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Changes in Synaptic Protein Content and Signaling in a Mouse Model of Fragile X Syndrome

Kelly Birch
University of San Diego

Peter W. Vanderklish PhD
The Scripps Research Institute

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Kelly A. Birch
Department of Psychological Sciences
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Fragile X Syndrome (FXS) is the most common inherited cause of intellectual disability (ID) in males and a significant cause of ID in females. In addition to ID, affected children may also exhibit hyperactivity, extreme anxiety in multiple forms, poor social communication (including poor eye contact and poor pragmatics), and other autism spectrum behaviors such as restricted interests and repetitive patterns of behavior. FXS is also characterized by a variety of distinct physical characteristics, including an enlarged head, protruding ears, and joint laxity (Learning About Fragile X Syndrome, 2013). Furthermore, a hallmark neuroanatomical abnormality of FXS brain is the overabundance of long, thin, and "tortuous" dendritic spines - the postsynaptic aspect of excitatory glutamatergic synapses - which suggests that a fundamental synaptic abnormality underlies the syndrome's many symptoms.

Nearly all cases of FXS are caused by transcriptional silencing of the Fragile X mental retardation I (*Fmr1*) gene on the X chromosome, which results from a CGG triplet repeat expansion of more than 200 copies within a non-protein coding segment of the gene corresponding to the 5' untranslated region of the transcribed messenger RNA (mRNA). However, some rare cases of FXS are caused by mutations in protein coding regions of *Fmr1* (Collins et al., 2010). This abnormally expanded DNA segment inactivates the *Fmr1* gene, leading to the lack of expression of the gene's encoded protein, the Fragile X mental retardation protein (FMRP) (Learning About Fragile X Syndrome, 2013). In accordance with this genetic inactivation, the *Fmr1* knockout mouse was developed for use as an important tool in FXS research. Knockout mice exhibit similar behavioral and molecular phenotypes to humans with FXS, including learning deficits, hyperactivity, audiogenic seizures (thought to be a parallel to

developmental seizures that present in many with FXS), and changes in dendritic spine density and shape (Kazdoba, Leach, Silverman & Crawley, 2014).

Though FXS is monogenic in nature, its underlying pathogenic mechanisms are likely to be incredibly complex because FMRP acts as a suppressor of activity-dependent translation of many mRNAs (Napoli et al., 2008). FMRP is an mRNA-binding protein that suppresses the translation of most of its direct target mRNAs. Recent estimates place the number of mRNAs bound by FMRP at approximately 842, encompassing proteins with a broad array of biological functions (Darnell et al., 2011). Thus, the absence of FMRP potentially results in the overexpression of a large number of proteins, impacting diverse cellular functions. Many mRNAs targeted by FMRP code for proteins that are highly expressed in neurons that regulate synaptic form and function, including proteins involved in molecular transport, cytoskeletal structure, and signaling (Napoli et al., 2008). However, though the mRNAs that associate directly with FMRP have been extensively studied, not much is known about the extent of protein expression changes that may be present in the FXS brain. This is largely because one cannot reliably infer protein expression levels from mRNA binding data, and because loss of FMRP can have secondary or downstream effects on protein expression that result from perturbations in key signal transduction pathways (Sharma et al., 2010). For example, recent research has shown that the mammalian target of rapamycin (mTOR) and extracellular signaling-regulated kinase (ERK) pathways show increased activation in the *Fmr1* knockout mouse (Michalon et al., 2012). Both of these pathways regulate translation, sculpting the proteome through effects on the rate and profile of mRNAs translated. The abnormal activity of these pathways may thus result in phenotypically relevant protein changes in the expression of proteins whose encoding mRNAs are not direct targets of FMRP.

The disinhibition of FMRP target mRNAs in FXS - and the broader dysregulation of key signaling pathways at synapses - have been linked to a multitude of synaptic changes that may underlie the neurodevelopmental delays and other symptoms present in individuals with FXS. Notably, morphological studies suggest abnormalities in the synaptic maturation process. In addition, humans affected with FXS have a higher density of dendritic spines, suggesting issues with the synaptic pruning process that occurs throughout development (Greenough, Klintsova, Irwin, Galvez, Bates & Weiler, 2001). Furthermore, past research has demonstrated altered synaptic plasticity in FXS (Sidorov, Auerbach & Bear, 2013). Synaptic plasticity refers to the ability of synapses to strengthen or weaken over time in responses to increases or decreases in their activity, or even specific temporal patterns of activity. Such changes in synaptic efficacy - which include long-term forms of synaptic potentiation ("LTP", strengthening of synapses) and depression ("LTD", weakening of synapses) - are thought to be neurophysiological substrates of memory formation, and thus critical to many neural network functions involved in cognition (Bear & Malenka, 1994). Importantly, the long-term maintenance of synaptic plasticity depends on the synthesis of new proteins in dendritic spines. To accomplish this, many mRNAs are trafficked to dendrites where they are translated to eventually become these crucial proteins. As previously discussed, FMRP is a crucial regulator and suppressor of mRNA translation. Thus, its absence in FXS likely leads to altered synaptic plasticity (Sidorov, Auerbach & Bear, 2013).

