novel 4E-binding protein involved in the repression of the translation of FMRP target mRNAs (Napoli *et al*., 2008). Thus while these studies highlight the complexity of the interactions at hand, they provide strong support for FMRP having a role as a regulator of mRNA translation and protein expression within neurons.

Yet the exact cellular mechanism that underlies the pathogenesis of FXS remains unclear. A hallmark of FXS animal models is aberrant and excessive protein synthesis in neurons; however, it is uncertain how FMRP can regulate global translation. The prevailing model was first mentioned in 2004 by Bear and his colleagues, and was named the "mGluR theory" of FXS (Bear *et al*., 2004). This refers to the idea of excessive group 1 metabotropic glutamate receptor (gp 1 mGluR) signaling being responsible for the translational dysregulation observed in a FXS neuron. The group 1 family of mGluRs consists of two subtypes, mGluR1 and mGluR5. Within this model, FMRP negatively regulates the downstream translation of mRNAs that are necessary for the facilitation of

the mGluR-activated cell signaling pathway. Thus within an *FMR1* KO paradigm, the absence of FMRP results in exaggerated signaling, despite basal levels of mGluR activity. This excess signaling has been linked to the various neuronal phenotypes associated with FXS such as enhanced long term depression of synaptic signaling and altered synaptic function and plasticity (Dolen and Bear, 2008). Reversal of some of these phenotypes has been demonstrated by use of gp1 mGluR antagonists such as 2-methyl-6-(phenylethyl)-pyridine (MPEP, specific to mGluR5) (McBride *et al*, 2005), and through genetic reduction of mGluR5 expression in *Fmr1* KO mice. Cross-breeding of FXS mice with mice which are heterozygous for the mGluR5 encoding gene resulted in the rescue of many FXS-associated phenotypes (Dolen *et al*., 2007). These studies reinforce the concept of FMRP regulating the mGluR pathway, but the means by which FMRP acts as a break on gp1 mGluR signaling of protein synthesis remain unclear. To develop an improved therapeutic strategy for FXS, it will be essential to understand the molecular mechanisms underlying exaggerated mGluR signaling. Of note, recently other membrane receptor signaling pathways have been shown to be dysregulated in the absence of FMRP, such as signaling through muscarinic acetylcholine receptors and dopamine receptors (Chang *et al*., 2008; Wang *et al*., 2008). This suggests that FMRP might regulate activity and/or translation of a common downstream signaling molecule, rather than acting on the membrane receptors themselves. Gp1 mGluRs regulate protein synthesis mainly through two pathways: the ERK1/2 pathway and the P13K/mTOR pathway (Banko *et al*., 2006). Preliminary data from the Bassell lab comparing the enzymatic activity between the two pathways indicated a dramatic 3-fold elevation of the PI3K (phosphoinositide 3-kinase) activity within the *Fmr1* KO mice synaptoneurosomes

in comparison to wild type, while there was no evidence for any increase in ERK activity assay (Figures 3A, 3B, 4). Furthermore, increased phosphorylated levels of a PI3K target protein, Akt, were detected in *Fmr1* KO samples in contrast to the unchanged phosphorylated levels of ERK (Figures 3C, 3D). These assays provide strong evidence that in the absence of FMRP, the PI3K pathway is upregulated, suggesting that the dysregulation of the PI3K pathway may play a significant role in the biological pathology of FXS.

Thus, given that FMRP is a protein controlling the translation, transport, and stability of its target mRNAs we hypothesized that FMRP might regulate mRNAs coding for crucial downstream signaling molecules within the PI3K pathway, and especially those proteins which function to provide a link between gp1 mGluR signaling and PI3K activity. Previous reports have suggested that two mRNAs encoding subunits of P13K, the regulatory subunit p85β and the catalytic subunit p110β, are associated with FMRP (Brown *et al*., 2001; Miyashiro *et al*., 2003). Preliminary data from the Bassell lab shows an enrichment of p110β in *Fmr1* KO synaptoneurosomes, as well as within the dendritic synapses of *Fmr1* KO neurons (Figures 5, 6). Quantification of the p110β levels showed a 20-30% increase within the KO samples, which did not correlate to the aforementioned increase in KO PI3K activity. The dramatic increase in the PI3K activity in comparison to the more moderate increase in the p110β levels within the FMRP knockouts directed us to investigate other possible proteins that control PI3K activity via FMRP and could induce an upregulation of the enzyme. This led us to our hypothesis that FMRP could regulate the translation and expression of different PI3K pathway mediating proteins. An unpublished study specified the PI3K enhancer (PIKE) protein as being a putative target

mRNA for FMRP (Jennifer Darnell, personal communication). PIKE is a recently discovered GTPase involved in activating PI3K via a complex formation with the adaptor protein Homer and mGluR1 (Rong *et al.,* 2003). It exists in two different isoforms, PIKE-L (long) and PIKE-S (short), referring to the protein sequence length. These are alternative splicing isoforms, with both forms expressing an N-terminal GTP-ase domain and PH (pleckstrin homology) domain, but with PIKE-L having a C-terminal extension that contains an additional Arf-GAP domain and two ankyrin repeat domains (Rong *et al.*, 2003). Apart from this insertion within the PIKE-L sequence, the mRNA sequences of PIKE-S and -L are identical. PIKE-L is known to localize to the cytoplasm of the neuron as well as to the nucleus, unlike PIKE-S, which is specific to the nucleus. Also, only PIKE-L associates in a complex with mGluR1 and Homer in the brain, making it a particularly interesting candidate which might be dysregulated in FXS, because it provides a link between gp1 mGluR- mediated signaling and PI3K activity.

We therefore hypothesized that FMRP may directly regulate the PI3K pathway through controlling PI3K activity at synapses (Figure 7). This regulation might be mediated in two non-mutually exclusive ways: by directly controlling protein levels of the PI3K catalytic subunit p110β, as suggested by our preliminary data (Figure 5), and by controlling upstream proteins that can regulate PI3K activity, such as the aforementioned PI3K enhancer, PIKE. To test our hypothesis that signaling through PI3K at synapses is indeed affected in *Fmr1* KO, we analyzed the dendritic and synaptic membrane levels of Akt, a protein kinase that is a downstream target of P13K and is localized to membranes via its PH domain by accumulation of phosphoinositoltriphosphate (PIP3), a product of PI3K activity. This was done *in vivo* in biochemically purified brain fractions and *in vitro*

within cultured hippocampal neurons. Furthermore, we used PIKE as an example to test our hypothesis that FMRP might regulate other PI3K-modulating proteins besides p110β. To validate PIKE mRNA as a potential FMRP target, we performed FMRP-specific co-immunoprecipitations and quantified the associated mRNAs. We also quantified the synaptic protein levels of PIKE by western blotting. We proposed that the results of these experiments would indicate whether PIKE is an FMRP target mRNA and may be dysregulated in FXS. Importantly, the analysis of PIKE as a potential FMRP target that is critical for the defects in protein-synthesis dependent synaptic plasticity observed in FXS was recognized as one of the outstanding questions in the field of local translation at the synapse in a very recent review article which is currently "in press" in *Trends in Neuroscience*, (Wang *et al*., 2010). This emphasis on the PIKE protein stresses the significance of my thesis project to analyze PIKE mRNA association with FMRP and to compare PIKE-L protein levels at wild type and *Fmr1* KO synapses.

The aim of this study was to elucidate the signaling pathway involved in FXS and to expand upon the possible role of FMRP in regulating several proteins that together control PI3K activity. This thesis provides evidence for our hypothesis that aberrant PI3K activity underlies neuronal deficits in FXS, and has important therapeutic implications. Currently, therapeutic strategies to treat FXS in patients are mainly targeted at gp1 mGluRs. However, my thesis research suggests that PI3K inhibitors, which are currently being used in clinical trials to treat cancer, might be a more specific and thus more potent target for FXS treatment.

