Distribution Agreement

In presenting this thesis as a partial fulfillment of the requirements for a degree from Emory University, I hereby grant to Emory University and its agents the non-exclusive license to archive, make accessible, and display my thesis in whole or in part in all forms of media, now or hereafter know, including display on the World Wide Web. I understand that I may select some access restrictions as part of the online submission of this thesis. I retain all ownership rights to the copyright of the thesis. I also retain the right to use in future works (such as articles or books) all or part of this thesis.

Nourine Ahmed April 20, 2011

Identification of Aberrantly Expressed Synaptic Proteins in Mouse Model of Fragile X Syndrome

by

Nourine Ahmed

Stephen T. Warren, PhD.
Adviser

Department of Neuroscience and Behavioral Biology

Stephen T. Warren, PhD.

Adviser

Michael Crutcher, PhD.

Committee Member

Kristen Frenzel, PhD.

Committee Member

April 20, 2011

Identification of Aberrantly Expressed Synaptic Proteins in the Mouse Model of Fragile X Syndrome

By

Nourine Ahmed

Stephen T. Warren, PhD.

Adviser

An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
of the requirements of the degree of
Bachelor of Sciences with Honors

Department of Neuroscience and Behavioral Biology

2011

Abstract

Identification of Aberrantly Expressed Synaptic Proteins in the Mouse Model of Fragile X Syndrome

By Nourine Ahmed

Fragile X syndrome (FXS) is the most commoly inherited form of intellectual disability. A mutation in the FMR1 gene of the X chromosome results in the loss of fragile X mental retardation protein (FMRP), an RNA-binding protein. The mechanism by which disability results, however, is unclear. Mouse Models of FXS exhibit altered synaptic plasticity that contributes to enhanced long-term depression (LTD) induced by mGluR activation inthw hippocampus. This mGluR-dependent LTD (mGluR-LTD) requires new protein synthesis to be induced and maintained. The mGluR theory of FXS proposes that FMRP is a regulatory agent that suppresses protein synthesis at the synapse. Activation of mGluR in the synapse initiates local protein synthesis, the products of which induce LTD via endocytosis of AMPA receptors. Consequently, in the pathological state, the loss of translation inhibition from FMRP results in increased synthesis of LTD-inducing proteins and enhanced LTD. However, the LTD-inducing proteins that are regulated by FMRP are not yet identified. Using high throughput proteomic analysis and Western blotting on synaptoneurosome (SNS) preparation of hippocampi, our study identified 3 synaptic proteins differentially expressed between wild-type (WT) and Fmr1 knockout (KO) mice: Eukaryotic elongation factor 1a (eEF1A), Neuronal axonal protein 22 (NAP22) and cyclin G associated kinase (GAK), suggesting these are regulated by FMRP and may contribute to the FXS pathophysiology.

Identification of Aberrantly Expressed Synaptic Proteins in the Mouse Model of Fragile X Syndrome

By

Nourine Ahmed

Stephen T. Warren, PhD.

Adviser

A thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
of the requirements of the degree of
Bachelor of Sciences with Honors

Department of Neuroscience and Behavioral Biology

2011

Table of Contents

Abstract

Fragile X syndrome (FXS) is the most commonly inherited form of intellectual disability. A mutation in the *FMR1* gene of the X chromosome results in the loss of fragile X mental retardation protein (FMRP), an RNA-binding protein. The mechanism by which disability results, however, is unclear. Mouse models of FXS exhibit altered synaptic plasticity that contributes to enhanced long-term depression (LTD) induced by metabotropic glutamate receptor (mGluR) activation in the hippocampus. This mGluR dependent LTD (mGluR-LTD) requires new protein synthesis to be induced and sustained. The mGluR theory of FXS proposes that FMRP is a regulatory agent that suppresses protein synthesis at the synapse. Activation of mGluR in the synapse initiates local protein synthesis, the products of which induce LTD via endocytosis of AMPA receptors. Consequently, in the pathological state, the loss of translation inhibition from FMRP results in increased synthesis of LTD-inducing proteins and enhanced LTD. However, the LTD-inducing proteins that are regulated by FMRP are not yet identified. Using high throughput proteomic analysis and Western blotting on synaptoneurosome (SNS) preparation of hippocampi, our study identified 3 synaptic proteins differentially expressed between wild-type (WT) and Fmr1 knockout (KO) mice: Eukaryotic elongation factor 1A (eEF1A), Neuronal axonal protein 22 (NAP22) and Cyclin G associated kinase (GAK), suggesting that these are regulated by FMRP and may contribute to the FXS pathophysiology.

Introduction

Fragile X Syndrome

Fragile X syndrome (FXS) is the most commonly inherited form of intellectual disability and one of the few identified causes for autism (Bassell and Warren, 2008). Most patients carry a trinucleotide repeat expansion mutation in the *FMR1* gene of the X chromosome, which results in the loss of fragile X mental retardation protein (FMRP) (Pieretti et al., 1991; Verkerk et al., 1991). FMRP is an RNA-binding protein found ubiquitously throughout tissues and within cells, including in dendritic spines, where it regulates protein synthesis (Ashley et al., 1993; Hinds et al., 1993). Patients lacking this protein exhibit developmental delay and intellectual disability (Boyle and Kaufmann, 2010). How the loss of FMRP contributes to the FXS phenotype, however, is not well understood.

FMRP Regulates Synaptic Plasticity

Previous studies have established FMRP as a critical agent in modulating synaptic plasticity. Activation of group I metabotropic glutamate receptors (mGluRs) is known to induce LTD. A selective agonist of group I mGluR, (RS)-3,5-dihydroxyphenlglycine (DHPG), induces α-amino-3-hydroxy-5-mehtyl-4-isoxazole propionic acid (AMPA) receptor endocytosis and Long-Term Depression (LTD) in hippocampal Schaffer collateral synapses of CA1 area (Oliet et al., 1997). In *Fmr1* KO mouse neurons both the AMPA receptor endocytosis and LTD are exaggerated, demonstrating aberrant synaptic plasticity in the region (Huber et al., 2002). Furthermore, FXS patients and *Fmr1* knockout (KO) mouse models lacking FMRP exhibit immature dendritic spines, suggesting impaired synaptic transmission (Coomery et al., 1997; Irwin et al., 2000; Grossman et al., 2006). Given the critical role of the hippocampus and

synaptic plasticity in learning and memory, FMRP deficit in dendritic spines is believed to underlie the intellectual disability phenotype observed in FXS.

mGluR Theory of FXS

The molecular mechanism by which FMRP absence leads to cognitive impairment is unclear. The mGluR theory of FXS, a widely-supported model of FXS pathophysiology, is based on the observation that group I mGluR activation initiates local protein synthesis, the products of which induce LTD (mGluR-dependent LTD) via endocytosis of AMPA receptors (AMPARs) (Bear et al., 2004). This theory proposes that FMRP acts as a regulatory factor on the translation machinery, suppressing synthesis of LTD-inducing proteins at the synapse by binding specific mRNA ligands, except when the synapse fires (Figure 1a). Consequently, in the pathological state of FXS, the loss of translation inhibition from FMRP results in a constitutive increase in protein synthesis of certain proteins and enhanced LTD (Figure 1b).

The mGluR theory of FXS has been supported by electrophysiological studies that show enhanced mGluR-LTD (Huber et al., 2002), immunocytochemical studies that demonstrate enhanced AMPA receptor endocytosis and mGluR-antagonist treatment that rescues AMPA receptor endocytosis in *Fmr1* KO mice (Nakamoto et al., 2007). The signaling cascade that is initiated by mGluR-I activation has also been studied in this system (Bassell and Warren, 2008; Gross et al., 2010).

LTD-inducing proteins regulated by FMRP are unidentified

Though much progress has been made in unveiling the molecular mechanism of FXS, the LTD-inducing synaptic proteins products that are regulated by FMRP are not yet identified.

Identifying these proteins and their functions is critical for a better understanding of both the FXS pathophysiology and the molecular mechanisms underlying LTD, a phenomenon critical in learning and memory. It should be noted that though LTD is a widely studied phenomena and aberrant AMPA-R composition has been associated with multiple cognitive impairment disorders such as Alzheimer's Disease, Schizophrenia and FXS (Luscher and Huber, 2010), the proteins involved in the LTD-inducing pathway are unknown.

Our study aims to identify the proteins that are regulated by FMRP and thus induce mGluR mediated LTD. We used high throughput proteomic analysis on synaptoneurosome (SNS) preparation of hippocampi to identify synaptic proteins differentially expressed between wild-type (WT) and *Fmr1*KO mice. We expect that synaptic proteins regulated by FMRP will be overexpressed in the absence of FMRP in the KO state as compared to WT. Then using Western blotting, we verified the results of proteomics, and identified synaptic proteins that are both constitutively overexpressed in *Fmr1* KO mice and upregulated in WT SNS samples treated with DHPG. Since DHPG is an agonist of group I mGluRs and mGluR activation leads to LTD, we expect proteins that are differentially expressed between WT and WT+DHPG samples to be involved in the mGluR mediated LTD pathway.