Both LTP and LTD are well-characterized forms of synaptic plasticity in which the *de novo* synthesis of proteins at synapses plays a critical role (Bear & Malenka, 1994). Consistent with a role of FMRP in suppressing translation at synapses, *Fmr1* knockout mice demonstrate both enhanced hippocampal and cerebellar metabotropic glutamate receptor (mGluR)-mediated LTD. In the absence of FMRP, the proteins responsible for LTD are over-expressed, leading to

LTD enhancement (Sidorov, Auerbach & Bear, 2013). Prior work in our lab demonstrated that activation of mGluRs with the selective agonist DHPG results in a translation-dependent elongation of dendritic spines such that they resemble the synaptic phenotypes that are the hallmark neuroanatomical abnormality of FXS brain (Vanderklish & Edelman, 2002). Considering that longer, thinner spines have less room for glutamate receptors, this observation provides a plausible basis for the enhancement of LTD seen in *Fmr1* KO mice. Importantly, early work by Weiler et al. (1997) demonstrated that stimulation of mGluRs at normal synapses results in an increase in FMRP expression, suggesting that FMRP may act as part of a negative-feedback loop to limit mGluR-induced translation. These three observations prompted the "mGluR theory" of FXS, which posits that exaggerated translation downstream of mGluR signaling leads to the changes in synaptic form and plasticity that are the proximal cause of many of the deficits present in FXS (Bear, Huber & Warren, 2004). Supporting this theory, acute pharmacological inhibition of mGluR5 and genetic reduction of mGluR normalize LTD and rescue a broad set of FXS-like phenotypes in mouse and fly models of FXS (Dolen & Bear, 2008; Aschrafi, Cunningham, Edelman & Vanderklish, 2005).

In brief, although research has shown that the absence of FMRP leads to widespread synaptic changes, the syndrome's complex pathogenic mechanisms mean that is not clear exactly which and how many synaptic proteins may be over-expressed in the FXS brain. Identifying and characterizing these proteins may be a crucial step towards developing mechanism-based pharmacological therapies for FXS. The current study expands upon previous research to examine a particular set of a proteins that exhibit altered expression in the *Fmr1* knockout mouse compared to wild-type.

Previous Research

In recent work conducted by the Vanderklish lab in collaboration with Dr. Lujian Liao and others, high-throughput proteomic methods were used to survey protein expression in *Fmr1* knockout and wild-type cortical synaptic fractions at two developmental time points (Tang et al., 2015). In this study, they used a method termed stable isotope labeling in mammal (SILAM) - whereby mouse whole brain fully labeled with 15N-enriched amino acids is used as a common internal standard - to achieve a broader and more precise quantification of protein changes in *Fmr1* knockout brain than had yet been achieved. Proteomic analysis refers to the systematic identification and quantification of proteins on a large scale. Using this method, the researchers identified nearly 5000 proteins, approximately 1000 of which were found to be altered in the *Fmr1* knockout mouse compared to wild-type, with the vast majority of changes being upregulation. In order to identify the most crucial components of this large set of proteins, the findings were subsequently compared with with databases of autism risk genes, postsynaptic proteins, and putative mRNA targets of FMRP.

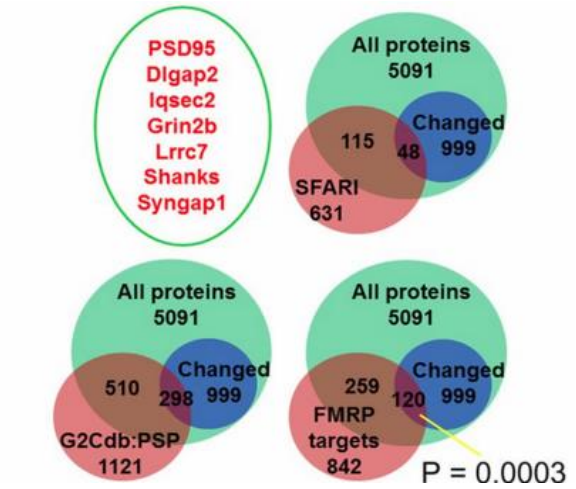

Figure 1. From Tang et al., 2015. Comparison of all proteins and significantly changed proteins in post-natal day 17 mice to SFARI autism risk gene database, gene-to-cognition database (G2Cdb:PSP) and to FMRP targets. P-value represents enrichment odds ratio determined by Chi-square test.

After utilizing each of these databases individually, the researchers crossed all three data sets with their own data set from the post-natal day (P17) mice. The result was a core set of synaptic proteins, all of which are encoded by known autism risk genes, are confirmed synaptic proteins, and whose mRNAs are known targets of FMRP. Importantly and strikingly, all postsynaptic proteins in this set belong to a common protein complex defined by (i) common interactions with the postsynaptic density scaffolding protein PSD-95, and (ii) coordinated regulation of the enzymatic activity of a protein called IQSEC2. This subset of proteins is shown in red font in Figure 1, and the functions of each member of the complex are summarized below. As IQSEC2 has been implicated in the regulation of mGluR LTD and LTP, the proteomic data prompted us to examine the "core complex" in greater detail.