Methods

Synaptoneurosome Preparation

Synaptoneurosome preparation was performed as described in a previous publication (Muddashetty *et al*., 2007). Wild type and *Fmr1* knockout mouse cortices (P16-18), provided by Christina Gross, PhD, were homogenized with a pestle (12x) in 2ml per mouse brain of synaptoneurosome (SNS) homogenization buffer (20mM Tris-HCl, 118mM NaCl, 4.7mM KCl, 1.2mM $MgSO_4$, 2.5 CaCl2, 1.53mM KH_2PO_4, 212.7mM glucose, and 1mM DTT, pH 7.4) containing protease inhibitors. The homogenate was filtered with a syringe through 100 μm and 11 μm filters, respectively. The synaptoneurosomal pellet was generated by centrifugation at 1000 g for 20 min at 4°C. The supernatant was discarded and the pellet was resuspended in membrane homogenization buffer (150 mM NaCl, 10 mM HEPES, 1 mM EGTA, and 0.1 mM MgCl2, pH 7.4) to form the SNS fraction. At this point, samples were taken for protein analysis.

Membrane Fractionation

The membranes were prepared from the SNS by homogenization with a syringe (25G, 3x), followed by centrifugation in a SW41Ti rotor at 100,000 *g* for 1 h at 4ºC (28500 rpm). The resultant pellet was solubilized in membrane homogenization buffer supplemented with 1% triton and 0.5% DOC (deoxycholate) to produce the synaptic membrane fraction.

Measurement of protein concentration

The Bradford protein assay was utilized to determine the protein concentration of the sample fractions. A standard concentration curve was formed using known concentrations of BSA in PBS. Each protein sample was diluted 1:100 μl in PBS, and the Bradford reagent was then added to all the samples. The absorbance at 595 nm was measured and quantified by spectrophotometry.

Western Blotting

Protein concentrations of the SNS and the synaptic membrane fractions were determined by the Bradford method. 15 μg of each protein sample was loaded and run on an 8% SDS polyacrylamide gel, and then transferred to PVDF membranes. The membranes were probed using specific antibodies for pan-Akt (rabbit monoclonal, Cell Signaling Technology), PIKE (generous gift from Keqiang Ye, Emory University), Kv1.2 (Antibodies Incorporated, NeuroMAB) and tubulin (Milipore) and subsequently incubated with peroxidase-labeled secondary antibodies. The bands were visualized by chemiluminescence detection of the substrate luminol through autoradiography. Densitometric analysis of the detected bands was performed by using ImageJ (NIH). As a control for the protein loading, tubulin, a housekeeping gene which is not changed in the absence of FMRP, was detected on the membranes to determine variances in the amount of protein in the lanes. Akt concentrations were determined in both the synaptoneurosomes and the synaptic membrane, and the ratio was taken between these levels. The PIKE concentration was determined within the synaptoneurosome fractions.

Immunocytochemistry of cultured hippocampal neurons

Cultured primary hippocampal neurons from WT and *Fmr1* KO embryos (E17, DIV17) were fixed in 4% PFA, and then incubated for an hour in a blocking buffer (2% BSA heat shock, 2% Fetal Bovine Serum, 0.1% Triton-X-100). The cells were then incubated for an hour with the pan-Akt antibody (1:100 dilution) and an antibody for the synaptic marker protein synapsin (1:500, mouse monoclonal, Millipore). Another set of hippocampal rat neurons (DIV17) were incubated with the PIKE antibody (1:100) to detect the PIKE within the dendrites. In order to visualize the signal, Cy2-coupled anti-rabbit and Cy3-coupled anti-mouse secondary antibodies were applied to the cells for Akt and synapsin, respectively. PIKE was visualized with a Cy2-coupled anti-rabbit secondary antibody. DAPI was then used to stain the nuclei. Images were acquired with a wide-field fluorescence microscope (Nikon TE2000) as Z stacks (21 stacks, 0.15 µm steps), and the stacks were deconvoled using AutoQuantX (Cybernetics). The fluorescent signal intensities in dendrites were measured and analyzed using Image J, with the region of interest being approximately 50 µm distant from the cell body. Akt levels were determined in both the cell body and the ROI, in which the ratio was taken between these levels in order to measure the shift of Akt localization.

Co-immunoprecipitation

40 µl of Protein A agarose beads in two separate Eppendorf tubes were washed in 1 ml of PBS three times, in which centrifugation at 1000 g was done in between the washes to collect the beads at the bottom of the tube, and the PBS was subsequently discarded. 20 µl of the 7g1 antibody and the mouse serum were each incubated with the beads in 200 µl PBS overnight. Wild type mice cortices were homogenized with a pestle (12x) in 1.5ml

per cortex of lysis buffer (50mM Tris, 150mM NaCl, 1% NP40, 5mM $MgCl_2$), with protease inhibitors and RNAse inhibitors. The cortical homogenate was centrifuged at max (21,000 g) for 20 min at 4°C. The supernatant was collected, in which a sample was taken to analyze for total mRNA concentration. The rest of the supernatant was divided by half and incubated with either the mouse serum or 7g1 antibody coupled beads for 2 hours. After the incubation, the beads were washed three times for 10 minutes each in the lysis buffer using the previously mentioned washing technique. The mRNA was extracted from the samples using Trizol® and Trizol® LS (Invitrogen), following the manufacturer's protocol. The associated mRNAs were then reverse transcribed into cDNA (SuperScript® Reverse Transcriptase III kit, Invitrogen, according to the manufaturer's protocol, using random hexamers as primers), and quantified by real time PCR using the SYBR Green Master mix on the Lightcycler® 480 (Roche Applied Sciences). 2 μl of the cDNA from the samples were used for the amplification and detection of each targeted mRNA, and every PCR was run in duplicates. We detected for mRNA of PIKE-L, PSD95, β-actin, and γ-actin, with PSD95 acting as the positive control (Muddashetty *et al*., 2007), and γ-actin as the negative control. The ratios between the mRNA levels in the mouse serum incubated sample and the 7g1 incubated sample were determined in order to detect a possible enrichment of the PIKE mRNA that interacts with FMRP.

Statistics

Two-tailed student's t-tests were performed on variables measuring protein levels in western blot analyses or immunostainings between WT and *Fmr1* KO mice. Significance

was defined at $p=0.05$ or less. An ANOVA was performed on the qrt-PCR quantification of targeted mRNAs, in which the post-hoc Dunnett's test was used to determine the significance of PIKE-L, PSD95, and β-actin mRNA levels against the negative control, γ-actin mRNA.

Results

To test the first part of our hypothesis that the PI3K pathway is upregulated in *Fmr1* KO mice, we determined protein levels of the PI3K downstream target Akt in (1) synaptic membranes and (2) dendrites of cultured neurons from *Fmr1* KO mice.

Increased Akt levels in the synaptic membranes from Fmr1 KO cortex

In order to detect for possible dysregulation within the PI3K pathway of *Fmr1* KO mice, we analyzed membrane levels of Akt, an important target molecule of PI3K. Akt is a major signaling molecule that has been shown to translocate to the membrane in response to the formation of PIP3, a transmembrane secondary messenger product of PI3K (Downward, 1997; Song *et al*., 2005). Therefore, western blotting for Akt was performed on equal amounts of protein of both total synaptoneurosome fractions as well as the membranes of these synaptoneurosomes within WT and *Fmr1* KO mice cortices. Synaptoneurosome fractions of cerebral cortex were purified from mice at postnatal days 16-18 by a filter method as described previously (Muddashetty *et al*., 2007). Membranes from these fractions were prepared using a protocol modified from McLendon *et al*. (2000). The bands which reflected the synaptic membrane levels of Akt were normalized to the bands of the synaptoneurosome levels as a quantitative control for natural variances of Akt levels in the samples. The signal was also normalized to tubulin as a control for the protein loading. The membrane protein Kv1.2 was increased within the membrane fractions, while barely discernable in the synaptoneurosome samples, indicating an enrichment of membranous fractions in the preparations (Figure 8).

Quantification of the resultant bands showed a twofold elevation of Akt levels in the synaptic membrane compared to the synaptoneurosomes within the *Fmr1* KO mice (Figure 9; n=3, p= 0.25, paired t-test). The results of the different experiments showed considerable variability, but increased Akt membrane levels in *Fmr1* KO could be detected in all three experiments (synaptic Akt levels in *Fmr1* KO/*Fmr1* WT: 3.28, 1.45, 1.31). This illustrates a possible upregulation of the PI3K pathway in response to the absence of FMRP, in which greater enzymatic activity due to the lack of translational control of PI3K catalytic and regulating subunits results in greater PIP3 formation and thus an increased translocation of Akt to the plasma membrane.