Animals

All procedures were performed in accordance with the Institutional Animal Care and Use Committee (IACUC) guidelines. Wild type (WT) C57BL6 mice and their *Fmr1* knockout (KO) littermates were used in each experiment. Mice were sacrificed at 8 weeks of age with cervical dislocation, and hippocampi were collected and pooled by genotype (WT vs. KO) after dissection. Only male mice were used to maintain gender-match control.

SNS samples for mass spectrometry (MS) were prepared from 2 WT and 2 KO littermates. A duplicate set of samples was prepared for MS using 2 WT and 2 KO littermates from a second set of parents.

SNS samples were prepared from 8 WT and 8 KO mice for verification experiments using Western blotting.

Frozen cortical tissue of Stable Isotope Labeling with Amino acids in Mouse (SILAM) (Krueger et al., 2008) was obtained from the Junmin Peng Lab of Emory University Department of Human Genetics. Heavy isotope-labeled SILAM tissue was combined with freshly dissected hippocampal samples at 1:1 ratio by mass and used as internal control for MS analysis.

Synaptoneurosome (SNS) Sample Preparation

Synaptoneurosomes (SNS) from WT and Fmr1 knockout samples were prepared separately. Tissue samples were homogenized in homogenization buffer [containing 118 mM NaCl, 4.7mMKCl, 1.2 mM MgSO4, 2.5 mM CaCl2, 1.52mM KH2PO4, 21.7 mM glucose, and 1mM DTT, pH 7.4], supplemented with Complete protease inhibitors (Roche), 30 U/ml RNase-Out RNase inhibitor (Invitrogen)]. The homogenate was then filtered twice through three 100

μm nylon mesh filters, followed by once through 10 μm MLCWP 047 Millipore (Bedford, MA) filter, then centrifuged at 1000x g in 4ºC. The pellet was subsequently resuspended in the homogenization buffer.

DHPG Stimulation

A subset of SNS samples obtained from WT mice were incubated in 37°C waterbath for 15 minutes. Half of the samples were then incubated with 1 mM DHPG and the other half with equal volume of water in 37°C water bath for another 15 minutes. All samples were then frozen and stored in -80°C.

Electron Microscopy (EM)

SNS samples were fixed with 2.5% glutaraldehyde in 100 mM cacodylic acid, pH 7.4, pelleted at 48,000 × g for 30 min in fixative, secondary-fixed in osmium tetroxide, dehydrated, embedded in resin, and processed for electron microscopy. Examination was done using JEOL (Peabody, MA) 1200 EX TEM at the Robert P. Apkarian Integrated Electron Microscopy Core at Emory University.

Coomassie Staining

Gel electrophoresis was performed using SNS samples and BSA standards. Samples were allowed to run until ~1cm below loading well. Gel was stained with GelCode Blue (Thermo Scientific, Pierce Protein Research Products), and relative concentrations of SNS samples to BSA standards were determined using Image J software.

Mass Spectrometry (MS)

SNS samples were incubated with SDS buffer [containing 20 mM Tris pH 8.0, 20 mM DTT, and 4% SDS) and were treated with iodoacetamide [50 mM] to separate and protect peptide strands, then submitted to Emory Proteomics Core Service Center. Heavy isotope-labeled tissue was combined with hippocampal samples at 1:1 ratio by mass and used as internal control for mass spectrometry analysis (MS).

Western blotting (WB)

Equal amounts of proteins were resolved on 7-15% polyacrylamide gels, transferred to PVDF membrane (Amersham), and probed with antibodies against PSD-95 (mouse anti-PSD-95 antibody, Chemicon International LV1359450), β-tubulin (mouse anti-β-tubulin antibody, Novus Biologicals 6001018), eEF1A (mouse anti-eEF1A antibody; Millipore 05-235), Nap22 (rabbit anti-Nap22; Millipore AB9306), RAS associated protein 2a (Rap2a) (mouse anti-RAP2 antibody; BD Transduction Laboratories 610215), RAB7 member RAS oncogene family(Rab7) (rabbit anti-Rab7 antibody; Sigma 105K4764) GAK (rabbit anti-GAK antibody;Abcam ab86397), Hu antigen R (rabbit anti Hu antigen R antibody; Millipore 07468), PKC (rabbit anti-PKC antibody; Cell Signaling Technology 2683S), ALG2 (rabbit anti-ALG2 antibody; Millipore AB15854), Keratin 18 (mouse anti-keratin-18 antibody; Millipore 04-586), Fatty Acid Synthase (rabbit anti-fatty-acid-synthase antibody; Cell Signaling Technology 3180S)followed by horseradish peroxidase-conjugated secondary antibodies. The immunoreactivity was revealed by enhanced chemiluminescence reaction (Amersham Bioscience) and captured on autoradiography films.

Densitometric analysis was performed on Western blots using ImageJ software. T-test was performed using replicates to determine significance of differential density bands.

Data Analysis

Protein expression between WT and KO samples was considered different if all of the following criteria were met:

1) Expression level of protein as determined by MS differs between WT and KO by a minimum of 2 standard deviations (corresponding to $\geq$1.5 fold difference in protein concentration) in both preparations.
2) Signal to noise ratio in MS reading is $\geq$10.
3) Western blotting shows differential density between WT and KO SNS samples by densitometric analysis.

Protein expression between WT samples and DHPG-treated WT samples was considered different if Western blotting shows differential density between samples as measured by densitometric analysis.

Results

To determine differential synaptic protein expression between WT and *Fmr1* KO hippocampi, we used high-throughput mass spectrometry on isolated hippocampal synapses.

SNS isolation verified with electron microscopy and western blot

First, to confirm successful synaptoneurosome (SNS) preparation, we performed electron microscopy and Western blotting on purified SNS samples. Electron microscopy showed the presence of abundant intact synapses, with unambiguous pre-synaptic and post-synaptic elements (Figure 3).

For Western blotting, we used antibodies against post-synaptic density 95 (PSD95) and β-Tubulin as a loading control. PSD95 is a protein constitutively expressed in glutamatergic synapses (Beique et al., 2006). If synaptoneurosomes are successfully isolated from hippocampal tissue, we expect to see an enrichment of PSD95 in purified samples as compared to tissue homogenate, in which PSD95 concentration would be diluted amongst many non-synaptic elements. In comparing homogenized hippocampal tissue before SNS purification to purified SNS of duplicate samples, PSD95 signal was increasd by 142% and 107% respectively, indicating successful enrichment of SNS (Figure 4 & Table 1). These results indicate that our method proficiently enriches SNS containing intact synapses from neuronal cell bodies.

Preparation of SNS samples for mass spectrometry

The possibility of direct comparison between WT and KO SNS samples is compromised since the two samples are prepared independently and, as a result, MS-detected differences in protein expression level may be due to differential SNS yield between samples and not a true

representation of inherent differences in protein expression. Heavy isotope-labeled SILAM tissue was combined with freshly dissected hippocampal samples at 1:1 ratio by mass and used as internal control for MS. As mass spectrometry analysis (MS) distinguishes proteins of different isotopes, the heavy-isotope-labeled SILAM tissue serves as an internal standard, facilitating comparison of WT and KO samples (Ong and Mann, 2005).

To submit samples to mass spectrometric analysis, proteins should be prepared as individual soluble peptide strands. To this end, we incubated SNS samples in SDS buffer and treated with iodoacetamide to separate and protect peptide strands. After treatment we performed gel electrophoresis; however, the sample remained in the gel well while the ladder entered and ran successfully, indicating aggregation of the samples (Figure 5). Mass spectrometry analyzes individual protein strands and cannot be performed if samples are aggregated.

We had previously performed gel electrophoresis of SNS samples without aggregation when we resuspended SNS samples in homogenization buffer, froze samples after preparation, thawed samples before gel electrophoresis, and did not incubate with SDS buffer and iodoacetamide. Keeping these factors in mind, we compared various conditions to solve the problem of aggregation in the treated samples. Changing the resuspension buffer or adding a freeze-and-thaw process for complete protein lysis did not lessen aggregation. Lowering the temperature of incubation from 95°C to 75°C, however, did resolve aggregation (Figure 6). We could not remove the incubation step completely because incubation with SDS buffer followed by treatment with iodoacetamide is critical to separating peptides for MS analysis. We used this modified condition to prepare samples for mass spectrometric analysis.