Background on Proteins of Interest

PSD-95

The PSD (postsynaptic density) as a whole is a protein-dense region located at the postsynaptic membrane of excitatory synapses (Westmark, 2013). It organizes hundreds of proteins that are responsible for signaling, scaffolding, and otherwise modulating synaptic structure and activity. Postsynaptic density protein 95 (PSD-95) in particular is a scaffolding protein whose synthesis is modulated by mGluRs (Westmark, 2013). Additionally, it plays an important role in stabilizing synaptic changes that result from synaptic plasticity (Meyer, Bonhoeffer & Scheuss, 2014). Some research has also shown that PSD-95 is needed for developmentally appropriate synapse degradation and elimination, a process which is regulated by FMRP and autism risk genes (Tsai et al., 2012). Thus, in the absence of FMRP in FXS, PSD-95 may be abnormally expressed, leading to altered synaptic morphology and function.

DLGAP2 (SAPAP2)

The protein DLGAP2 (also known as SAPAP2) is a main component of postsynaptic scaffolding and directly interacts with PSD-95 and multiple SHANK proteins; this complex plays a crucial role in synaptic function. Rare mutations in DLGAP2 have been associated with autism spectrum disorders (Jiang-Xie et al., 2013). Furthermore, one study reports that DLGAP2 knockout mice display lower dendritic spine density and a reduction of scaffolding proteins in synapses (Jiang-Xie et al., 2013). Thus, up-regulation of DLGAP2 in FXS may be associated with the opposite phenotypes (recall that the FXS brain presents with higher than normal dendritic spine density).

IQSEC2

The gene for IQSEC2 encodes a guanine nucleotide exchange factor (GEF) for the ADP-ribosylation factor (Arf) of small GTPases. IQSEC2 also associates with PSD-95, and some research suggests that activity-dependent translation of IQSEC2 mRNA may contribute to synaptic plasticity (Sakagami et al., 2008). Thus, considering the role of FMRP as a translational suppressor, up-regulation of IQSEC2 in the knockout mouse may partially underlie the aberrations in synaptic plasticity present in FXS. Furthermore, mutations in IQSEC2 have been shown to cause non-syndromic ID (Shoubridge et al., 2010).

The IQSEC2-Arf6 pathway. IQSEC2 contains a particular domain that catalyzes the exchange of GDP for GTP on small GTP-binding proteins known as ADP ribosylation factors. ADP ribosylation factor 6 (Arf6) is a small GTPase that plays a role in membrane trafficking at excitatory synapses. This membrane trafficking - particularly the movement of neurotransmitter receptors to and from the postsynaptic membrane - is critical for synaptic plasticity (Sakagami et

al., 2008). Thus, the IQSEC2-Arf6 pathway may play a role in the synaptic plasticity dysfunctions present in FXS and ID in general.

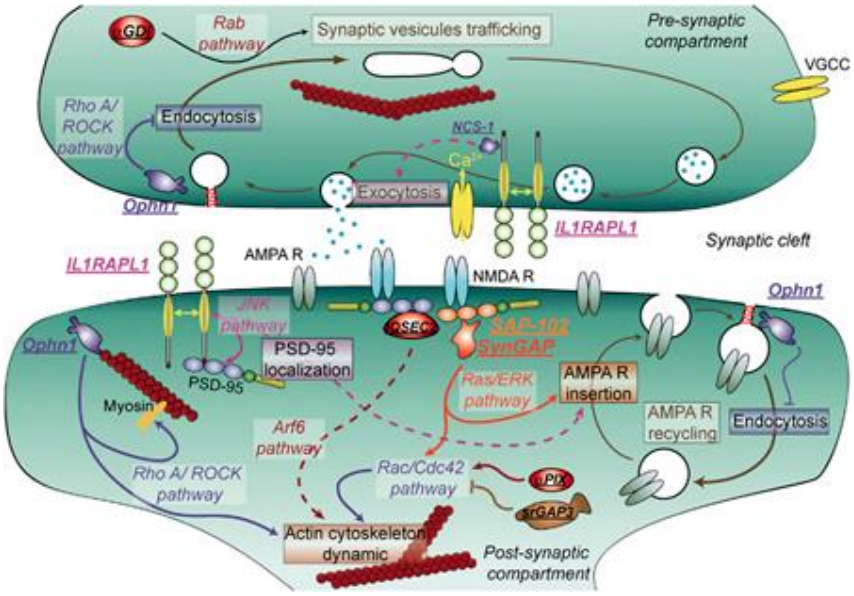

Figure 2. From Pavlowsky, Chelly & Billuart, 2012. Major molecular pathways found to be involved in intellectual disability. The IQSEC2-Arf6 pathway is visible in red.