Increased Akt levels in the dendrites of Fmr1 KO mice

In order to further expand on the effects that the absence of FMRP may have on Akt, we compared Akt levels within the dendrites of WT and *Fmr1* KO cultured hippocampal neurons through immunocytochemistry. Akt was detected with a monoclonal rabbit Akt-specific antibody and a secondary Cy2-coupled rabbit antibody and visualized in green, and synapsin with a monoclonal mouse synapsin-specific antibody and a Cy3-coupled mouse antibody and visualized in red (Figure 10). Quantification of the fluorescent Akt-specific signal revealed an increase of Akt levels within the dendrites relative to Akt levels in the soma of the *Fmr1* KO neurons (Figure 11; n=12, p=0.002, paired t-test). The significance of the relative increase of Akt within the *Fmr1* KO dendrites results from the translocation of Akt in response to the activation of the PI3K signaling pathway. The proposed upregulation of the PI3K pathway will result in greater Akt recruitment to the synaptic membrane, as evidenced by the previous experiment. This increased recruitment may possibly be reflected in the increased

dendritic Akt levels, in which the shuttling of Akt from the soma to the synapses may be occurring.

We have so far provided strong evidence for our hypothesis of aberrant PI3K signaling in *Fmr1* KO mice. However, there remain questions concerning the exact mechanism of this altered signaling, of which FMRP clearly plays a significant role. Preliminary data established the relationship between the absence of FMRP, increased protein levels of PI3K and increased PI3K activity (Figure 4). The discrepancy between this exaggerated increase and the subtle increase observed in the protein levels of PI3K catalytic subunit, p110β, points us to the prospect of FMRP regulating the translation of other targeted mRNAs, which might modulate PI3K signaling. Various mRNAs have already been identified to be associated with FMRP (Brown *et al*., 2001), thus its function in controlling the expression of different proteins has already been established. PIKE-L is a particularly interesting protein where the translation of its mRNA may be regulated by FMRP, because it links mGluR1 activity with PI3K activation. PIKE-L is a facilitator of PI3K that associates with mGluR1 via binding to the scaffolding protein Homer. It initiates the recruitment of PI3K to synaptic membranes, thus activating its enzymatic function (Rong *et al*., 2003). Furthermore, PIKE was hypothesized to be a potential FMRP target of high significance for the pathobiology of FXS (Wang *et al*., 2010). To test the second part of our hypothesis and to analyze whether PI3K-modulating proteins might be regulated by FMRP, we therefore analyzed PIKE-L, as a potential FMRP target by (1) determining PIKE protein levels in synaptoneurosomes, and (2) examining whether PIKE mRNA is associated with FMRP in brain cortices.

Increased PIKE levels in Fmr1 KO synaptoneurosomes

Levels of the PIKE-L protein were determined by western blot under the same conditions as the Akt analysis. Due to the reported association of PIKE-L with mGluR1 and Homer, we decided to analyze PIKE-L levels in synaptoneurosome fractions to test our hypothesis that PIKE protein levels might be changed in *Fmr1* KO (Figure 12). Quantification of the western blots revealed that there was a significant enrichment of PIKE-L in the Fmr1 KO synaptoneurosomes in relation to WT (Figure 13; $n=5$, $p= 0.002$, paired t-test). This supports our hypothesis of an upregulated PI3K pathway in response to the lack of FMRP, in which the increase of the PI3K activator PIKE-L is indicative of greater PI3K activation.

PIKE-L observed in hippocampal rat dendrites

Immunocytochemistry with a PIKE specific antibody and a Cy-2 coupled rabbit secondary antibody was also performed on hippocampal rat neurons. Visualization of the immunostaining clearly detects PIKE as a prominent protein within the dendrites (Figure 14), consistent with the known cytoplasmic localization of PIKE-L.

Association of PIKE-L mRNA with FMRP

In order to detect an association between PIKE-L mRNA and FMRP, we co-immunoprecipitated FMRP using an anti-FMRP antibody, 7g1, and using mouse serum as control. Quantification of the immunoprecipitated mRNA by qRT-PCR with specific primers for PIKE-L showed an increase of the PIKE-L mRNA within the sample incubated with 7g1 compared to the mouse serum (Figure 15; $n=6$, $p=.05$, Dunnett's

posthoc test). The somatic γ-actin mRNA was chosen as a negative control and the PIKE-L enrichment was shown to be significantly higher than y-actin enrichment by a Dunnett's test. The PSD95 mRNA was used as a positive control, and the β-actin was another negative control, and while both values were not statistically significant, they were both consistent with their position as controls. Thus the increased level of coimmunoprecipitated PIKE-L mRNA in the 7g1 incubated sample suggests an association of PIKE-L mRNA and FMRP.

Overall, these results present a compelling argument for our hypothesis of an upregulated PI3K signaling pathway in the *Fmr1* KO mice, and propose a molecular mechanism for the role of FMRP as a negative regulator of this pathway. Increased PI3K signaling downstream of gp1 mGluRs might provide a mechanistic explanation for the mGluR theory. Furthermore, by showing the association of PIKE-L mRNA with FMRP, as well as increased PIKE-L protein levels in the absence of FMRP, we have demonstrated that FMRP may not only regulate the expression of the PI3K catalytic subunit p110β (as shown in Figure 5) but may also regulate the expression of other proteins modulating PI3K signaling.

<u>Discussion</u>

The goal of this study was to elucidate a possible molecular mechanism leading to dysregulated cell signal transduction in fragile x syndrome. Our results support the hypothesis of a dysregulated PI3K pathway in the absence of FMRP, shedding new light on a possible molecular mechanism by which the cellular pathology of fragile X is manifested. Furthermore, we expand on the proposed function of FMRP as a negative regulator of an mGluR1 activated pathway, by demonstrating an association between FMRP and the PIKE-L mRNA, and up-regulated PIKE-L protein in the absence of FMRP. This not only promotes the hypothesis of the PI3K pathway having a key role in fragile X, but more importantly uncovers FMRP as a potential protein that regulates multiple mRNA targets within a signaling complex and thereby can control the activity of a specific signaling pathway.

A central characteristic of FXS animal models is exaggerated signaling through group 1 metabotropic glutamate receptors (gp1 mGluRs), in which several protein synthesis-dependent phenotypes associated with gp1 mGluR activation have been observed to be dysregulated (Chuang *et al*., 2005; Huber *et al.*, 2002). Consequently, current therapeutic strategies to treat FXS in patients are targeted mainly at gp1 mGluRs. However, recent studies indicate that a variety of receptor-mediated signal transduction pathways are dysregulated in FXS (Wang *et al*., 2008; Volk *et al*., 2006), suggesting that FMRP acts on a common downstream signaling molecule. In neurons, two main pathways that are downstream of gp1 mGluRs and have been identified to drive the activation of protein synthesis are the PI3K/mTOR pathway and the PKC/ERK pathway

(Banko *et al*., 2006). Preliminary results from the Bassell lab have shown that whereas ERK signaling appears to be normal in the *Fmr1* KO, PI3K activity is highly upregulated. The PI3K/mTOR pathway is important for the regulation of protein synthesis during synaptic plasticity in neurons (Banko *et al*., 2006). Furthermore, it was shown that it is necessary for cell survival and the prevention of cell apoptosis (Marte and Downward, 1997; Kennedy *et al*., 1997). It has been implicated in the oncogenesis of various cancers (Vogt, 2001), and current research on cancer treatments has been targeting specific components of the PI3K pathway with promising results (Hennessy *et al*., 2005; Yuan and Cantley, 2008). Similarly, in antagonizing the PI3K pathway by focusing on specific regulatory proteins, we may be able to determine a more efficient and precise therapeutic approach for FXS.