Mass Spectrometric Analysis

Next, using this optimized method, we prepared duplicate samples from hippocampi of WT and *Fmr1* KO littermates. At least 100 µg of total sample is needed to perform MS. To determine the mass of our prepared SNS samples, we calculated the protein concentration in known volume of sample using Coomassie staining and ImageJ software (Figures 7 and 8). Multiplying the protein concentration by total volumetric yield provided the total yield in mass. Total mass of samples ranged from 707µg to 1903µg, well over the minimal 100µg required for MS analysis (Tables 2 and 3).

We submitted the samples to Emory Proteomics Core Service Center for proteomic analysis using MS, which identified 3885 to 4520 peptides, corresponding to 1993 to 2230 proteins, in each sample. After normalizing each peptide using heavy-isotope labeled peptides provided from SILAM tissue, we statistically analyzed and identified 11 proteins as candidates that showed $\geq$2 standard deviation increase (corresponding to $\geq$1.5 fold difference in protein concentration) in expression levels in KO samples as compared to WT samples (Table 4): alpha-1,3 mannosyltransferase (ALG2), cyclin G associated kinase (GAK), ELAV (embryonic lethal, abnormal vision, Drosophila)-like 1 (Hu antigen R), Eukaryotic Elongation Factor 1A (eEF1A), Fatty acid synthase, Neuronal Axonal Protein 22 (Nap22), Protein kinase C (PKC), RAB7 member RAS oncogene family (Rab7), RAS related protein 2a (Rap2a), and Thimetoligopeptidase 1 (TOP). Though some proteins were less expressed in KO than WT samples, we were primarily interested in those that were overexpressed, as FMRP is a translation suppressor and loss of translational suppression from FMRP in FXS state would result in increased protein levels. It is possible that FMRP may be inhibiting the translation of a protein which in turn decreases the expression of a second protein; in such a case, loss of FMRP would

result in decreased expression of the second protein. Given the primary role of FMRP as a translation suppressor, however, we chose to focus on those that showed an increase in expression in this study.

MS results verified with western blot

In order to verify whether MS-identified proteins are indeed differentially expressed or not, we performed Western blotting of freshly prepared SNS from WT and KO hippocampi, using antibodies against candidate proteins. Ten of the 11 candidate proteins were assessed using this method, as antibodies were not available for thimetoligopeptidase 1 (TOP). In addition, no signals were observed for ALG2, Hu-Antigen R, Keratin 18 or RAB7. Consequently, only eEF1A, GAK, NAP22, PKC and RAP2 were successfully analyzed by western blot; eEF1A, Nap22, and GAK showed increased expression in KO samples as compared to WT samples (Figures 9-11 and Tables 5-7). Though these differences are not statistically significant, there is a trend of increase. Significance is compromised by varying ratio of increase among replicates. Possible sources of variance include inherent differences between samples and technical differences in sample handling and preparation. Also, small n numbers, such as those used in this study, provide a poor representation of sample distribution and compromise statistical significance.

Though the ratio of increase varies, an increase in eEF1A, NAP 22 and GAK expression has been observed in independently prepared duplicate samples by MS and another set of freshly prepared replicate samples by WB. No samples showed decreased expression or lack of expression as measured by either technique (MS and WB). This repeated observation of increased expression indicates that differential expression is likely to be inherent. These results

suggest that eEF1A, NAP 22, and GAK may be regulated by FMRP; the loss of FMRP in KO state would induce aberrant protein expression of those proteins that are regulated by FMRP.

Western blot shows increased protein expression with mGluR stimulation

To determine if proteins identified by MS and verified by WB as regulated by FMRP are synthesized upon mGluR stimulation, we compared protein expression level across WT and WT with DHPG stimulation SNS samples. We expected to see an increase in a protein's expression with DHPG stimulated samples if mGluR stimulation initiates its synthesis. eEF1A was significantly greater in WT+DHPG conditions as compared to WT (Figure 9 and Table 5). NAP22 and GAK also showed increased expression in DHPG-treated samples, though the ratio of increase varied among replicate samples (Figures 10, 11 and Tables 6, 7). These results indicate that eEF1A, NAP22, and GAK are synthesized upon mGluR stimulation and may be involved in the LTD pathway that is induced upon mGluR stimulation and is aberrant in FXS mouse model.

Figures and Tables

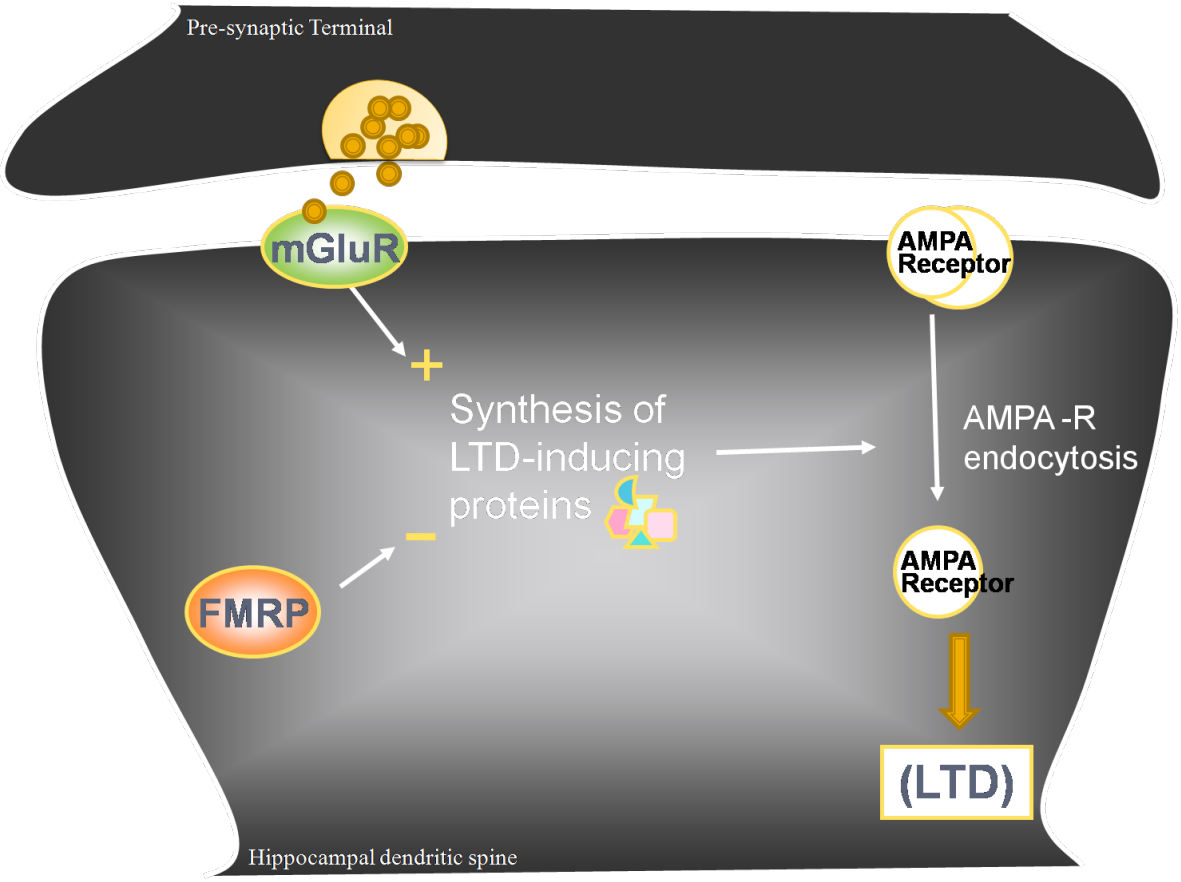


Figure 1a. mGluR theory of FXSis based on the observation where group I mGlu-R activation initiates local protein synthesis, the products of which induce LTD via endocytosis of AMPA receptors. The theory proposes that FMRP acts as a regulatory factor on translation machinery, suppressing synthesis of LTD-inducing proteins at the synapse.