GRIN2B (NR2B)

GRIN2B (also known as NR2B) encodes an NR2 subunit of N-methyl-D-aspartate receptors (NMDA-Rs), which are a major category of ionotropic glutamate receptors (Pan et al., 2015). GRIN2B also associates with PSD-95 and plays a crucial role in synaptic plasticity (Lim, Hall & Hell, 2002). Furthermore, large family studies of autism spectrum disorder have pointed to GRIN2B as a strong autism candidate gene (Iossifov et al., 2014; De Rubeis et al., 2014).

LRRC7 (Densin-180)

The LRRC7 gene (also known as Densin-180) codes for a brain-specific protein concentrated at the postsynaptic density. Research has shown that LRRC7 associates with PSD-95 in dendritic spines (Ohtakara et al., 2002). Furthermore, one study utilizing LRRC7 knockout mice found that the loss of the encoded protein resulted in abnormal behaviors characteristic of autism spectrum disorders (Carlisle et al., 2012).

SHANKS

SHANK proteins are multi-domain scaffold/adaptor proteins of the PSD that serve to connect neurotransmitter receptors and other membrane proteins to the neuronal cytoskeleton and signaling pathways. Additionally, SHANKS may play a role in regulating dendritic spine morphology, which is abnormal in FXS (Boeckers, Bockmann, Kreutz & Gundelfinger, 2002). Finally, like the proteins previously discussed, mutations in the Shank3 gene have been shown to result in autism spectrum disorder-like behaviors (Peca et al., 2011).

SynGAP1

SynGAP1 encodes a synaptic Ras GTPase-activating protein (RasGAP) concentrated in dendritic spines in cortical neurons. The protein acts to suppress signaling pathways linked to NMDA-R synaptic plasticity and AMPA-R membrane insertion (the IQSEC2-Ar6 pathway shares functions with SynGAP1) (Clement et al., 2012). Interestingly, SynGAP1 can either stimulate or suppress dendritic spine synapse function, thus possibly contributing to symptoms of ID (Clement et al., 2012). Finally, like the other proteins described, SynGAP1 associates with PSD-95 (Kim, Liao, Lau & Huganir, 1998).

The Current Study

Research Goals and Hypotheses

(1) Considering the fact that large sampling methods such as the proteomics method used in Tang et al (2015) have the inherent risk of yielding “false positives” - even when statistical measure to limit the false detection rate are applied - the first goal of this research is to confirm that this “core set” of proteins is in fact up-regulated in the *Fmr1* knockout mouse compared to wild-type via an independent measurement method.

(2) The second goal is to test the specific hypothesis that Arf6 activation by IQSEC2 is elevated in the *Fmr1* knockout mouse compared to wild-type.

(3) The third goal is to explore how Arf6 activation by IQSEC2 can be modulated by signaling through mGluRs using mGluR agonists and antagonists.

Methodology

Sample preparation. Brains of four *Fmr1* knockout and four wild-type mice at P17 were rapidly removed and plunged into a 15mL conical tube with cold, slushy Hank's Balanced Salt Solution (HBSS) (without calcium and magnesium) + 50 uM Leupeptin + 20 mM NaF + 100 uM Na_3V0_4. Cortex was dissected out in slushy HBSS + 50 uM Leupeptin + 20 mM NaF + 100 uM Na_3V0_4. Cortex was homogenized in 2 mL homogenization buffer + protease inhibitor + phosphatase inhibitor for 15 strokes. The resulting homogenate was spun at 700g for 5 minutes; the pellet (P1) was discarded and the supernatant (S1) was spun again at 13,000g for 10 minutes. The resulting pellet (P2) was resuspended in 300 uL of the homogenization buffer and frozen at -80 degrees Celsius. Finally, to create the lysed pellet fraction (LP2), P2 was resuspended in 500 uL lysis buffer, put through one freeze-thaw cycle, tip sonicated for 10 seconds, and spun at 14,000g for 20 minutes. The resulting LP2 was resuspended in 150 uL lysis buffer and frozen at -80 degrees Celsius.

Western blotting. Immunoblotting was used to independently measure the concentration of each protein of interest. Samples were equilibrated and boiled in a sodium dodecyl sulfate (SDS) buffer + 1.5 uL dithiothreitol (DTT) at 95 degrees Celsius for 5 minutes. Approximately 30 ug of protein was loaded per well on 4-12% NuPAGE Novex Bis-Tris Gels. Gels were run using 1X NuPAGE MES SDS Running Buffer, after which proteins resolved by molecular weight were transferred to PVDF membranes using a tank transfer method. After

blocking the membranes to prevent nonspecific binding of antibodies, membranes were probed with a 1:1,000 dilutions of antibodies to the proteins of interest, using alpha-Tubulin, Vinculin, or Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as normalization proteins. The blots were scanned and band intensities were measured using AlphaEaseFC software, followed by student's *t* tests to assess the statistical significance.

