Caprin1 and *Fmr1* Genetically Interact to Regulate the Development of the Larval *Drosophila* Neuromuscular Junction

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Abstract

Fragile X Syndrome (FXS) is the most prevalent inherited neurodevelopmental disorder and the most common single-gene cause of autism[1]. FXS occurs due to the loss of the *Fmr1* gene and its protein product, the Fragile X Messenger Ribonucleoprotein (FMRP). FMRP is an RNA-binding protein (RBP) with notable functions in synaptic development. Given that cellular processes often entail the collaborative actions of multiple proteins acting as binding partners to regulate mRNA metabolism, identifying FMRP's associates is essential for comprehending FXS mechanisms. Caprin1 was identified as a high-confidence interactor via its co-immunoprecipitation with FMRP in an IP/LC experiment done in the Barbee laboratory, among others[2,3]. The objective was to determine if *Caprin1* and *Fmr1* genetically interact to regulate synaptic development of the neuromuscular junction (NMJ) in *Drosophila melanogaster (fruit flies). D. melanogaster* is a well-understood model organism for studying FXS because it shares many similarities with human FXS[4]. To determine if a molecular interaction between the RBPs existed, a genetic cross of *D. melanogaster* with mutated copies of *Fmr1* and *Caprin1* was created. The analyzed synapses were analogous to the overgrowth seen in the FXS brain. *Fmr1* and *Caprin1* were determined to be genetic interactors in the regulation of synaptic development in the *D. melanogaster* NMJ. These findings offer a glimpse into the intricate molecular processes governing neurodevelopment in the presence of mutated *Fmr1* and *Caprin1* genes, providing valuable insights into their contributions to the molecular pathology of FXS. Future experiments should aim to determine the function of Caprin1 within neurons and examine the biochemical interaction between FMRP and Caprin1 proteins.

Keywords: *Fragile X Syndrome, Caprin1, FMRP, neuromuscular junction, synaptic development*

1 INTRODUCTION

1.1 Fragile X Syndrome

Fragile X Syndrome (FXS) is the most prevalent inherited cause of mild to severe intellectual disability. It is the leading single-gene cause of autism[1]. The syndrome results in lifelong neurological and behavioral impairment, affecting approximately 1 in 4,000 males and 1 in 7,000 females[1]. About one-half of X-linked intellectual disability cases are the result of FXS. FXS is associated with features such as intellectual disability, social and communication difficulties, hyperactivity, repetitive behaviors, and physical characteristics like an elongated face and large ears. FXS is caused by a mutation in the *Fmr1* gene. The mutation consists of a trinucleotide repeat expansion, CGG, which elicits epigenetic silencing. The expanded repeats cause hypermethylation extending from the 5′ untranslated region (UTR) of the *Fmr1* gene into the promoter region. This loss-of-function mutation prevents the production of Fragile X Messenger Ribonucleoprotein (FMRP), the protein product of Fmr1. The full mutation occurs when there are over 200 CGG repeats. A premutation carrier will have between 55-200 CGG repeat expansion and be at risk of developing fragile X tremor-ataxia syndrome (FXTAS) if male and fragile X-associated primary ovarian insufficiency (FXPOI) if female.

1.2 Function of FMRP

FMRP serves functions in mRNA regulation and metabolism, translation and ribosome stalling, RNA editing and modifications, regulated transport, and DNA damage response[1]. FMRP is classified as an RNA-

binding protein (RBP). RBPs regulate all aspects of gene expression including mRNA trafficking, splicing, stability, and translation[5]. While FMRP plays diverse roles, it is best characterized as a translational repressor[5]. FMRP possesses conserved RNA binding motifs that can directly interact with mRNA and physically obstruct ribosomes from accessing it[5]. Conversely, FMRP can also interact with various proteins involved in different stages of translation. FMRP can also sequester mRNA and ribosomal components thereby spatially repressing translation by removing the necessary constituents out of the available pool[6]. Adding an additional layer of control, FMRP can either enhance or suppress the silencing effects of miRNA[7]. FMRP is implicated in synaptic plasticity, dendritic spine development, and overall neuronal maturation. The constant formation and elimination of synapses during development requires thousands of proteins to cooperate to achieve proper neuronal communication and functional neural networks. Thus, FMRP's modulatory action on protein synthesis (and its potential interactors) is important for the pruning of synapses during development and for alterations via synaptic plasticity later in life. Its dysfunction in FXS can lead to impaired synaptic plasticity and contribute to the neurological and cognitive deficits associated with the disorder.