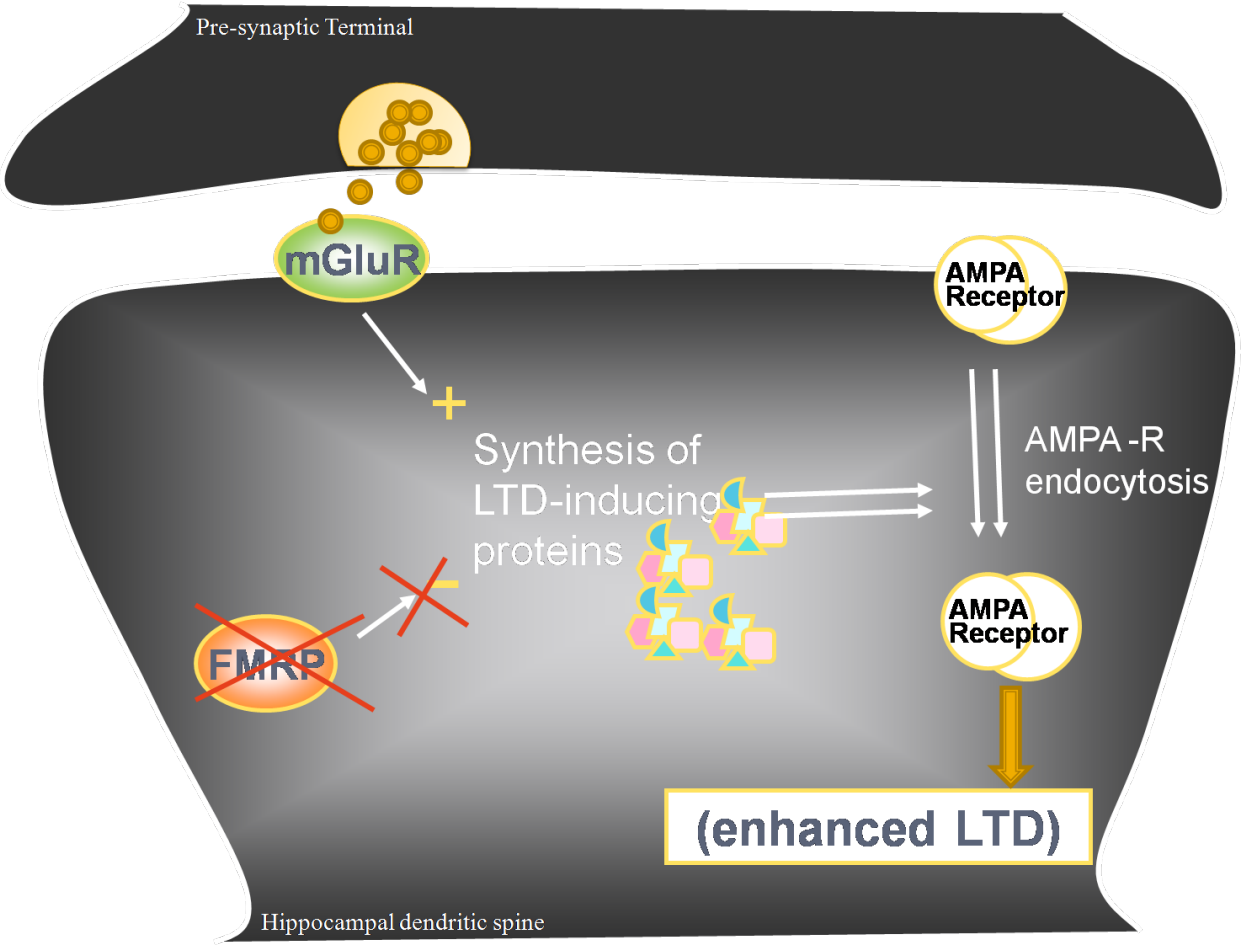
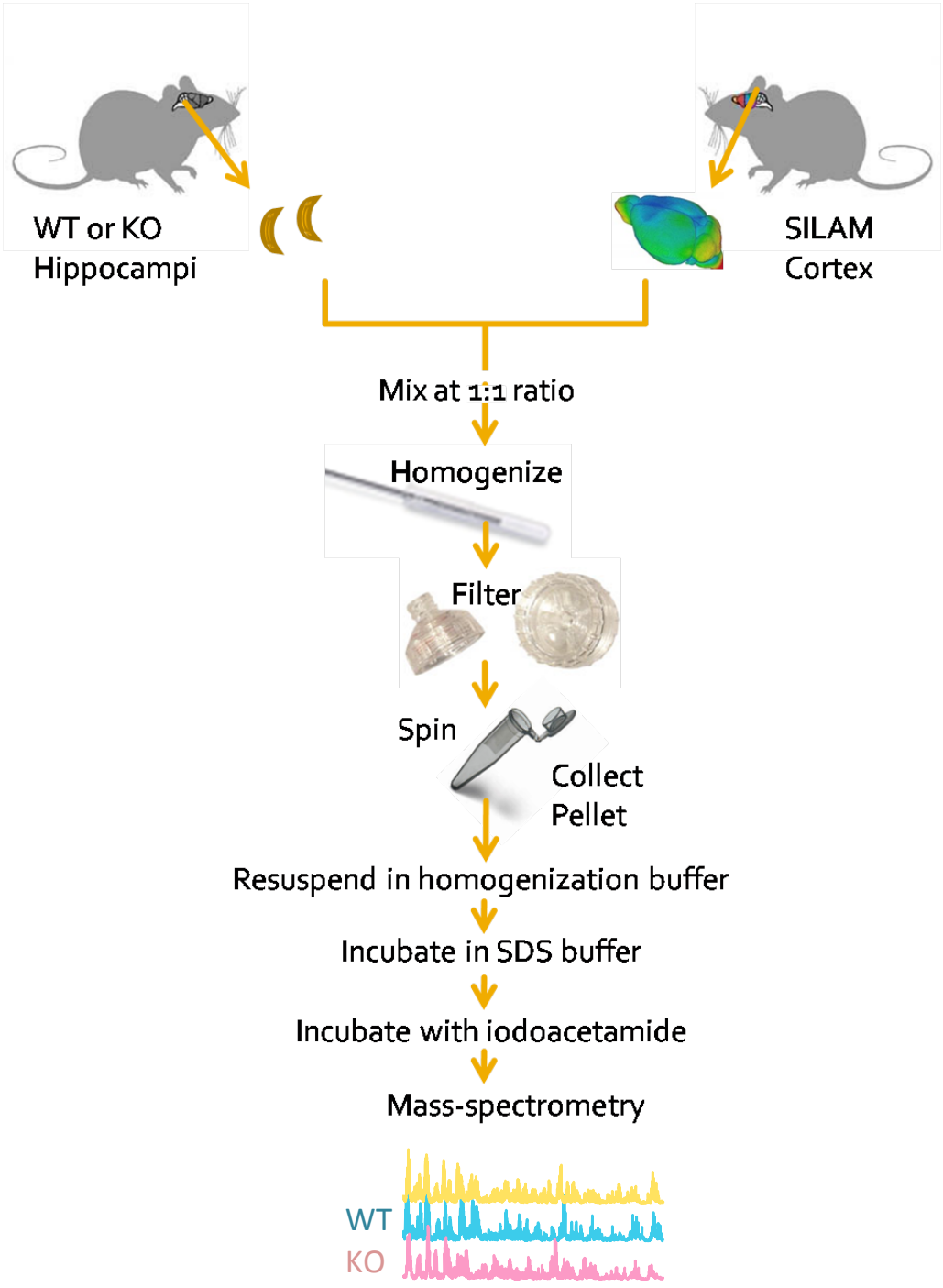


Figure 1b. mGluR theory of FXS In the pathological state of FXS, the loss of translation inhibition from FMRP results in a constitutive increase in protein synthesis of certain proteins, increased AMPA-R endocytosisand enhanced LTD.

Figure 2. Synaptoneurosome Preparation Scheme



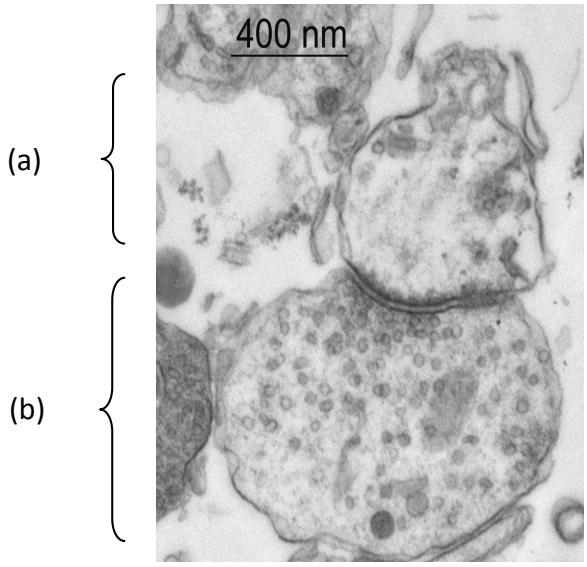


Figure 3. Electron microscopy images show intact synapses isolated with SNS purification methods employed. Post-synaptic (dendritic) element (a) is recognized by the presence of post-synaptic density bordering the synapse. Pre-synaptic (axonal) element (b) is characterized by the presence of vesicles and mitochondria.