Synaptoneurosomes. Synaptoneurosomes (SNS) are subcellular preparations enriched in both resealed presynaptic and postsynaptic structures. They are particularly useful for studying rapid receptor-mediated signal transduction events, and for investigation of synaptic events mediated by postsynaptic mechanisms. SNS preparation utilized a protocol based on prior work by Hollingsworth et al. (1985), with minor modifications. Briefly, SNS were prepared using a filtration method with and without density gradient centrifugation using Percoll. Following preparation, Western blotting was utilized to confirm enrichment of postsynaptic components.

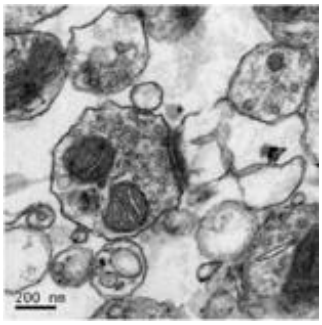

Figure 3. From Tang et al., 2015. Electron micrograph image of a synaptoneurosome. Presynaptic vesicles, the postsynaptic density, and other synaptic components are visible.

Filtration only method. Brains of 2 mice at P17 were immersed in iced homogenization buffer (HB) (124 mM NaCl + 4.25 mM KCl + 4 mM $MgSO_4$ + 0.5 mM $CaCl_2$ + 1.25 mM KH_2PO_4 + 10 mM glucose + 26 mM $NaHCO_3$ + 5 mM HEPES). Cortex was dissected out and chopped into small pieces before being placed in an iced homogenizer with 10 mL HB and homogenized for 20 strokes. The homogenate was then syringe-pressure filtered through 3 layers of 100 um membrane and a 10 um membrane. The resulting 10 um filtrate was spun at

1000g for 15 minutes before resuspending the pellet in 400 uL of the stimulation/experiment buffer (EB) (124 mM NaCl + 4.25 mM KCl + 1.25 mM $MgSO_4$ + 2.5 mM $CaCl_2$ + 1.25 mM KH_2PO_4 + 10 mM glucose + 26 mM $NaHCO_3$ + 5 mM HEPES).

Filtration with Percoll method. Following 10 um filtration, the filtrate was placed into microfuge tubes with 0.75 mL of 12% Percoll. Tubes were spun at 15,000g for 5 minutes. Membrane bands were extracted and all collected in separate tubes before being resuspended in EB to approximately 1.75 mL.

Stimulation with pharmacological agents. Dihydroxyphenylglycine (DHPG) is an agonist of group I metabotropic glutamate receptors (mGluRs) while 2-Methyl-6-(phenylethynyl)pyridine (MPEP) is an antagonist of mGluRs. SNS samples were pre-incubated for approximately ten minutes at 37 degrees Celsius using an O2, CO2 bubbler. 1 uM of tetrodotoxin (TTX) and 100 uM (2R)-amino-5-phosphonopentanoate (AP5) were added during the pre-incubation period.

In a thermomixer, stimulation tubes were set up each containing 75 uL of EB and either 100 uM DHPG or 40 uM MPEP. 75 uL of the appropriate SNS batch was aliquoted to each tube under gentle rotation. Samples were collected at 0 minutes, 1 minute, 3 minutes, and 10 minutes by adding 50 uL of an SDS buffer + 1 uL of 1M DTT and boiling at 95 degrees Celsius for 5 minutes.

Arf6 activation assay. The current study is still ongoing, and thus the Arf6 activation assay is still in progress. Arf6, like other small G-proteins, cycles between the inactive GDP-bound and the active GTP-bound states. The association of certain effector proteins with the GTP-bound state of Arf6 is the basis for Arf6's function. This specific association of effector proteins with Arf6-GTP can be exploited to monitor Arf6 activation (Cohen & Donaldson, 2010).