1.3 Structure of FMRP

FMRP contains several functional domains, consisting of two K homology domains (KH) and one arginine glycine-rich region (RGG) box[8]. These domains are involved in RNA binding and interactions with other RBPs. Through these interactions, FMRP plays a critical role in the localization of mRNAs within neurons, especially in dendritic spines, contributing to the regulation of local translation and synaptic plasticity. FMRP is known to engage in various RBP interactions, including associations with both nucleolar proteins FXR1P and FXR2P. FMRP also interacts with cytoplasmic proteins outside of the nucleus like CYFIP1 and CYFIP2[9]. The resulting complexes form heterodimers indicating a direct protein-protein interaction[10]. Forming larger functional complexes is typical of most RNA-binding proteins, such as FMRP, as they tend not to operate in isolation. Functional complexes are involved in various cellular processes, including mRNA translation and synaptic function[11]. The absence or malfunction of FMRP, along with altered interactions with its binding partners, can lead to synaptic and neuronal dysregulation contributing to the characteristics of FXS.

1.4 Localization of FMRP

The precise localization of FMRP plays a pivotal role in its ability to interact with RNA molecules, other proteins, and cellular structures. FMRP is known to exhibit a dynamic pattern of localization, reflecting its diverse functions in cellular processes. Predominantly, FMRP localizes in the cytoplasm, but also is detectable in the nucleolus[12]. The binding of specific mRNAs by FMRP can direct its transport to designated subcellular compartments along with activity dependent mechanisms. It is abundantly found in dendritic spines, the small protrusions on neurons where synaptic connections occur[12]. The precise positioning of FMRP at synapses is critical to its role of regulating translation, synaptic plasticity, and overall neuronal function.

1.5 Liquid-liquid Phase Separation Properties of FMRP and other RBPs

Recent studies have established that FMRP regulates the formation of several types of RNA/protein granules, including messenger ribonucleoprotein particles (mRNPs) transport granules, fragile X granules (FXG), P-bodies, and stress granules[7]. The ability of FMRP to undergo liquid-liquid phase separation (LLPS) may contribute to the formation and dynamics of stress granules, which are transient aggregates of mRNA, RBPs, and initiation factors that form in response to cellular stress[7]. The phosphorylation state of FMRP influences both stability and binding affinity, dictating associations with various RBPs and mRNA. Phosphorylated FMRP more readily phase separates with mRNA into the liquid droplets' characteristic of LLPS. LLPS is crucial for targeted delivery and localized translation of mRNAs. Within the neuron this is particularly important because specific proteins, like FMRP, need to be brought to specific locations, like dendrites and axons, to perform specific processes required for proper neuronal function. Thus, FMRP associates with mRNA and other RBPs inside of LLPS particles for trafficking to the synapse. The phase separation capability may enhance spatial and temporal control of FMRP's interactions with mRNA molecules, ribosomal components, and other binding partners ultimately influencing cellular processes such as translation and synaptic plasticity.

1.6 FMRP and Synaptic Plasticity

FMRP reportedly binds to approximately 4% of all mRNAs in neuronal tissue[13]. Thus, a lack of FMRP's repressive action leads to the enhanced translation of many messages in the central nervous system. One of these systems is the metabotropic glutamate receptor 5 (mGluR5), a Type 1 mGluR. An enhanced mGluR5 pathway contributes to long-term depression (LTD). Specifically, FMRP acts as a translational repressor of mRNA downstream of Type 1 mGluR activation. In its absence, there is excessive mGluR-dependent protein synthesis[14]. LTD causes weak and immature synaptic connec-

tions hindering synaptic plasticity, synaptogenesis, and pruning[13]. Synapses are the basis for neural circuitry and are essential to neuronal function, communication, and enabling of complex behaviors. Without FMRP regulating synaptic development, especially in the postnatal period, synapses become overgrown due to the lack of "connection pruning"[15]. Likewise, long-term plasticity (LTP) requires activation of Type 1 mGluRs and is dependent on protein synthesis under the control of FMRP just like LTD[11]. Thus, FMRP is known as a "master regulator" and "molecular switch"[16] as its regulatory activity is necessary for the processes of synaptic development and maintenance both early on and later in life. At its core, FMRP acts as a versatile regulator, exerting its influence at multiple levels of gene expression control, including RNA binding, translation regulation, mRNA transport, and synaptic plasticity.