To further analyze the signaling activity of the PI3K pathway in the absence of FMRP, this thesis first focused on a downstream target protein of PI3K, Akt, a prominent signaling molecule. Knowing that Akt translocates to the plasma membrane through the binding of the transmembrane phospholipid PIP3, a product that is formed when PI3K phosphorylates PIP2 in the initial stages of signal activation (Cantley, 2002), we focused our analysis of Akt levels to the synaptic membrane. Results of the western blots revealed an increase in the membrane levels of Akt in the *Fmr1* KO mice, and while the values were not statistically significant, each separate experiment reflected this increase and demonstrated a trend toward greater Akt recruitment in the *Fmr1* KO membrane. More trials would be needed in order to raise the statistical power of the data, but these initial results support our preliminary data of increased PI3K pathway signaling in response to the lack of FMRP. The immunocytochemical analysis of Akt levels revealed an increase

of Akt within the dendrites compared to the soma in the *Fmr1* KO neurons, indicating a possible shift of the Akt protein from the soma into the dendrites as Akt is being recruited to the synapse in response to greater PIP3 production.

Overall, these results support the idea of FMRP acting as an inhibitor of PI3K signaling at the synapse. The actual means of this inhibition is not well known, but recent data from the Bassell lab linked increased PI3K enzymatic activity in *Fmr1* KO mice with greater p110β protein levels at synapses, thus pointing to FMRP as a potential direct regulator of the PI3K catalytic subunit. The magnitude of the increase observed in the enzyme activity assay of the *Fmr1* KO SNS was not parallel to the increase seen in the levels of p110β, in which the PI3K demonstrated much higher activity (3.5-fold) in comparison to the increase in p110β (~20-30%) in the absence of FMRP. This indicated that FMRP may control or regulate the translation of other target mRNAs that are involved in the PI3K pathway. Possible targets include the PI3K regulatory subunit p85, which has been suggested to be an FMRP target (Brown *et al*., 2001), as well as PTEN, a negative regulator of the mTOR pathway which has been shown to have decreased activity in *Fmr1* KO mice (Sharma *et al*., 2010).

This thesis focused on another potential target of FMRP, the PI3-kinase enhancer protein, or PIKE. PIKE is a recently discovered GTPase protein that augments PI3K activity through the GTP binding activity of both p85 and p110β, the regulatory and catalytic subunit of PI3K (Chan and Ye, 2007). It exists in two isoforms, both of which are derived from the same gene and are formed by alternative splicing. Both are found prominently in the brain, but the short splice variant, PIKE-S, is localized to the nucleus

(Ye *et al*., 2000) while PIKE-L is detected within the nucleus as well as within the cytoplasm (Rong *et al*., 2003). This difference in the localization could be attributed to the variation in the PH domains, through which PIKE-L binds preferentially to PIP3, while PIKE-S, whose PH domain is composed only of the first 75 amino acids of the PIKE-L HP domain, does not bind to any phosphoinositol (Hu *et al.*, 2005).

PIKE-L was implicated in facilitating the activation of PI3K via mGluR1 by interacting with the adaptor protein Homer, which itself has been shown to associate with mGluR1 at the postsynaptic density to mediate signaling (Xiao *et al*., 1998). The formation of an mGluR-Homer 1c-PIKE-L-PI3K complex was found to be necessary in inducing mGluR-LTD in WT mice. However, exaggerated mGluR-LTD at the synapse is a known phenotype in *Fmr1* KO mice, and yet there was a decrease in the formation of this complex in KO (Ronesi and Huber, 2008). This points to an uncoupling of mGluR stimulation and PI3K activity, making PIKE-L an interesting protein for us to analyze. A possible dysregulation of PIKE-L in FMRP could explain the excessive upregulation (3.5-fold) of PI3K activity in FXS mice in the presence of normal mGluR function.

PIKE has been suggested as a putative target of FMRP (Jennifer Darnell, personal communication; Sharma *et al*., 2010), and the question of whether PIKE really is an FMRP target is of outstanding interest to the field of FXS research (Wang *et al*., 2010). In co-immunoprecipitation experiments, we observed that the PIKE-L mRNA is associated with FMRP, which supports the theory of FMRP having an immediate role in regulating PIKE-L expression, and advances the hypothesis of FMRP serving as a regulator of multiple proteins that contribute to the PI3K signaling cascade. This data is

supported by the increased levels of PIKE-L protein that we saw in *Fmr1* KO synaptoneurosomes, in which the lack of FMRP would result in the excessive translation and increased expression of PIKE-L at the synapse. Interestingly, a recent paper also investigating dysregulated signaling in FXS identified PIKE as a potential target of FMRP as well, but reported increased expression of PIKE-S in *Fmr1* KO mice, while PIKE-L levels were reported unchanged (Sharma *et al*., 2010), contrary to our findings.

Several neurotransmitter-induced forms of synaptic plasticity are dysregulated in FXS animal models, especially those mediated by gp1 mGluRs. Furthermore, FMRP has been suggested to regulate translation in synaptic compartments. A potential target of FMRP that functions at the synapse and regulates membrane receptor-mediated activation of intracellular signaling, as represented by PIKE-L, therefore seems to be a more biologically significant potential FMRP-target, than the exclusively nuclear PIKE-S. Our data reflects the FMRP-associated mRNA of the long isoform only, given that the position of the primers in the sequence falls within the PIKE-L splice (Figure 16). Future studies can focus on differentiating between these two PIKE isoforms. As the qRT primers used in our co-immunoprecipitation assays specifically recognize PIKE-L, but not PIKE-S, we can draw the conclusion that FMRP is certainly associated with PIKE-L mRNA. As the entire PIKE-S mRNA sequence is also present in PIKE-L, specifically addressing whether PIKE-S mRNA is also an FMRP target is more difficult and can be done only in indirect assays. A potential experiment might be repeating the co-immunoprecipitation using two different sets of primers, one specific for PIKE-L (primers within the PIKE-L splice sequence) and one specific for both PIKE-L and PIKE-S. From the FMRP-associated mRNA value quantified using the primers specific

for both PIKE isoforms, you can subtract out the mRNA value detected from using the PIKE-L specific primers and see if there is a significant difference (= PIKE-S mRNA). If no difference is detected, this would support the hypothesis that only PIKE-L mRNA binds to FMRP. As qRT-PCR is a quantitative method to measure mRNA levels, this experiment will be challenging, but should be feasible. Furthermore, in vitro pulldowns with overexpressed FMRP and specific mRNA reporters in secondary cell lines which do not have endogenous PIKE could be performed. mRNA sequences to be tested could for example just include the spliced "L" sequence, and possibly the common 3'UTR as a negative control. If association with FMRP could only be detected for the PIKE-L specific sequence, but not for the sequence present in both isoforms (e.g. the 3'UTR, which is an mRNA region commonly bound by FMRP), this would indicate that just PIKE-L, but not PIKE-S is an FMRP target.

The thesis presented here have expanded upon the mGluR theory by identifying the PI3K cell signaling pathway as a possible mechanism for FXS, in which aberrant signaling ultimately results in producing the many neuronal phenotypes associated with FXS. FMRP's role in inhibiting this pathway has also been expanded upon by the demonstration of an association between FMRP and PIKE-L mRNA. These findings highlight FMRP as a multi-complex entity that interacts with many diverse proteins in order to properly regulate translation and protein synthesis. We have yet to discover the full extent of how this regulation is done, and if it indeed is only specific to mGluR pathways. The attempted genetic rescue of major FXS phenotypes performed by Bear and colleagues could reverse many, but not all of the phenotypes seen in the *Fmr1* KO mouse model through reduced genetic expression of mGluR5 (Dolen *et al.*, 2008), and

other groups have had difficulty reproducing these results (personal communication, Richard Paylor and colleagues). This indicates that mGluR is not the only signaling affected, and it provides an argument favoring a downstream signaling pathway that is dysregulated in FXS, which may be downstream of different membrane receptors (Volk, *et al*., 2006).

Gaining a deeper understanding of these affected pathways is necessary in order to provide better therapeutic options for those suffering from FXS. FXS encompasses a wide range of symptoms and conditions and it is the most common genetic cause of autism. Interestingly, autism has been linked with the dysregulation of the PI3K pathway (Serajee *et al*., 2003), hinting at a possible connection between the molecular pathway involved both fragile X and autism.