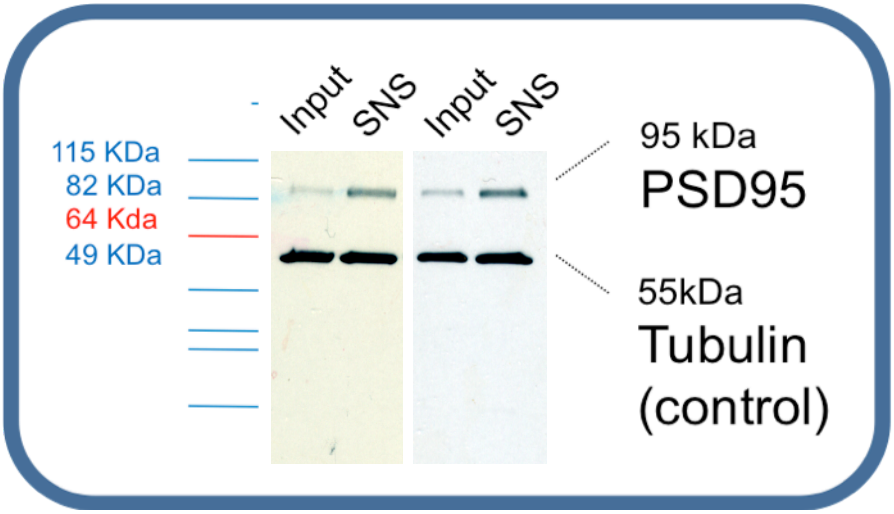


Figure 4. Western bloting demonstrates enrichment of synaptoneurosome (SNS). Western Blotting was performed using samples attained from freshly homogenized hippocampal tissue (labeled *Input*) and purified SNS (labeled *SNS*). Blot was probed with antibodies against PSD95 and β-Tubulin as a loading control. Increased PSD95 signal is observed in SNS samples.

Table 1. Densitometry Data for PSD-95 Enrichment in SNS

	Density Ratio PSD95:Tubulin	Increase compared to Input
Sample 1		
Input	0.24	1.0
SNS	0.58	1.42
Sample 2		
Input	0.33	1.0
SNS	0.67	1.07

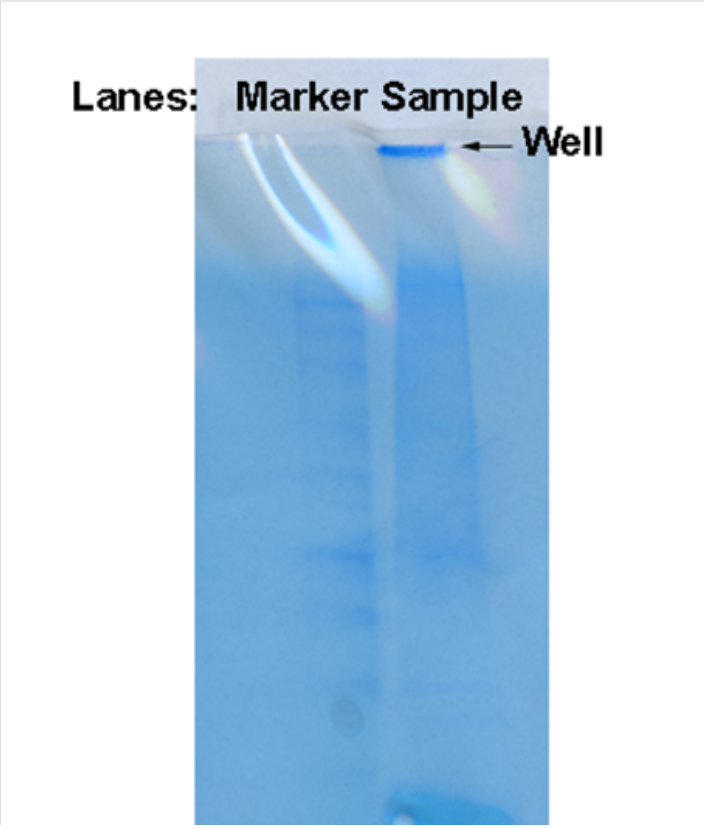


Figure 5. SNS sample aggregates after SDS and iodoacetamide treatment. After incubation with SDS buffer and addition of iodoacetamide, SNS sample remained in a gel well, suggesting aggregation of the sample.

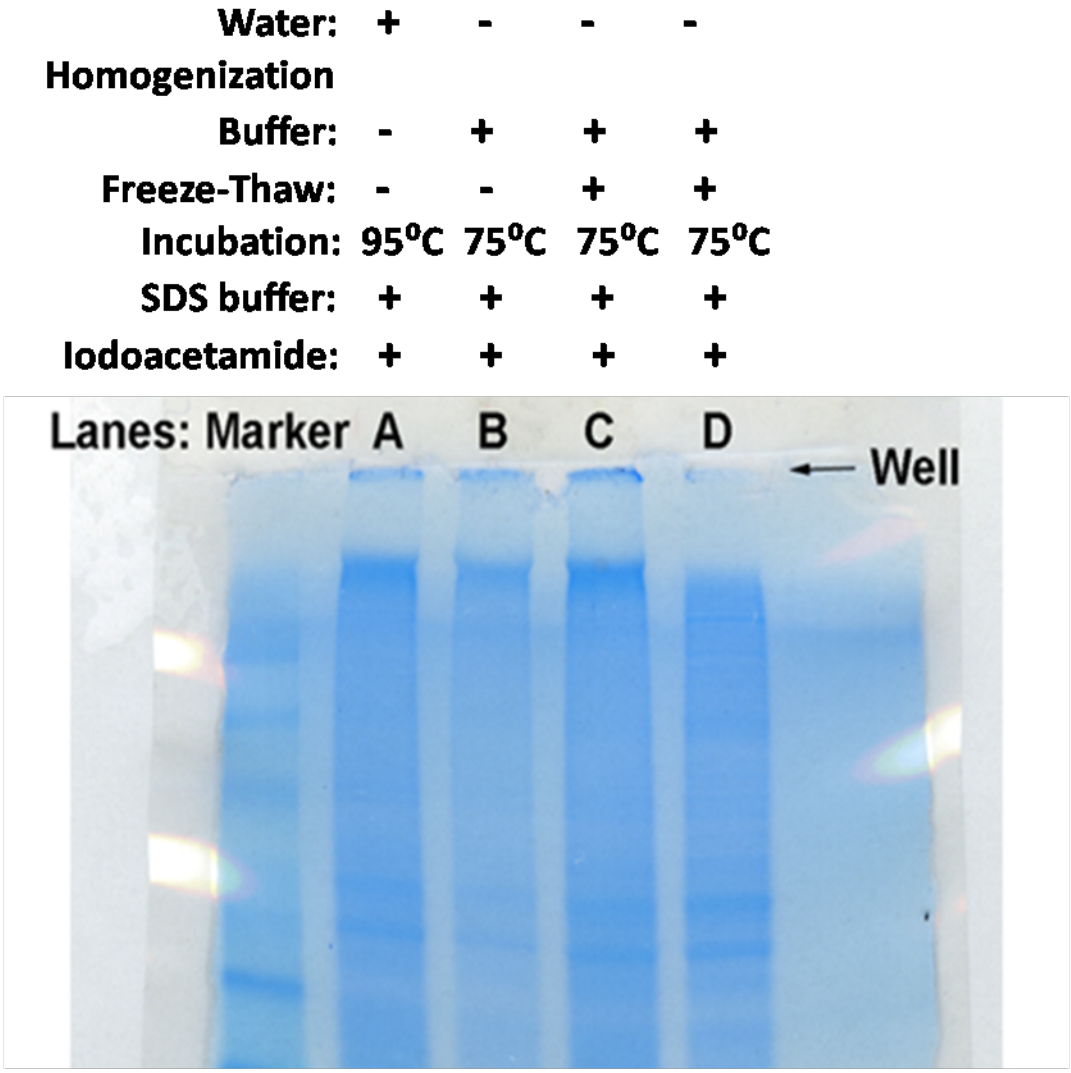


Figure 6. Optimal conditions for preparation of soluble samples: Alternative resuspension solution, additional process of protein lysis by freeze and thaw, and various incubation temperature were tested. All other combination of conditions, except "D," caused aggregation of proteins and retention of the samples in the gel wells.