1.7 Regulatory Control of FMRP

Another feature of FMRP and its regulatory control is its heavy influence on dendritic spine development via FMRP-mediated mRNA transport. Consequently, the deficiency of FMRP in individuals with FXS results in abnormal synaptic morphology[17]. A distinctive pattern of immature synapses manifests at the dendritic spine, marked by increased density, abnormal morphology, altered synaptic plasticity, and impaired synaptic maturation. The FXS synapse appears longer, thinner, and more filopodia-like compared to mature, mushroom shaped spines observed in typical development. These structural changes are thought to be related to impaired synaptic pruning, a process crucial for the refining and optimizing of neuronal circuits during development[17]. In FXS, this process is disrupted, and synapses remain in a more immature state impacting the overall functioning of neurons. Increased synaptic terminal bouton count and extensive branching occur within the overgrown, immature synapses compared to the pruned and distinctly mature synapses of a healthy wildtype. These features contribute to the neurobiological basis of the cognitive and behavioral challenges observed in individuals with FXS. Understanding the specific characteristics of immature synapses is crucial for developing targeted interventions that address synaptic abnormalities.

1.8 Determining RBP Interactors of FMRP

The function of FMRP can be determined by its interactions with other RBPs. However, most of these other interacting RBPs are not known. It is important to understand the associations of proteins because it provides insights into their specific cellular functions. Immunoprecipitation is a widely used laboratory technique that allows for selective precipitation of a specific protein or protein complex from a mixture. This technique is the standard for studying protein-protein interactions and identifying possible binding partners. After the proteins are eluted by immunoprecipitation and found to be bound together, they are then identified using techniques such as mass spectrometry. The Barbee laboratory at the University of Denver performed an FMRP-co-immunoprecipitation experiment of the adult *D. melanogaster* head, enriched for neuronal tissue. The precipitated proteins bound to FMRP were identified by liquid chromatography-mass spectrometry (LC/MS). Caprin1 was identified as a high-confidence interactor.

1.9 Caprin1

Caprin1 is an evolutionary-conserved, ubiquitously expressed cytoplasmic phosphoprotein. It is classified as an RBP indicating its role in binding and is characterized by different domains including an RNA binding domain and a coiled-coil domain. These domains suggest its involvement in RNA metabolism and protein-protein interactions. Importantly, Caprin1 has been implicated in several cellular processes including the regulation of mRNA translation and stress granule formation like FMRP[7]. It is thought that the Caprin1/G3BP1 complex regulates the transport and translation of mRNAs. These mRNAs encode proteins involved in synaptic plasticity, as well as cellular migration and proliferation across several cell types[18]. Caprin1 is specifically found packaged with other RBPs in postsynaptic granules located at neuronal dendrites[18]. As such, Caprin1-mediated dendritic localization of mRNAs has been suggested as an underlying mechanism for AMPA receptor scaling that occurs in response to varying increases and decreases in neuronal activity[19]. Altering the density of AMPA receptors contributes to overall synaptic strength, plasticity, and proper function. In essence, Caprin1 serves as a key player in the orchestra of synaptic plasticity, shaping the brain's ability to learn, adapt, and remember by acting as a central regulator and molecular taxi.

1.10 FXS Modeling

D. melanogaster, commonly known as the fruit fly, is a well-established model organism for the study of neurodevelopmental disorders including FXS. Many fundamental genetic pathways and molecular processes are conserved between *D. melanogaster* and humans. The *Fmr1* gene and its protein product FMRP have functional equivalents in *D. melanogaster*. When the *D. melanogaster* ortholog of FMRP is lost it exhibits many shared cellular and behavioral phenotypes with human patients[4]. One of these shared phenotypes is significant overgrowth of the larval NMJ, a phenotype characteristic of hippocampal synapses in human FXS patients[17].

Since synaptic dysfunction is a hallmark of FXS, the *D. melanogaster* NMJ proves useful for investigating altered synaptic structure and function in a non-human model[4]. Henceforth, this phenotypic trait allows insights into precisely how FMRP may influence synaptic plasticity at the molecular level. Zooming in, the A3 segment NMJ along muscle 6 and 7 is a well-studied model system for investigating the development and function of synapses in the *D. melanogaster* nervous system. It is easily accessible for experimental manipulation due to its positioning on the ventral side of larvae. The transparency of *D. melanogaster* larvae allows for visualization of NMJ structures using light microscopy and fluorescent staining.