Future Goals

In order to validate and further test our hypothesis, additional experiments can be performed. A colocalization analysis with synapsin and Akt or PIKE immunostainings can be done in WT and *Fmr1* KO neurons to quantify the localization of Akt and PIKE at the synapse. We would predict increased localization of Akt and PIKE at synapses. Immunohistochemical analysis of these proteins can be done to determine the localization of these proteins in vivo using WT and *Fmr1* KO mice neuronal sections, where we might observe increased PIKE levels in dendrites. In situ hybridization will allow us to observe the localization of the PIKE mRNA in vivo as well, which we predict may be present in dendrites, yet translated in excess in the absence of FMRP. Finally, other regulatory mRNAs such as PTEN and p85 should be identified and pursued as a possible

target of FMRP. Although we suspect that FMRP may regulate a number of mRNA molecules in the PI3K pathway, we envision that further work will show that PIKE is a critical component to the dysregulated phenotype of fragile x syndrome. This thesis could lead, for example, to efforts to rescue FXS phenotypes by reduction of PIKE expression or activity in Fmr1 KO mice.

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<u>Figures</u>

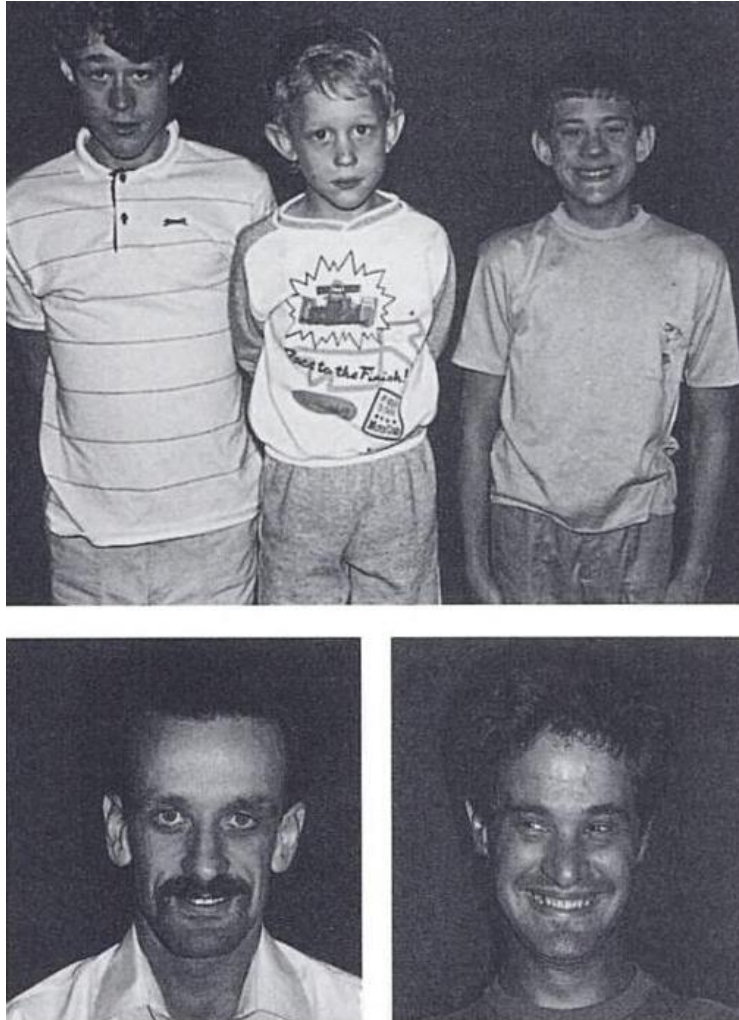

Figure 1: Males with fragile X syndrome display typical physical phenotypes such as prominent forehead, prominent ears, and/or an elongated face. *Fragile X syndrome: diagnosis, treatment, and research,* 3rd ed., Hagerman and Hagerman.

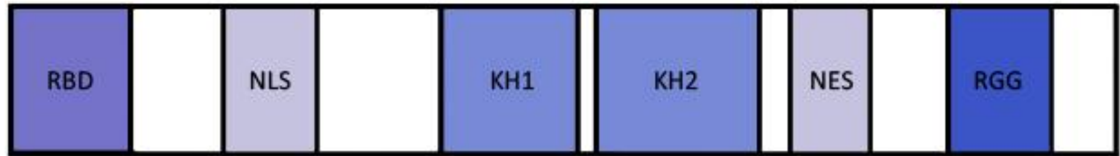

Figure 2: The FMRP domain structure: RBD = RNA Binding domain. NLS = Nuclear Localization Sequence. KH = K Homology domain (RNA-binding motif). NES = Nuclear Export Sequence. RGG = RGG Box (RNA-binding motif).

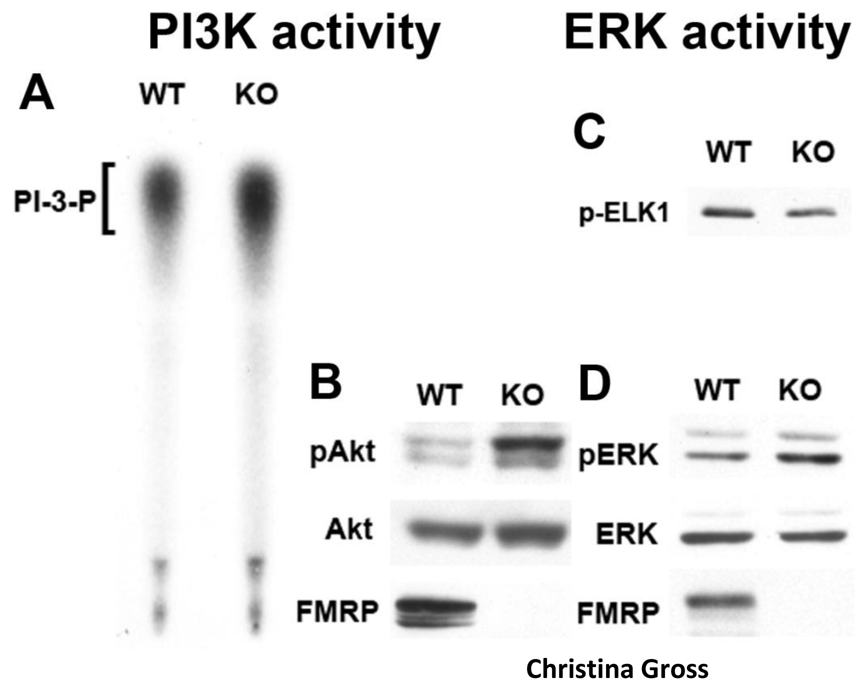

Figure 3: **(A)** This autoradiography of a PI3K activity assay shows that the catalytic PI3K subunit p110β immunoprecipitated from *Fmr1* KO synaptoneurosomes produces more PI-3P when incubated with phosphoinositide compared to WT, indicating higher enzymatic activity. (**B)** Western blot analyses using phospho-Akt and Akt antibodies show that phosphorylation levels of Akt are markedly increased in KO synaptoneurosomes. **(C)** An ERK1/2 activity assay shows that phospho-ERK1/2 immunoprecipitated from the same samples as shown in (A) does not have increased efficiency to phosphorylate recombinant ELK1, a major substrate of ERK1/2. **(D)** In addition, phosphorylation levels of ERK1/2 are not increased in the absence of FMRP. FMRP-specific western blots are shown to confirm the genotype. These results strongly corroborate the hypothesis that PI3K signaling, but not ERK1/2 signaling is increased in *Fmr1* KO.

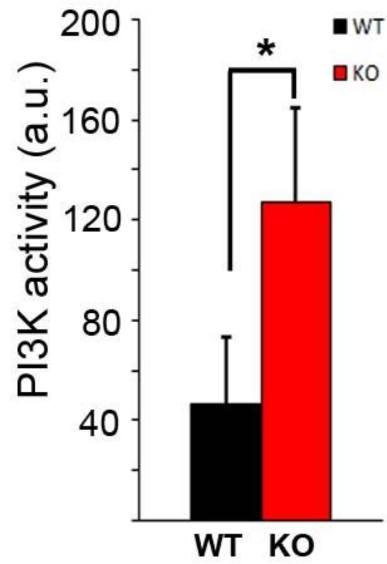

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Figure 4: Quantification of the PI3K activity assay (Fig. 3A) reveals an approximate 3-fold increase of PI3K activity in the *Fmr1* KO SNS (n=8, *p=0.035; paired t-test).