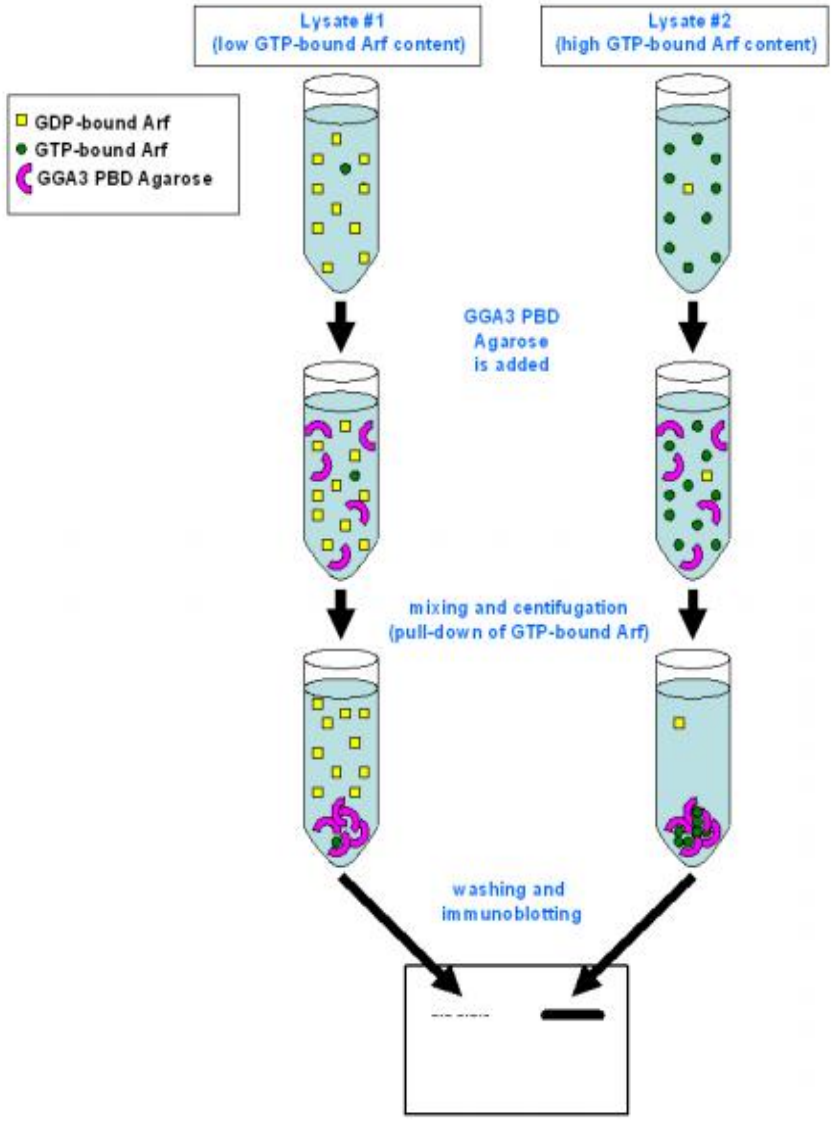

Figure 4. From Cell BioLabs, 2010. The Arf6 activation assay process is pictured here. Agarose beads selectively isolate and pull-down the active form of Arf6 for quantification by Western blotting.

Results

Western blot for validation. The following figures represent average band densities (+/- SEM) quantified in AlphaEaseFC and analyzed using Student's' *t* tests to compare expression of the proteins of interest between the *Fmr1* KO and the WT mice cortical synaptic fractions.