1.11 FMRP and Caprin1 as Potential Interactors

A plethora of literature has established that Caprin1 and FMRP colocalize and directly interact in non-neuronal tissue[20;21;22;2;23]. Caprin1 and FMRP interact in systems such as the *D. melanogaster* ovary, mammalian embryo, and in vitro. Namely, the analysis of Caprin1 and FMRP crystal structures shows that they have a direct protein-protein interaction that is not RNA-dependent. It appears they bind to RG-rich sequences in the RGG box located on both proteins[22]. Equally important, Caprin1 was co-immunoprecipitated with FMRP in multiple laboratories beyond the Barbee laboratory[2;3]. It is believed that the interaction between Caprin1 and FMRP is important in the control of mRNA metabolism, particularly its translation into proteins[3]. Defects in these processes are directly linked to FXS. However, there is a gap in knowledge of how precisely the dysregulation of translation causes the neurodevelopmental defects that are characteristic of FXS. Thus, Caprin1 is an RBP of interest as it may have important interactions with FMRP at the genetic level in *D. melanogaster* neurons.

Here we ask if a genetic interaction between *Caprin1* and *Fmr1* exists. Secondly, if an interaction does exist, is it of biological relevance within the *D. melanogaster* neuron? Lastly, does their combined genetic interaction control for FXS phenotypes such as synaptic overgrowth. These experiments are based on the prediction that no effect on NMJ development will be observed when a single copy of *either* gene *Fmr1* or *Caprin1* is lost within a single mutant, as the remaining copy will be enough to rescue the phenotype, as is typically observed. Therefore, to determine if there is a genetic interaction, a double mutant cross that has lost *both* a copy of *Fmr1* and *Caprin1* will be reared and examined for genetic interplay predicted to result in immature, overgrown synapses. Thus, it is hypothesized that *Fmr1* will genetically interact with *Caprin1* to regulate synaptic development of the larval NMJ. This interaction will be discerned by observable altered morphology in comparison to both wildtype and single mutant lines.

2 METHODOLOGY

2.1 *D. melanogaster* Stocks and Crosses

Fly lines and genetic crosses were maintained at 25ºC on standard Bloomington media. Genetic lines used included *Iso31,w*Sb/Tm^{3}SerGFP, FRT80B,capr^2/Tm^2, Fmr1$^{\Delta 50}$/Tm^{6}TbSb,FRT80B,capr^2/Tm^{3}SerGFP*. The *Caprin1* line was created by crossing *w*Sb/Tm^{3}SerGFP* and *FRT80B,capr^2/Tm^2*. A male and virgin female with the genotype *FRT80B,capr^2/Tm^{3}SerGFP* were selected and crossed from the F1 generation to establish the *Caprin1* line. The double mutant was selected from virgin *Caprin1* females with the genotype *FRT80B,capr^2/Tm^{3}SerGFP*, and males from the *Fmr1* line with the genotype *Fmr1$^{\Delta 50}$/Tm^{6}TbSb*. The denoted double mutant genotype is *FRT80B,capr^2/ Fmr1$^{\Delta 50}$*. All crosses contained 15 virgin females and 5 males. There were three negative controls used: a wildtype, a single *Caprin1* mutant over wildtype, and a single *Fmr1* mutant over wildtype. They are denoted as follows *Iso31 (+/+), FRT80B,capr^2/Iso31 (Caprin1/+), and Fmr1$^{\Delta 50}$ /Iso31 (Fmr1$^{\Delta 50}$/+)*.

2.2 Neuromuscular Junction Microdissections and Immunofluorescent Staining

Larvae of *D. melanogaster* at the wandering third instar stage were dissected in Jan and Jan buffer, a calcium-free saline solution. The larval body wall preps were fixed with 3.5% paraformaldehyde (PF) and then washed in 1x-phosphate buffered saline (PBS). The preps were incubated overnight with mouse -DLG, the primary antibody, for immunofluorescent tag attachment. The 1° antibody to block dilution was 1:100. The next day the preps were washed in PBS containing 10% Triton X-100 (PBS-T) and incubated for 1 hour with the secondary antibodies anti-mouse IgG Alexa488, a green-fluorescent tag, and anti-HRP Alexa633, a blue-fluorescent tag. The 2° antibodies to block dilution was 1:500. Antibodies were sourced from Developmental Studies Hybridoma Bank, anti-discs large. Lastly, preps were mounted in DAPI Fluoromount-G (Southern Biotech) and stored at -20°C until imaged.