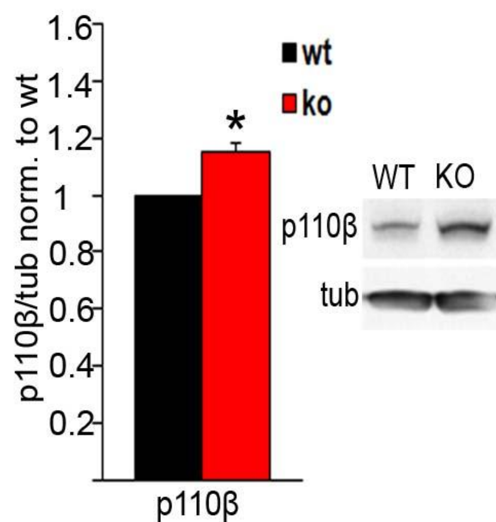

Figure 5: Western blots show an increase of p110β protein levels in *Fmr1* KO SNS. Quantification reveals this to be a 20% elevation in the KO SNS relative to WT (n=6, *p=0.018; paired t-test).

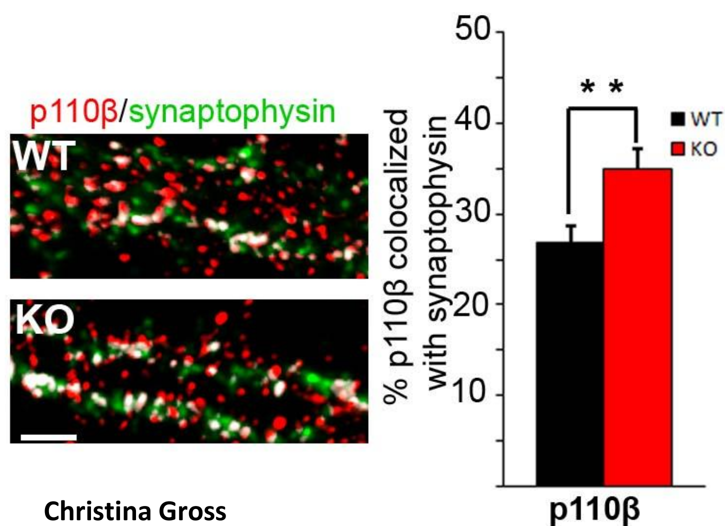

Figure 6: Coimmunostainings of p110β (red) with synapsin (green) show regions of colocalization (white). Quantification reveals a 30% increase of p110β that is localized to the synapse in *Fmr1* KO dendrites (n=43 dendrites each for WT and KO, 3 independent hippocampal cultures, **p=0.008; independent t-test).

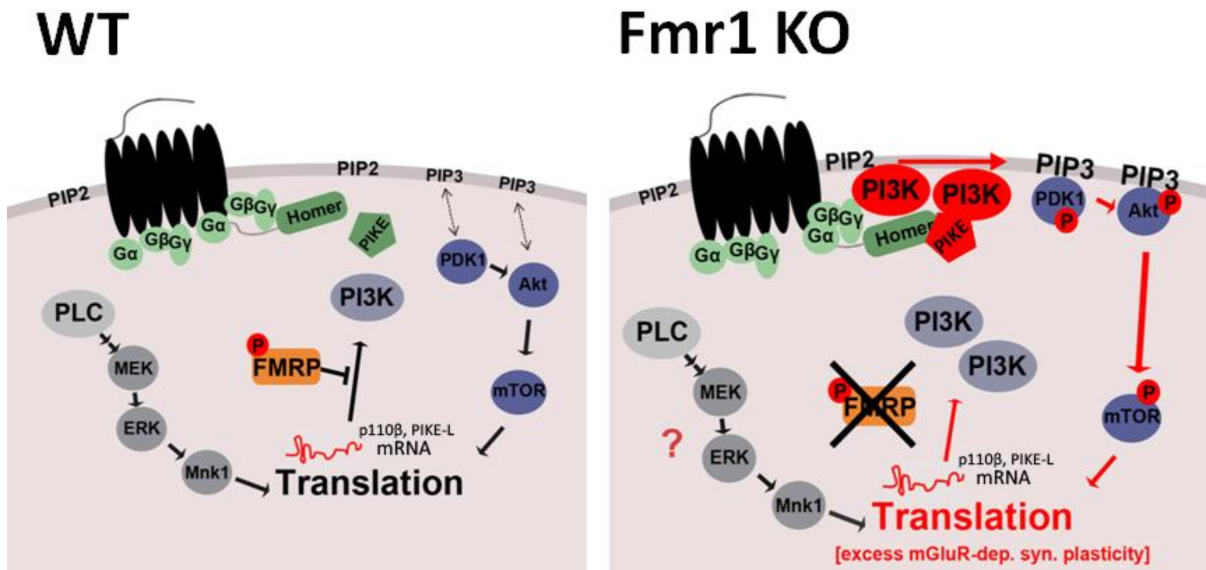

Figure 7: Proposed model of PI3K regulation by FMRP within WT and KO synapses. In the WT model, FMRP is present to actively inhibit the translation of specific target mRNAs such as p110β and PIKE-L, resulting in normal levels of PI3K activation. In the *Fmr1* KO model, the absence of FMRP induces increased translation of the aforementioned mRNAs, resulting in exaggerated downstream signaling of thePI3K pathway.

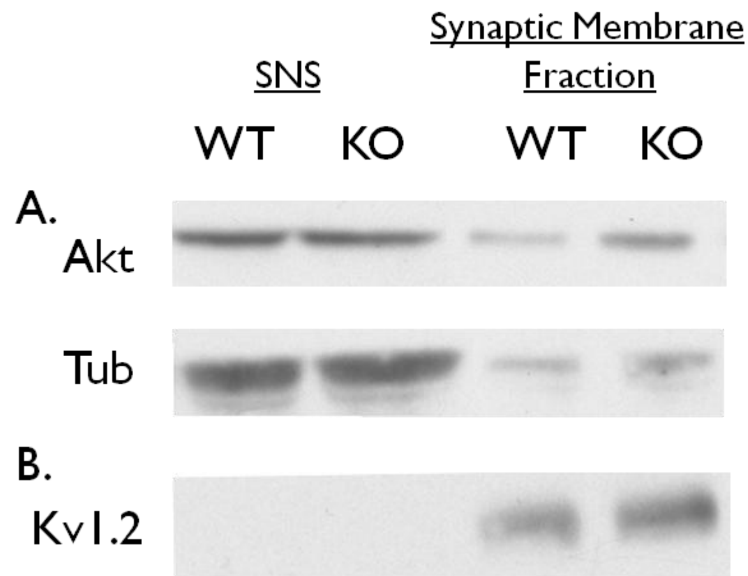

Figure 8: **(A)** Western blots of WT and *Fmr1* KO cortical SNS and synaptic membrane fractions using a rabbit monoclonal pan Akt antibody (Cell Signaling Technology) and a mouse monoclonal Tubulin antibody (Sigma) as loading control suggest enrichment of Akt in synaptic membranes from *Fmr1* KO mice . The same amount of protein (15 g) was loaded for each lane. (**B)** Western blot analysis with an antibody specific to the membrane protein Kv1.2 demonstrates high levels of Kv1.2 within both WT and *Fmr1* KO synaptic membrane fractions, whereas Kv1.2 is hardly detectable in the synaptoneurosomal fractions, indicating enrichment of membranous fractions in the preparations.