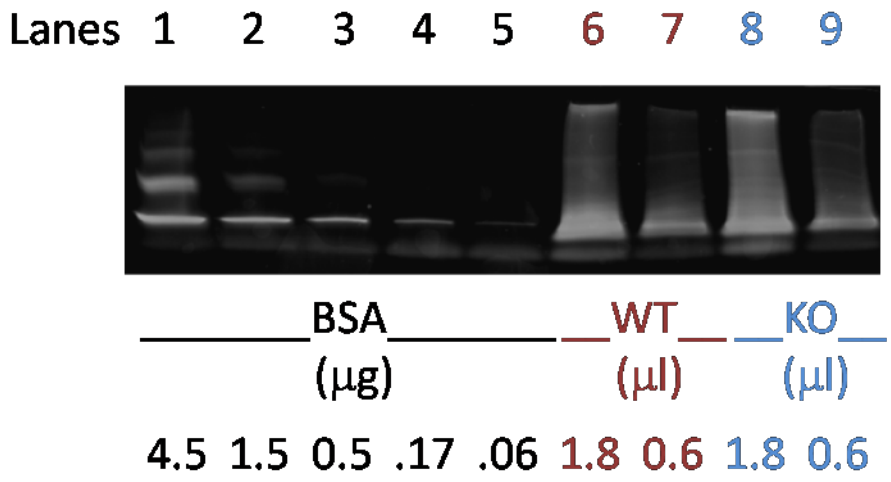


Figure 7. Coomassie Staining used to determine protein concentration of MS sample 1: WT= 5.66 µg/µl and KO= 6.65 µg/µl. The concentration of each sample was multiplied by the volume to determine mass of sample.

Table 2. Mass, Volume and Protein Concentration of MS sample 1

Property	WT	KO
Total Yield (Volume)	125 µl	125 µl
Protein Concentration	5.66 µg/µl	6.65 µg/µl
Total Yield (Mass)	707 µg	830 µg

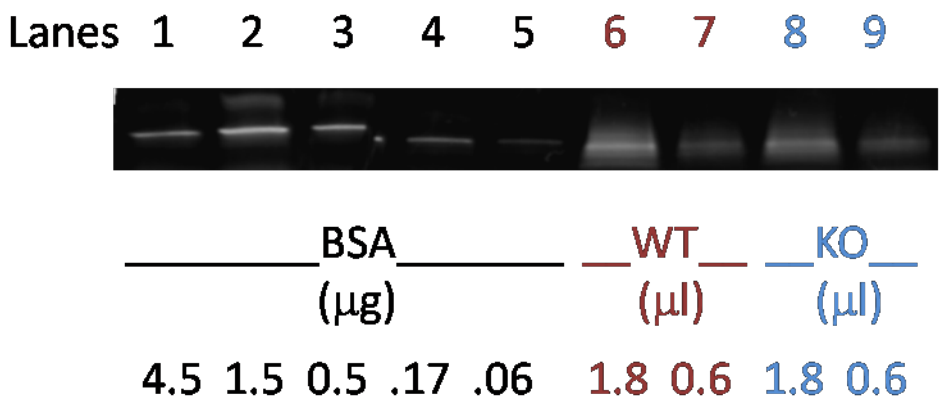


Figure 8. Coomassie Staining used to determine protein concentration of MS sample 2: WT= 11.2 µg/µl and KO= 10.0 µg/µl. The concentration of each sample was multiplied by the volume to determine mass of sample.

Table 3. Mass, Volume and Protein Concentration of MS sample 2

Property	WT	KO
Total Yield (Volume)	170 µl	135 µl
Protein Concentration	11.2 µg/µl	10 µg/µl
Total Yield (Mass)	1903 µg	1353 µg

Table 4. MS-Identified Candidate Proteins

Description	Prep 1 KO:WT	Prep 2 KO:WT
Alpha-1,3-mannosyltransferase (ALG2)	1.5	1.9
Cyclin G associated kinase (GAK)	4.2	2.2
Eukaryotic elongation factor 1A (eEF1A)	9.3	6.8
ELAV (embryonic lethal, abnormal vision, Drosophila)-like 1 (Hu antigen R)	25.8	3.0
Fatty acid synthase	1.9	2.9
Keratin 18	1.9	2.2
Neuronal axonal protein 22 (NAP 22)	3.0	3.8
Protein kinase C, epsilon (PKC)	1.6	1.9
RAB7, member Rasoncogenefamily (RAB7)	2.7	3.7
RAS related protein 2a (Rap2a)	3.5	2.8
Thimetoligopeptidase 1 (TOP)	3.2	1.5

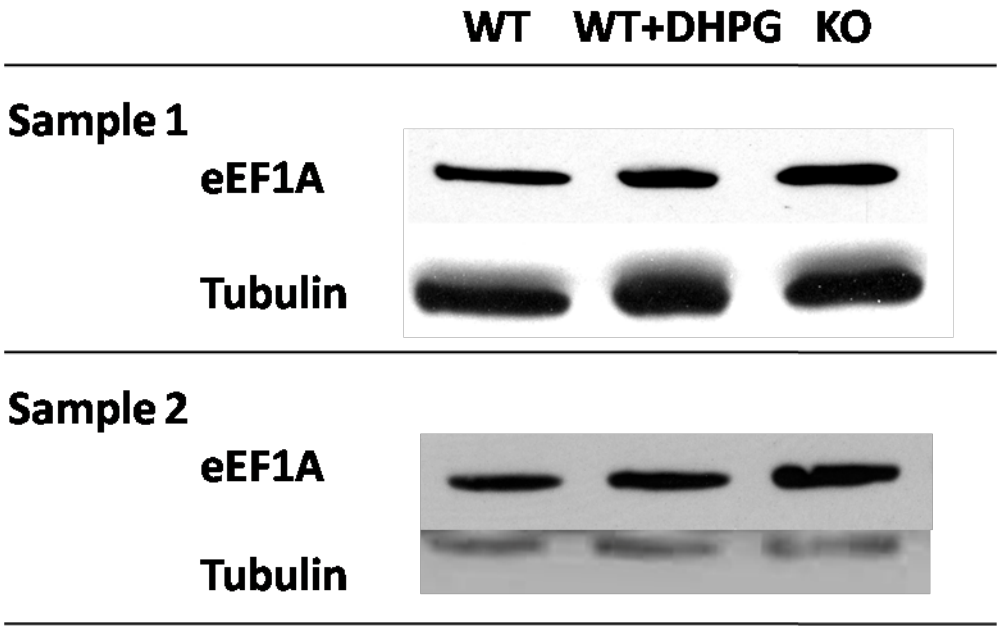


Figure 9. Western blotting verifies over-expression of eEF1A in KO as compared to WT SNS samples and shows over-expression of eEF1A in WT+DHPG samples as well. Duplicate samples were prepared. Tubulin was used as loading control.

.

Table 5. Densitometry Data of eEF1A Western Blot

Protein	Density Ratio eEF1A:Tubulin	Percent Increase compared to WT
Sample 1		
WT	1.94	1.0
WT+DHPG	2.09	1.08
KO	2.26	1.27
Sample 2		
WT	0.85	1.0
WT+DHPG	0.91	1.08
KO	1.07	1.22

	WT+DHPG	KO
P-value of increase	p=.009*	p=.14

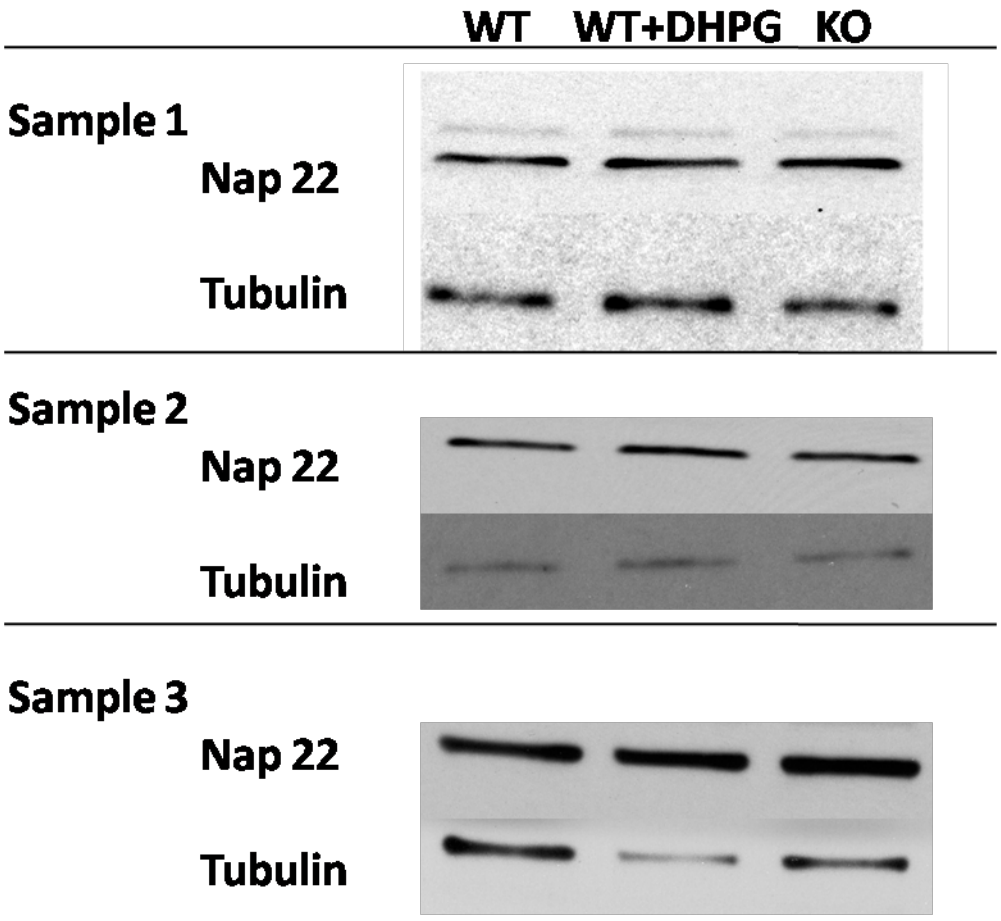


Figure 10. Western blotting verifies over-expression of NAP 22 in KO as compared to WT SNS samples and shows over-expression of NAP 22 in WT+DHPG samples as well. Three replicate samples were prepared. Tubulin was used as loading control.