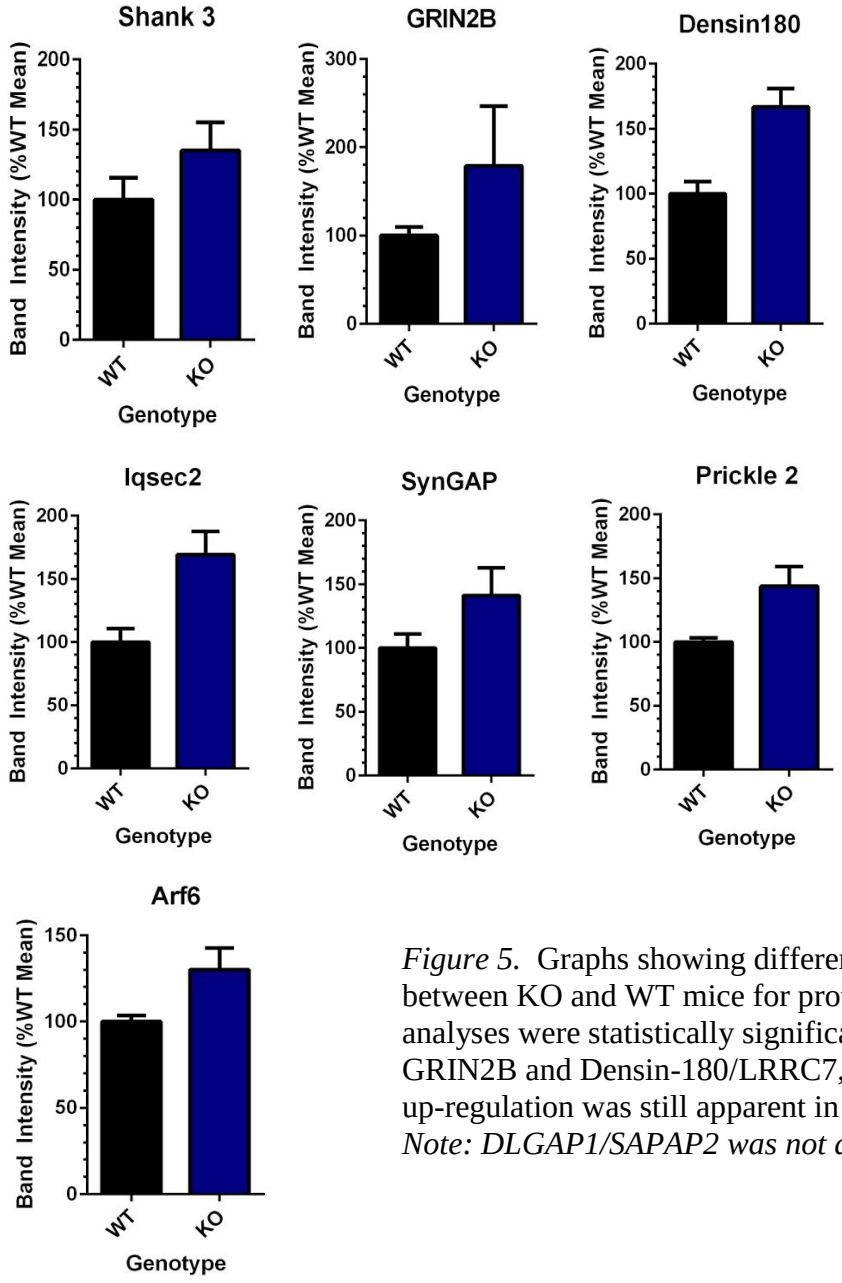

Figure 5. Graphs showing differences in band intensities between KO and WT mice for proteins of interest. All analyses were statistically significant ($p < 0.05$) except for GRIN2B and Densin-180/LRRC7, though a trend towards up-regulation was still apparent in these two proteins. *Note: DLGAP1/SAPAP2 was not analyzed.*

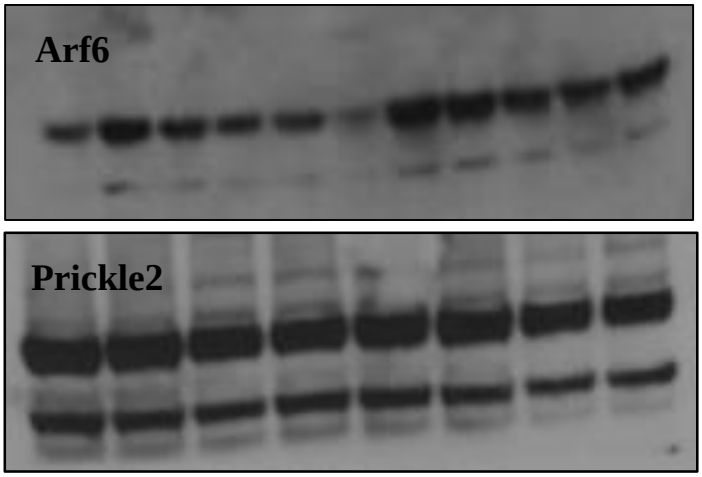

Figure 6. Images of Western blot films for Arf6 and Prickle2. The five bands to the left of center represent WT samples while the five bands to the right of center represent KO samples. Arf6 and Prickle2 were both normalized to the protein GAPDH.

Synaptoneurosome enrichment. In order to determine if the SNS preparations were suitable for use in stimulation experiments and the Arf6 activation assay, Western blotting was conducted to analyze the expression of common pre and postsynaptic proteins in the SNS samples. Increased band density in the final SNS samples compared to the homogenate alone would indicate that the SNS preparation procedure was successful in creating samples enriched in synaptic components.

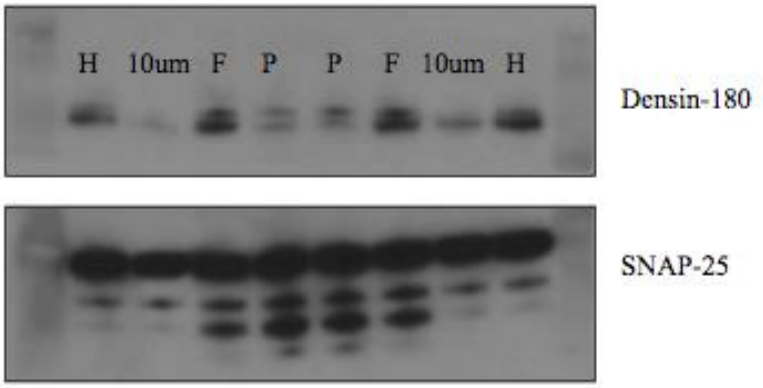

Figure 7. Western blot film showing enrichment of synaptic components in SNS preparation. The H lane denotes the homogenized brain without further processing, the 10um lane denotes the sample after filtration through 10um filter (but without further centrifugation and processing), the S lane denotes the final product of the filtration only preparation, and the P lane denotes the final product of the filtration with Percoll preparation. Densin-180 was used due to its high concentration in the postsynaptic density, and SNAP-25 was used due to its role as a crucial presynaptic protein. The band intensities demonstrate that the S lane was the most enriched with Densin-180, while the P lane appears most enriched with SNAP-25. Though it is unclear which SNS preparation is more effective, it is clear that both methodologies were effective in enriching synaptic components; band intensities of the final preparations are darker than the band intensities of H and 10um.

Stimulation experiments and Arf6 activation assay. Experiments utilizing SNS to study Arf6 activation in *Fmr1* knockout and wild-type mice (both at baseline and in response to mGluR stimulation) are still ongoing, and thus results are not available at this time.