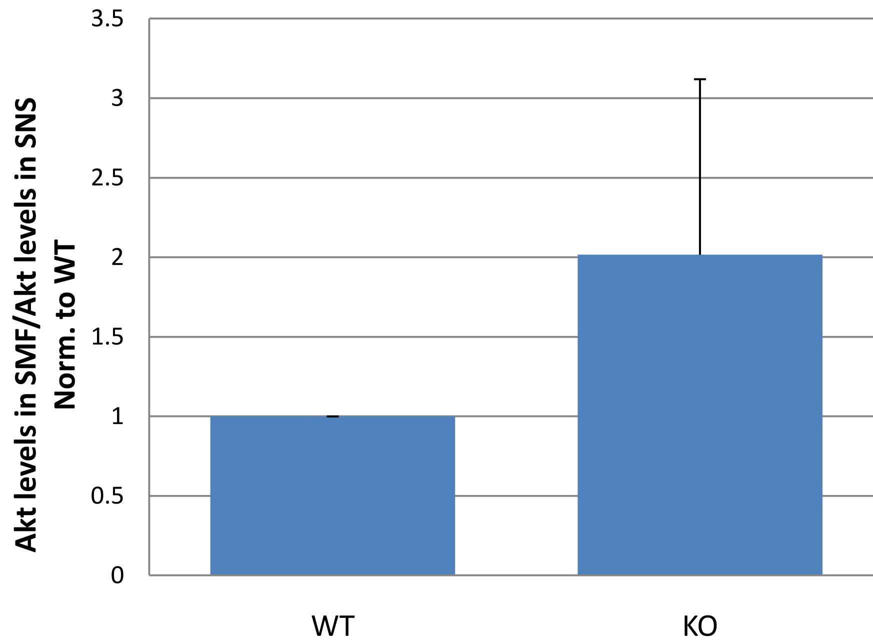

Figure 9: Densitometric quantification of western blots shows an increased ratio of Akt levels in the synaptic membrane fraction relative to the Akt levels in synaptoneurosomes from *Fmr1 KO* cortices (n=3, student's t-test p-value of 0.25; error bar represents standard deviation). The optical densities of Akt-specific signals were quantified using Image J software (*NIH*) and normalized to tubulin-specific signal on the same western blot.

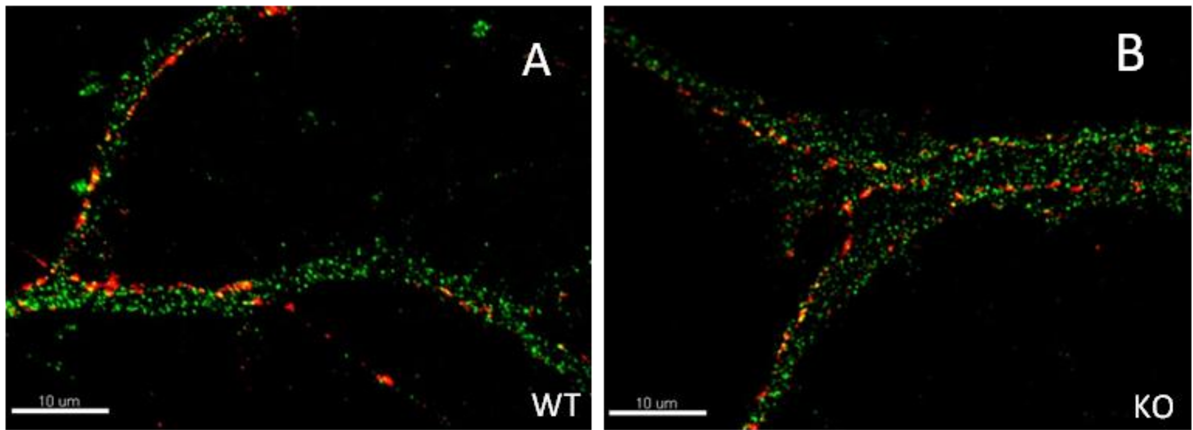

Figure 10: Fluorescent coimmunostaining of **(A)** WT and **(B)** *Fmr1* KO cultured hippocampal mouse neurons using Akt- and Synapsin-specific antibodies. The signal for Akt is visualized in green using a Cy2-coupled anti-rabbit antibody, and synapsin in red using a Cy3-coupled anti-mouse antibody.

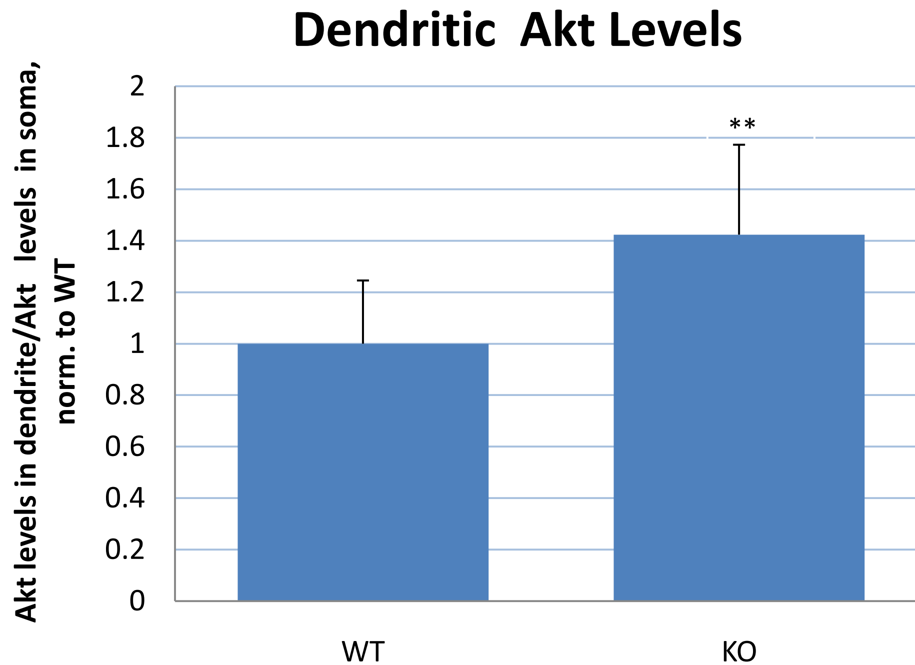

Figure 11: Quantitative analysis of Akt immunostainings revealed an increase of Akt levels within dendrites relative to Akt levels in the soma of the *Fmr1* KO neurons. (n=12, ** = student's t-test p-value of 0.002; error bars represent standard deviations). Images were taken as z-stacks using a Nikon TE2000 inverted microscope and captured with a cooled CCD camera. Prior to quantification with Image J software, images were deconvolved using AutoquantX (*Cybernetics*). Fluorescent intensity was measured in a dendritic segment (>50µm apart from the cell body) within 5 consecutive sections of the stack. Fluorescent intensity in the cell body of the same cell was determined the same way and dendritic signal was normalized to somatic.

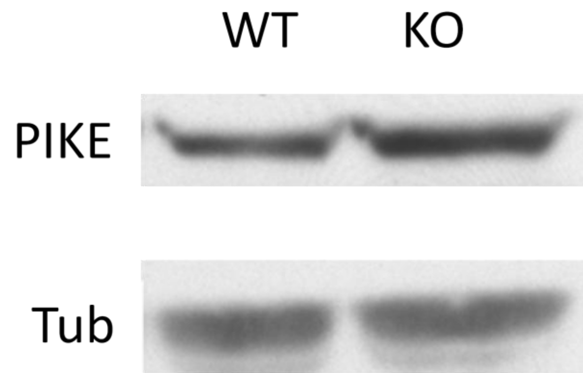

Figure 12: Western blots of WT and *Fmr1* KO mice synaptoneurosomes (SNS) using a rabbit monoclonal PIKE antibody (Keqiang Ye, Emory University) and tubulin antibody show an increase of PIKE-L in the absence of FMRP.