Table 6. Densitometry Data of NAP22 Western Blot

Protein	Density Ratio Nap22:Tubulin	Percent Increase compared to WT
Sample 1		
WT	0.88	1.00
WT+DHPG	0.97	1.10
KO	0.99	1.13
Sample 2		
WT	0.80	1.00
WT+DHPG	0.86	1.09
KO	0.83	1.04
Sample 3		
WT	1.11	1.00
WT+DHPG	1.93	1.74
KO	1.48	1.33

	WT+DHPG	KO
P-value of increase	p=.29	p=.19

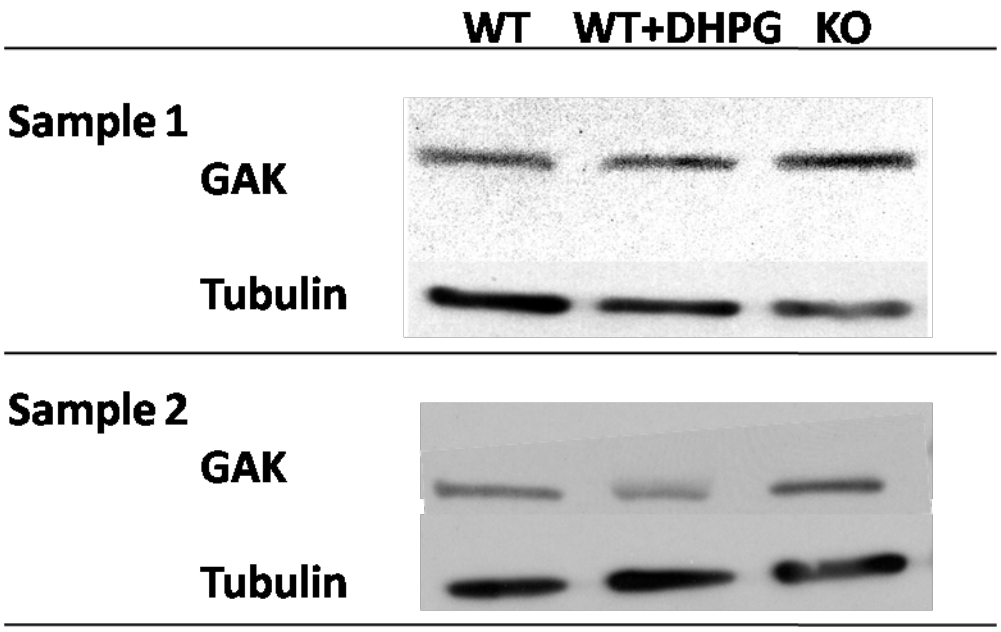


Figure 11. Western blotting verifies over-expression of GAK in KO as compared to WT SNS samples and shows over-expression of GAK in WT+DHPG samples as well. Dublicate samples were prepared. Tubulin was used as loading control.

Table 7. Densitometry Data of GAK Western Blot

Protein	Density Ratio GAK:Tubulin	Percent Increase compared to WT
Sample 1		
WT	0.54	1.00
WT+DHPG	0.62	1.13
KO	0.69	1.27
Sample 2		
WT	0.90	1.00
WT+DHPG	0.93	1.03
KO	1.05	1.16

	WT+DHPG	KO
P-value of increase	p=.38	p=.17

Discussion

The objective of this study was to identify proteins regulated by FMRP that may contribute to enhanced mGluR-dependent LTD phenotype of FXS model mouse. With the use of mass spectrometric analysis followed by Western blotting, we determined three proteins to be deferentially expressed between WT and *Fmr1* KO SNS samples, suggesting a lack of FMRP in KO littermates has contributed to their aberrant expression between samples: Eukaryotic translation elongation factor 1A (eEF1A), Neuronal Axonal Protein 22 (NAP22), and Cyclin G Associated Kinase (GAK).

Our findings are in agreement with previous literature that report eEF1A and NAP22 as mRNA ligands of FMRP, also suggesting their expression regulation by FMRP (Sung et al., 2003; Brown et al., 2001; Darnell et al., 2001). In addition, the eEF1A protein is reported overexpressed in lymphoblastoid cells of FXS patients, further suggesting that its translation and consequent protein expression is negatively regulated by FMRP (Sung et al., 2003). The GAK transcript, however, has not been listed as an FMRP ligand. Aberrant expression of GAK has been associated with Parkinson's disease (PD), and the protein has been suggested as a therapeutic target for PD (Dumitriu et al., 2011). Our data, however, may be the first report of GAK associated with FXS.

In addition, Western blotting analyses indicate eEF1A, NAP 22 and GAK are overexpressed in WT samples treated with DHPG, indicating that their translation is activity-dependent and suggesting involvement of these proteins in the LTD pathway that is initiated upon mGluR stimulation with DHPG.

Eukaryotic translation elongation factor 1A (eEF1A)

eEF1A is an abundant protein synthesis factor that plays a critical role during the elongation stage of protein synthesis. eEF1A functions to deliver appropriate tRNA-amino acid complexes to the A site of the ribosome when a codon match is detected. The elongation factor then allows a peptide bond to form and moves the peptide to the P site in order to bring the next tRNA-amino acid complex to the A site (Mateyak and Kinzy, 2010). Although the relationship between eEF1A and FXS is unclear, these data suggest it might play an important role in activity dependent local protein synthesis in neurons.

Neuronal Axonal Protein 22 (NAP 22)

NAP 22 is also known as brain abundant membrane attached signal protein 1 (basp1). NAP22 is expressed throughout the brain, with localization to the synaptic terminals, dendritic spines, and thin nerves fibers associated with synaptic vesicles, presynaptic and postsynaptic membranes, and microtubules (Iino et al., 1999).

NAP22 appears to be important for neuronal sprouting and plasticity (Frey et al., 2000), which are abnormal in FXS. It is not clear whether NAP22 is related to mGluR-dependent LTD or not. Our data may be the first evidence of NAP22 involvement in mGluR-dependent LTD, but further studies are needed to establish this relationship.

Nap22 is present in kidneys and testes (Mosevitsky and Silicheva, 2011), which is interesting because FMRP is known to be expressed also in the testes and macroorchidism is observed in FXS patients and model mice (Butler et al., 1993; Bakker et al., 1994). FMRP perhaps regulates translation of Nap22 in neuronal and testicular tissue. Future studies are

needed to determine if aberrant expression of NAP22 contributes to FXS-associated macroorchidism.

Cyclin G Associated Kinase (GAK)

GAK is the ubiquitous form of the neuronal-specific protein, auxilin 1 and is involved in clathrin-mediated endocytosis (Eisenberg and Greene, 2007; Lee et al., 2005). Interestingly, it is known that AMPA receptor endocytosis is clathrin-mediated. Given the aberrant GAK expression observed in *Fmr1* KO and DHPG-stimulated WT mice of this study, GAK is likely involved in the AMPA receptor endocytosis underlying mGluR-dependent LTD and FXS. Future investigation is needed to assess the contribution of GAK to the enhanced AMPA receptor endocytosis phenotype associated with FXS.

Other MS-Identified Proteins

ALG2, Hu-Antigen R, Keratin 18 and RAB7 also showed differential expression between WT and KO SNS as measured by MS. No signal was observed, however, with western blotting. Increased sensitivity of antibodies and/or increased sample quantity is needed in future western blotting attempts.

Conclusion

The mGluR theory of FXS proposes that mGluR stimulation and FMRP regulate the translation of LTD-inducing proteins. These proteins, however, are unidentified. With the use of MS and Western blotting analyses, we have determined eEF1A, NAP 22 and GAK to be overexpressed in hippocampal SNS both in *Fmr1* KO mice and in DHPG-stimulated WT; though

most increase were not statistically significant. Overexpression of these proteins determined by different techniques and measured in replicate samples may suggest their involvement in the molecular mechanism underlying FXS and LTD. Furthermore, the known functions of these proteins include translation activity, synaptic plasticity, and AMPAR endocytosis, which offer support for the mGluR theory of FXS. Future studies are needed to elucidate the role of these proteins in FXS pathophysiology and the mGluR-dependent LTD pathway. One or more of them may be responsible for AMPA receptor internalization following synaptic stimulation, a key event of neuronal plasticity and the most proximal defect in fragile X syndrome.