2.3 Imaging and Statistical Analysis

Images of the NMJ at segment A3 m 6/7 were obtained from scanning confocal microscopy using the Olympus Fluoview FV3000 with 60x objectives (N.A. = 1.42). Using the Olympus FV software, the entire NMJ was imaged using 0.4-micron optical sections. All channels were manually adjusted to the threshold prior to imaging. A Z-projection was assembled from all optical sections in ImageJ. Analysis was performed using Image J software with the cell counting plugin. Type 1s glutamatergic boutons and type 1b glutamatergic bou-

tons were manually counted and differentiated based on size and intensity of DLG staining. Type 1b (big) boutons are distinguished from type 1s (small) boutons due solely to their difference in size. Additionally, their respective branch tips were quantified. The immature phenotype associated with FXS, characterized by overgrown synapses with extended branching and increased bouton count, is expected to be observed in the double mutant. The bouton analysis was blinded. All data were first recorded in Microsoft Excel. Statistical analysis was performed in GraphPad Prism. Because there was a significant difference in standard deviation between groups, the statistical test used was the non-parametric Kruskal-Wallis ANOVA. Secondly, Dunn's multiple comparisons test was performed. Such tests were necessary to delineate a true relationship between variables since the collected data is not of normal distribution. Results were considered significant at $p < 0.05$. The results shown are mean ± SEM. n.s. = not significant, * $p < 0.05$, **$p < 0.01$, ***$p<0.001$, ****$p < 0.0001$. Fifteen NMJs (n=15) were analyzed in each set for a total of 60 NMJs.

3 RESULTS

3.1 *Caprin1* Genetically Interacts with *dFmr1* to Regulate Development of the Larval NMJ

The dysregulation of FMRP-mediated mRNA translation is a prominent feature in fragile X syndrome. However, the detailed mechanistic understanding of how this dysregulation occurs remains incomplete. FMRP is known to engage in documented interactions with other RNA-binding proteins (RBPs) and general cytoplasmic proteins, yet not all its interactors have been determined[9]. Caprin1 was identified as a possible binding partner of FMRP during their combined immunoprecipitation. Since they precipitated together and FMRP has notable roles in synaptic plasticity, the objective became to determine if *Caprin1* and *Fmr1* interact genetically to regulate the typical FXS *D. melanogaster* NMJ phenotype. The *D. melanogaster* FXS model uses loss-of-function mutants[24]. *Fmr1* nulls commonly exhibit enlarged synaptic terminals and increased frequency, indicating structural abnormalities associated with altered synaptic transmission[24]. To investigate this in the case of *Caprin1* and *Fmr1* genetic interactions, a double mutant animal was reared. The double mutant is defined by single deficient copies of both *Fmr1* and *Caprin1* within the same animal, a trans-heterozygote. The procedure began with NMJ dissections, immunofluorescent staining, then imaging by scanning confocal microscopy. The double mutant of interest was compared to three negative control lines: solely single mutant *Fmr1* and *Caprin1* sets and a wild-type set. Completely deficient *Caprin1* and *Fmr1* homozygotes were found to be lethal before the 3rd in-