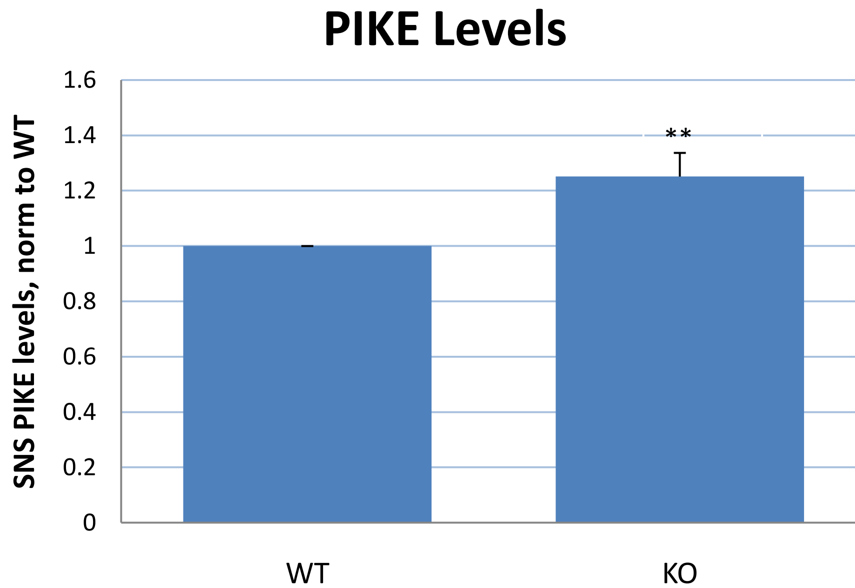

Figure 13: Densitometric quantification of western blots shows increased PIKE levels within the *Fmr1* KO synaptoneurosomes relative to WT (n=5, ** = student's t-test p-value of 0.002; error bar represents standard deviation). PIKE-L-specific signals were normalized to tubulin-specific signal on the same western blot.

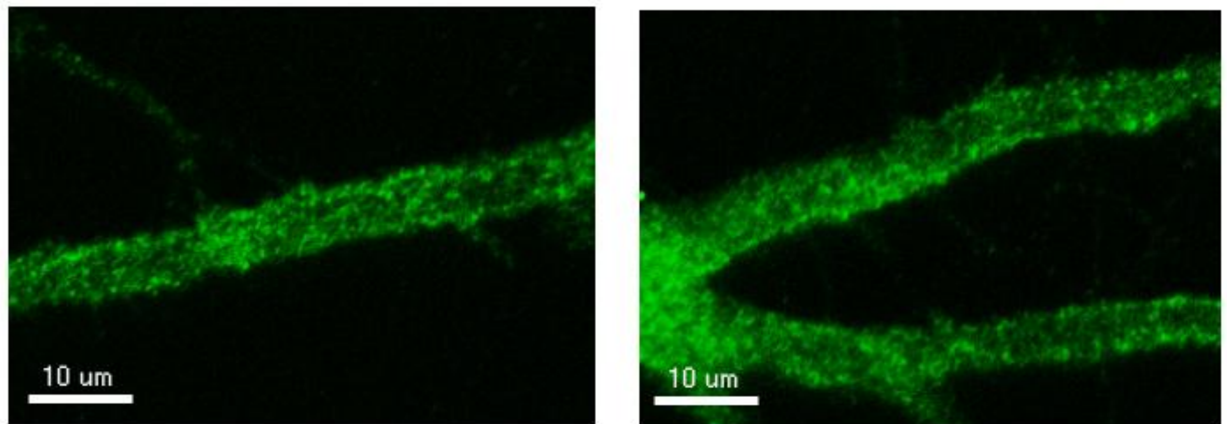

Figure 14: Immunofluroescent staining of rat hippocampal neurons (DIV17) using PIKE antibody and Cy2-coupled rabbit secondary antibody demonstrates punctuate staining of PIKE in dendrites. Two examples of dendrites are shown.

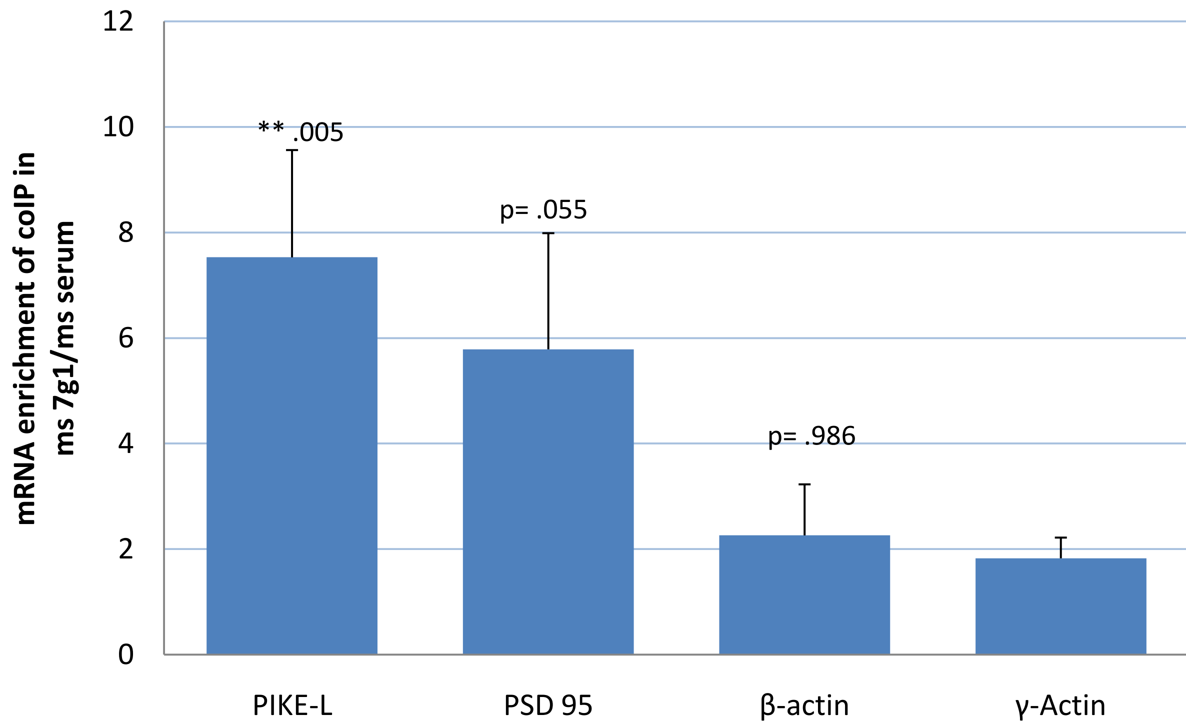

Figure 15: Quantification of mRNA shows increased levels of PIKE-L mRNA immunoprecipitated with FMRP compared to serum (γ-Actin is defined as the negative control, 1-way ANOVA with Dunnet's posthoc analysis, p-values against γ-Actin, n=6, error bars = SEM). The mRNAs were co-immunoprecipitated with FMRP using Protein A agarose beads coupled an anti-FMRP antibody, 7g1, and mouse serum. Specific qRT-PCR was used to quantify the FMRP associated mRNA levels.

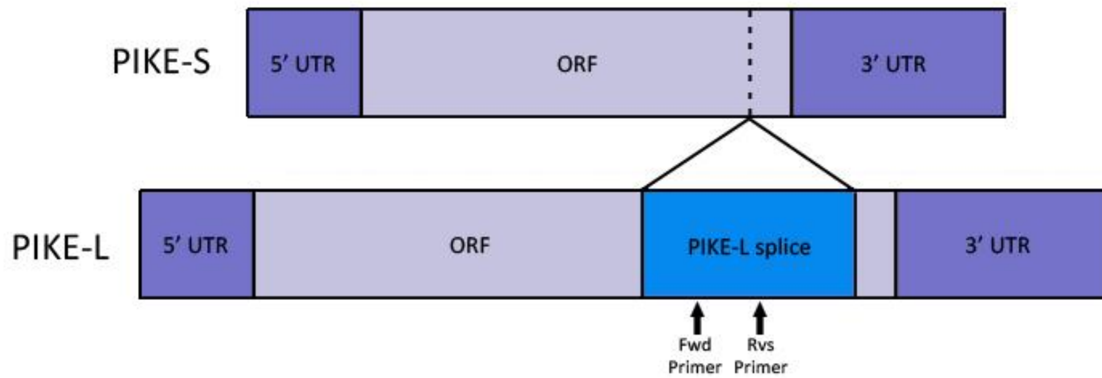

Figure 16: Schematic for DNA sequence of PIKE-L and PIKE-S. ORF= Open Reading Frame; UTR=untranslated region. The PIKE-L sequence is identical with PIKE-S, but has an additional fragment in the open reading frame (bp 2737-4033), which results from alternative splicing. The primers used for the quantitative real-time PCR in this experiment were located in this spliced fragment, thus resulting in the specific detection of PIKE-L mRNA.