Sources

Ashley CT Jr, Wilkinson KD, Reines D, Warren ST (1993) FMR1 protein: conserved RNP family domains and selective RNA binding.Science 262(5133):563-6.

Bakker CE, Verheij C, Willemsen R, van der Helm R, Oerlemans F, Vermey M, Bygrave A, Hoogeveen AT, Oostra BA, Reyneirs E, de Boule K, D'Hooge R, Cras P, van Velzen D, Nagels G, Martin JJ, de Deyn PP, Darby JK, and Willems PJ (1994) *Fmr1* knockout mice: A model to study fragile X mental retardation. Cell 78:23-33.

Bassell GJ and Warren ST (2008) Fragile X syndrome: loss of local mRNA regulation alters synaptic development and function. Neuron 60(2):201-14.

Bear MF, Huber KM, and Warren ST (2004) The mGluR theory of fragile X mental retardation. Trends Neurosci 27:370-7.

Beique JC, Lin DT, Kang MG, Aizawa H, Takamiya K and Huganir RL (2006) Synapse-specific regulation of AMPA receptor function by PSD95. Proc NatlAcadSci USA 103:19535-40.

Boyle L and Kaufmann W (2010) The behavioral phenotpye of *FMR1* mutations. AJMG 154C:469-476.

Brown V, Jin P, Ceman S, Darnell JC, O'Donnell WT, Tenenbaum SA, Jin X, Feng Y, Wilkinson KD, Keene JD, Darnell RB and Warren ST (2001) Microarray Identification of FMRP-Associated Brain mRNAs and Altered mRNA Trnaslational Profiles in Fragile X Syndrom. Cell 107:477-487.

Butler MG, Pratesi R, Watson MS, Breg WR, Singh DN (1993) Anthropometric and craniofacial patterns in mentally retarded males with emphasis on the fragile X syndrome. Clinical Genetics 44:129-38.

Comery TA, Harris JB, Willems PJ, Oostra BA, Irwin SA, Weiler IJ and Greenough WT (1997) Abnormal dendritic spines in fragile X knockout mice: maturation and pruning deficits. Proc. Natl. Acad. Sci. USA94:5401–5404.

Darnell JC, Jensen KB, Jin P, Brown V, Warren ST and Darnell RB (2001) Fragile X Mental Retardation protein targets G quartet mRNAs important for neuronal function. Cell 107**:** 489–499

Dumitriu A, Pacheco CD, Wilk JB, Strathearn KE, Latourelle JC, Goldwurm S, Pezzoli G, Rochet JC, Lindquist S, Myers RH (2011) Cyclin-G-associated kinase modifies {alpha}-synuclein expression levels and toxicity in Parkinson's disease: results from the GenePD Study. Hum Mol Genet 20:1478-1487.

Eisenburg E and Greene LE (2007) Multiple roles of Auxilin and Hsc70 in Clathrin-Mediated Endocytosis. Traffic 8:640-646.

Frey D, Laux T, Xu L, Schneider C, Caroni P (2000) Shared and unique rolesof CAP23 and GAP43 in actin regulation, neurite outgrowth, and anatomicalplasticity. J Cell Biol 149:1443–1454.

Gouraud SS, Heesom K, Yao ST, Qiu J, Paton JF and Murphy D (2007) Dehydration-induced proteome changes in thr rat hypothalamo-neurohypophyseal system. Endocrinology 148:3041-52.

Greene LE and Eisenberg E (2007) Multiple roles of auxilin and hsc70 in clathrin-mediated endocytosis. Traffic 6:640-646.

Gross C, Nakamoto M, Yao X, Chan CB, Yim SY, Ye K, Warran ST and Bassell GJ (2010) Excess phosphoinositide 3-kinase subunit synthesis and activity as a novel therapeutic target in fragile X syndrome. 30:10624-38.

Grossman AW, Aldridge GM, Weiler IJ and Greenough WT (2006) Local protein synthesis and spine morphogenesis: Fragile X syndrome and beyond. J Neurosci 26:7151-7155.

Hinds HL, Ashley CT, Sutcliffe JS, Nelson DL, Warren ST, Housman DE, and Schalling M (1993) Tissue specific expression of FMR-1 provides evidence for a functional role in fragile X syndrome. Nature 3:36-43.

Huber KM, Gallagher SM, and Warren ST (2002) Altered synaptic plasticity in a mouse model of fragile X mental retardation. Proc NatlAcadSci USA 99(11):7746-50.

Iino S, Kobayashi S, Maekawa S (1999)Immunohistochemical localization of a novel acidic calmodulin-binding protein, NAP-22, in the rat brain. Neuroscience 91:1435–1444.

Irwin SA, Galvez R, and Greenough WT (2000) Dendritic spine structural anomalies in Fragile X Mental Retardation Syndrome.Cereb. Cortex 10:1038-1044.

Krueger M., Moser M., Ussar S., Thievessen I., LuberCh.A, Forner F., Schmidt S., Zanivan S., Faessler R. and Mann M.(2008). SILAC Mouse for Quantitative Proteomics Uncovers Kindlin-3 as an Essential Factor for Red Blood Cell Function, Cell 134, 353–364.

Lee DW, Zhao X, Zhang F, Eisenberg E, Greene LE (2005) Depletion of GAK/auxilin 2 inhibits receptor-mediated endocytosis and recruitment of both clathrin and clathrin adaptors. J Cell Sci 118:4311-21.

Luscher C and Huber KM (2010) Group 1 mGluR-dependent synaptic long-term depression: mechanisms and implications for circuitry and disease. Neuron 65:445-59.

Maekawa S, Maekawa M, Hattori S, Nakamura S (1993) Purification and molecular cloning of a novel acidic calmodulin binding protein from rat brain. J BiolChem 268:13703–13709.

Maekawa S, Murofushi H, Nakamura S (1994) Inhibitory effect of calmodulin on phosphorylation of NAP-22 with protein kinase C. J BiolChem 269:19462–19465.

Maekawa S, Sato C, Kitajima K, Funatsu N, Kumanogoh H, Sokawa Y (1999) Cholesterol-dependent localization of NAP-22 on a neuronal membrane microdomain (raft). J BiolChem 274:21369–21374.

Man HY, Lin JW, Ju WH, Ahmadian G, Liu L, Becker LE, Sheng M, Wang YT (2000) Regulation of AMPA receptor-mediated synaptic transmission by clathrin-dependent receptor internalization. Neuron 25:649-62.

Mateyak MK and Kinzy TG (2010) eEF1A: thinking outside the ribosome. J BiolChem 285(28):21209-13.

Mosevitsky M and Silicheva I (2011) Subcellular and regional location of "brain" proteins BASP1 and MARCKS in kidney and testis. ActaHistochem. 113(1):13-8.

Nakamoto M, Nalavadi V, Epstein MP, Narayanan U, Bassell GJ, Warren ST (2007) Fragile X mental retardation protein deficiency leads to excessive mGluR5-dependent internalization of AMPA receptors. Proc NatlAcadSci USA 104:15537-42.

Oliet SH, Malenka RC and Nicoll RA (1997) Two distinct forms of long-term depression coexist in CA1 hippocampal pyramidal cells *Neuron* 18: 969–982.

Ong SE and Mann M (2005) Mass spectrometry-based proteomics turns quantitative. Nat ChemBiol 1:252–262.

Pieretti M, Fu Y-H, Warren ST, Zhang F, Oostra BA, Caskey CT and Nelson DL (1991) Absence of expression of the FMR-1 gene in fragile X syndrome.Cell 66:817-822.

Sung YJ, Dolzhanskaya N, Nolin SL, Brown T, Curriel JR, Denman RB (2003) The fragile X mental retardation protein FMRP binds elongation factor 1A mRNA and negatively regulates *in Vivo*. Journal of Biological Chemistry 278:15669-15678.

Verkerk AJMH, Pieretti M, Sutcliffe JS, Fu Y-H, Kuhl DPA, Pizzuti A, Reiner O, Richards S, Victoria MF, Zhang F, Eussen BE, van Ommen GLB, Blonden LAJ, Riggins GJ, Chastain JL, Kunst CB, Gakljaard H, Caskey CT, Nelson DL, Oostra BA and Warren ST (1991) Identification of a gene (FMR-1) containing a CGG repeat coincident with a breakpoint cluster region exhibiting length variation in fragile X syndrome. Cell 65:905-914.