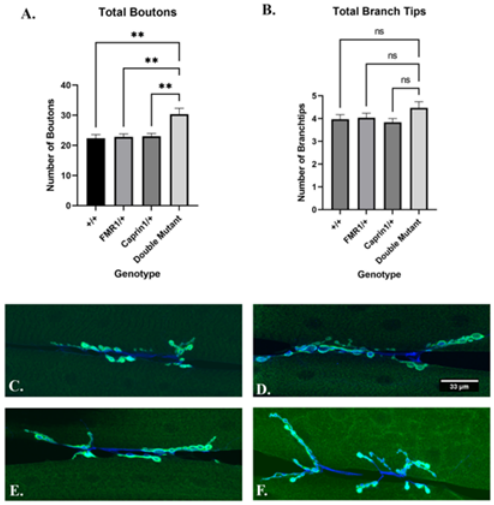

Figure 1. Post-synaptic glutamatergic boutons stained with αDLG appear green. The intensity of the green anti-mouse IgG Alexa488 staining is dependent on bouton type; Type 1b versus Type 1s. Type 1b appears more intense and Type 1s appears less intense. Blue stain represents anti-HRP Alexa633 pre-synaptic terminal staining. Fifteen NMJs (n = 15) were analyzed in each of the four sets, total (n = 60). The scale bar denotes 33 microns. (A) Graphical representation of total glutamatergic boutons present, Type 1b and 1s. The double mutant was significantly overgrown with a higher total count of both types of synaptic boutons, **p = 0.0004. (B) Graphical representation of total branching quantified by branch tip count. There was no significant difference found in branch tip complexity. (C) Wildtype, +/+ (D) *Fmr1* heterozygote, *Fmr1*/+ (E) *Caprin1* heterozygote, *Caprin1*/+ (F) Double mutant, trans-heterozygote, *Fmr1/Caprin1*.

star larval stage and could not be dissected nor used as positive controls. A Kruskal-Wallis non-parametric ANOVA test and Dunn's multiple comparison test were performed for both total synaptic bouton and branch tip count. Fifteen NMJs were imaged in each set n = 15; +/+, *Caprin1*/+, *Fmr1*/+, and the double mutant. A total of 60 NMJs were analyzed. The difference between total synaptic boutons, indicative of synaptic overgrowth, of the negative controls and double mutant was found to be significant with **$p < 0.0004$ (Panel 1A). The images produced showed a distinct morphological difference between the double mutant and single mutant controls (Panel 1C-F). Including increased synaptic bouton frequency and overall shrunken size. Both Type 1b and 1s boutons were visibly smaller in the double mutant compared to controls (Figure 2). Increased branching of the NMJ is another phenotype observed in Fmr1 homozygotes[24]. Although, branch tip complexity was not found to be significantly different between the double mutant and single mutants of this experiment (Panel 1B).

4 DISCUSSION

Fragile X Syndrome is a well-characterized disease; however, its underlying molecular mechanism remains

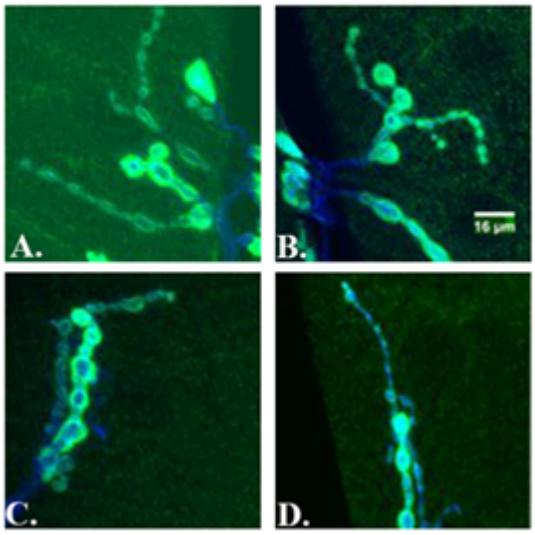

Figure 2. Above displays both Type 1b and 1s boutons located on the terminal branches of each genotype. The double mutant had visibly smaller Type 1b and 1s boutons, a distinct morphological difference. All images are zoomed to 16 microns in scale. (A) Wildtype, +/+ (B) *Fmr1* heterozygote, *Fmr1*/+ (C) *Caprin1* heterozygote, *Caprin1*/+ (D) Double mutant, trans-heterozygote, *Fmr1/Caprin1*.