Discussion

This study expands upon proteomic research investigating cortical synaptic protein changes in the *Fmr1* knockout mouse. Through this research, a core set of postsynaptic proteins operating in a common signaling cascade associated with PSD-95 was shown to be up-regulated in the *Fmr1* knockout compared to wild-type in P17 mice. Considering these proteins' roles in synaptic structure, organization, and plasticity, their abnormal expression in synapses lacking

FMRP provides some insight into how the abnormal molecular, morphological, and behavioral phenotypes in FXS are formed. Additionally, the fact that these proteins have also all been identified as autism risk genes indicates that abnormalities in PSD-95-based signaling modules may underlie some autism phenotypes as well. However, though up-regulation of proteins in adolescent mice has been characterized, research has shown that these protein changes are diminished in adult mice (Tang et al., 2015). Thus, the influence of FMRP on the synaptic proteome appears to be age dependent. However, one electrophysiological study found that in young adult knockout mice, NMDA-R-mediated LTP is normal, while in older mice this LTP is impaired (Martin, Lassalle, Brown & Manzoni, 2016). Clearly, more investigation is needed into how age is associated with synaptic changes in FXS.

Expanding upon the finding that the proteins of interest in the current study are up-regulated in FXS, the IQSEC2-Arf6 pathway may be overactive in *Fmr1* knockout synapses. If confirmed, this finding would lend more credence to the hypothesis that altered synaptic plasticity via skewed AMPA-R internalization may be an important underlying molecular mechanism in both FXS and autism. Previous research has already shown that up-regulated synaptic proteins in the knockout mouse exhibit higher rates of *de novo* synthesis as a result of stimulation of mGluRs (Tang et al., 2015). Ideally, the current research will expand upon this finding by stimulating synaptoneurosome preparations with mGluR agonists prior to analyzing activation of Arf6. This particular procedure will ideally shed light on how glutamatergic signaling may modulate the IQSEC2-Arf6 pathway.

Considering the continued prevalence of the mGluR theory of FXS, most research has focused on the pathologies of glutamatergic synapses. Some research has shown that mGluR inhibition can correct some FXS phenotypes in mice. For example, Michalon et al. (2012)

utilized the potent mGlu5 inhibitor CTEP. Results showed that acute treatment with CTEP in adult knockout mice corrected elevated hippocampal LTD, some protein synthesis changes, and audiogenic seizures. Furthermore, chronic CTEP treatment corrected aberrant dendritic spine density and cognitive deficits. Following these findings, multiple pharmaceutical companies began aggressively developing mGlu5 inhibitors and related compounds that might be efficacious in the treatment of humans with FXS (Landhuis, 2012). Unfortunately, most of these trials have been unsuccessful. Most notably, as of 2014, the company Novartis has discontinued the development of its forerunning mGlu5 antagonist, AFQ056, following negative results in a large international clinical trial in adults (Clapp, 2014). Disappointingly, it seems that promising preclinical studies do not necessarily translate to effective treatments; it is clear that more research investigating possible drug therapies is needed. Furthermore, considering the age-dependent findings, the developmental period should be considered in pharmacological research.

As previously discussed, the IQSEC2-Arf6 pathway is hypothesized to influence mGluR-mediated synaptic plasticity via regulation of intracellular membrane trafficking. Despite the fact that mGluR inhibition with drugs has been largely unsuccessful in humans with FXS, this pathway provides a further opportunity for pharmacological intervention that may influence glutamatergic signaling without targeting mGluRs directly. In order for Arf6 to cycle between its GDP and GTP-bound states, cytohesins - classified as guanine nucleotide exchange factors (GEFs) - must also be active. Interestingly, research has implicated a cytohesin inhibitor known as Sec 7 inhibitor H3 (secinH3). Azreg, Garceau, Harbour, Pivot-Pajot and Bourgoin (2010) found that cytohesin inhibition with secinH3 resulted in Arf6 but not Arf1 inhibition, demonstrating the molecule's specificity for Arf6. Though FXS research has not yet utilized secinH3, other research has demonstrated the molecule's role in inhibiting tumor angiogenesis.

Mice treated with secinH3 in Hongu et al. (2015) also appeared healthy during the 16 days of treatment, preliminarily suggesting that secinH3 does not have short-term toxicity. Utilizing secinH3 in FXS research could possibly be an important step towards developing mechanism-based drug therapies to FXS. A similar protocol as described in the current study (i.e. using synaptoneurosome preparations and Arf6 activation assays) after treatment of samples with secinH3 could be employed.

Overall, the current research confirms preliminary findings that particular synaptic proteins are abnormally expressed in the *Fmr1* knockout mouse compared to wild-type. In addition to mediating various aspects of synaptic structure and transmission, these proteins are also autism risk gene products and known targets of FMRP. More intriguingly, they operate in a common signaling cascade associated with PSD-95 in glutamatergic synapses that may mediate intracellular membrane trafficking. The ongoing investigation will shed light on how this signaling cascade may function abnormally in FXS, thus providing insight into resulting synaptic plasticity abnormalities. Furthermore, this study points to multiple avenues for further research, including the investigation of age-dependent changes and novel pharmacological therapies.

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