largely unknown. There is still a lack of knowledge surrounding the relationship between genes that control synaptic development in *D. melanogaster* neurons. The study at hand aimed to reveal a possible genetic interaction between RBPs and their role in FXS within *D. melanogaster* neurons. The majority of Caprin1 research has been studied in mammalian models. Prior to the results presented in this thesis, it was unknown whether Caprin1 served a function in *D. melanogaster* neurons. The current study has established *Fmr1* and *Caprin1* as genetic interactors that co-regulate the neurodevelopment of synapses at the neuromuscular junction in *D. melanogaster* neurons. Changes in bouton size and frequency are common morphological differences observed in FXS. On the genetic level, the pathology of FXS is caused by two mutant copies of *Fmr1* and the subsequent inability to produce FMRP. When FMRP is not produced alterations in synaptic morphology are observed. However, the double mutant in this experiment was only given one deficient copy of each of the *Caprin1* and *Fmr1* genes. Therefore, under typical circumstances, the offspring would not be considered to have FXS because it does not have two deficient copies of *Fmr1*. Nevertheless, if there is a genetic interaction between *Caprin1* and *Fmr1*, one deficient copy of each should be sufficient to induce a phenotype characteristic of abnormalities witnessed in FXS. Since there was synaptic overgrowth (Panel 1E), measured by the increased number of synaptic boutons compared to negative controls (Panel 1A), a genetic interaction between *Caprin1* and *Fmr1* must be occurring. Differences in bouton size were also noted within the double mutant displaying smaller type 1b and 1s boutons (Figure 2). The overall increase in bouton number may suggest a compensatory response or regulatory mechanism aimed at maintaining synaptic connectivity despite the reduction in size. This phenomenon could be indicative of various processes occurring at the synaptic level, such as synaptic pruning, changes in plasticity, or alterations in neurotransmitter release and reception. It was possible that branching would be affected as it is typically increased in FXS, but that was not observed as an overall trend here (Panel 1B). Therefore, by quantification of boutons it was determined that the synapses were overgrown, as is expected in FXS. The overgrown synapses observed suggest alterations in synaptic development and impaired neuronal functioning characteristic of FXS. In conclusion, *Caprin1* and *Fmr1* genetically interact to regulate synaptic development of the *D. melanogaster* NMJ. This finding is in line with other research showing *Fmr1*'s and *Caprin1*'s interaction and co-regulation of different processes in other cell types and systems[20;21;22;2;23].

Many Caprin1 and FMRP immunoprecipitation experiments have already strongly supported a genetic interaction between these genes and their products[2;3]. Subsequently, it has been consistently proposed that protein interactors modulate FMRP functions[22]. Since both FMRP and Caprin1 have previously been implicated in synaptic plasticity separately, their combined role specifically in *D. melanogaster* synaptic growth is within reason and would describe the interaction observed here[18;7;19;21]. Understanding the interactions between *Caprin1* and *Fmr1* in the regulation of larval *D. melanogaster* NMJ development can provide insights into the molecular mechanisms governing synaptic connectivity and plasticity. This research may contribute to our understanding of neurodevelopmental processes and potentially bring clarity to conditions linked with synaptic dysfunction, such as fragile X syndrome.

The literature provides further intriguing insights into the potential interaction between Caprin1 and FMRP, as revealed by nuclear magnetic resonance spectroscopy (NMR) studies. These investigations indicate that phase-separated condensates containing FMRP and Caprin1 proteins interact via the arginine-rich and aromatic-rich regions of both proteins[22]. Caprin1's structure includes two HR-2 binding domains and three arginine-rich (RGG) boxes[18]. Upon binding FMRP, an integral α-helix is formed. In the context of the research at hand, these structural alterations highlight the dynamic nature of Caprin1 and FMRP interaction, suggesting a potential regulatory role in the cellular processes under investigation. The synergy observed between FMRP, Caprin1, and G3BP1 forming a functional complex that is evolutionary conserved, underscores

the complexity of their interplay[21]. Moreover, the observed phase separation of phosphorylated FMRP with Caprin1 into neuronal stress granules adds a layer of complexity to their interaction dynamics, which may be pertinent to the stress response in the context of this paper[7]. These findings contribute to a deeper understanding of the molecular intricacies that underlie the functions of and connections between Caprin1 and FMRP in cellular processes.

Studies have revealed a collaborative relationship between FMRP and Caprin1 in the regulation of the mid-blastula transition (MBT). Both were found to associate with *CycB* and *frs* mRNAs and function as activators of their translation, while at the same time repressing the translation of other cell cycle modulates. The cooperative interaction of FMRP and Caprin1 ensured the correct timing of the MBT[20]. Caprin1's role in controlling follicle stem cell fate in the *D. melanogaster* ovary, where it acts as a positive translational regulator, further underscores its significance. A one-copy reduction of *Fmr1* exacerbated the Caprin1 encapsulation phenotype suggesting that *Caprin1* and *Fmr1* regulate a common process[23;20]. In the context of this study, a one-copy reduction of Caprin1 and FMRP also appears to be regulating a common process, synaptic growth at the *D. melanogaster* NMJ. Caprin1 has been identified in other immunoprecipitation experiments whose primary focus was to investigate FMRP associations with messenger-RNA-containing ribonucleoprotein (mRNPs) complexes.[2] Within that experiment at least two RNA targets were found to be shared by Caprin1 and FMRP. These mRNAs were identified as *CaMKII-alpha* and *Map1b*, both have established functions in the control of synaptic plasticity.

Limitations of this study include the inability to produce both *Fmr1* and *Caprin1* homozygous mutants even though they have been shown to be producible in some cases before. The use of these homozygous mutants as positive controls would have been preferred. The production of homozygotes was attempted but ultimately failed. *D. melanogaster* crosses were placed in a lower temperature environment to slow the lifecycle so that larvae could be obtained before lethality occurred; this was not successful. There are further limitations to consider when translating findings from *D. melanogaster* to humans. These include evolutionary differences, central nervous system complexity, lack of brain structures comparable to mammals, behavioral differences, absence of splice variants, drug metabolism differences, disease complexity, and limited modeling of synaptic plasticity. Despite these limitations, *D. melanogaster* remains a powerful tool for investigating the basic cellular and molecular mechanisms of disease before translation to mammalian models and eventually human clinical studies.

Understanding the interplay between FMRP and Caprin1 is essential for unraveling the complexities of mRNA regulation in neuronal cells and its implications in neurological disorders. The dynamic nature of their interactions provides insights into the molecular mechanisms underlying synaptic plasticity and in the pathogenesis of FXS. Here, *Fmr1* and *Caprin1* have been shown to genetically interact and work in tandem to co-regulate synaptic growth at the *D. melanogaster* neuromuscular junction. The intricate processes of mRNA transport and translation are fundamental for maintaining synaptic plasticity and functional neural circuitry. Both Caprin1 and FMRP are implicated in the dysregulation of mRNA transport and translation in various diseases and are likely at play here. A genetic interaction is evident as a double mutant containing one deficient copy each of *Fmr1* and *Caprin1* caused synaptic overgrowth and altered dendritic morphology. These structural alterations are in line with the synaptic phenotype observed in full mutation FXS caused via two copies of deficient *Fmr1*, strongly suggesting a genetic interaction.

5 FUTURE DIRECTIONS

The present study has established a biologically relevant interaction between *dFmr1* and *Caprin1* at the genetic level within *D. melanogaster*. To substantiate these findings, future experiments should focus on validating a physical interaction of their gene products via biochemical methods. Potential experiments could involve investigating whether these proteins colocalize within the same neuronal structures and assessing their role in regulating neurite morphogenesis in other neuron types affected by *dFmr1*: larval sensory neurons, mushroom body neurons, and so forth. Expanding the scope of investigation to include diverse neuronal populations affected by dFmr1 will provide a comprehensive understanding of their role in neuronal function. Exploring their combined impact on FXS behaviors would also prove beneficial. This includes observing the behavior of larvae via crawling assays and examining various behaviors in adults, such as circadian rhythms, grooming, climbing, flight, and other relevant patterns. These proposed experiments will not only validate the observed genetic interaction but also shed light on the broader implications of Fmr1 and Caprin1 in neuronal function and behavior regulation. Such endeavors pave the way for a more comprehensive understanding of their roles in neurobiology and potential therapeutic avenues for FXS.

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7 ABBREVIATIONS

1. FXS: Fragile X Syndrome
2. FMRP: Fragile X Messenger Ribonucleoprotein
3. RBP: RNA-binding protein
4. NMJ: Neuromuscular Junction
5. UTR: Untranslated region
6. CGG: Cytosine-guanine-guanine
7. mRNA: Messenger RNA
8. KH: K homology
9. RGG: Arginine glycine-rich
10. LC/MS: Liquid chromatography-mass spectrometry
11. mGluR5: Metabotropic glutamate receptor 5
12. LTD: Long-term depression
13. LTP: Long-term plasticity
14. NMR: Nuclear magnetic resonance spectroscopy
15. MBT: Mid-blastula transition
16. CaMKII: Calcium/calmodulin-dependent protein kinase II
17. Map1b: Microtubule-associated protein 1B
18. IP/LC-M: Immunoprecipitation-mass spectrometry
19. αDLG: Drosophila discs large homolog
20. LLPS: Liquid-liquid phase separation
21. RISC: RNA-induced silencing complex
22. miRNA: micro-RNA
23. FXTAS: Fragile X tremor-ataxia syndrome
24. FXPOI: Fragile X-associated primary ovarian insufficiency
25. PBS-T: Phosphate-buffered saline containing 10% Triton X-100
26. PBS: Phosphate-buffered saline
27. PF: Paraformaldehyde

8 EDITOR'S NOTES

This article was peer-reviewed.

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