

Examining the Roles of the Conserved mRNA Deadenylation Complex on *Drosophila* Neuronal Structures

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Abstract

The most common cause of inherited mental deficiency and monogenetic cause of autism is Fragile X Syndrome (FXS). There is little known about the origins of this disease which will be investigated in the present study. The series of experiments conducted examined the potential role of mRNA deadenylation proteins as contributing factors to the pathogenesis of FXS using *Drosophila melanogaster* as a model organism. One of the main complexes involved in deadenylation is the CNOT complex, which is comprised of many proteins, including POP2, TWIN, and NOT3. Each protein plays a unique role within the CNOT complex. This goal of this study was to further characterize these genes. Previous research in the Barbee lab has shown that these genes influence synapse development of the pre-synaptic terminal at the larval neuromuscular junction in *D. melanogaster*. However, it had not been tested whether POP2, TWIN, and NOT3 also have a post-synaptic effect. The localization of these genes at the neuromuscular junction was also examined and they were found to be concentrated in the pre-synaptic terminal. Finally, this study looked at whether these genes had any role in the development of sensory neurons. There was a significant increase in sensory neuron dendritic growth and a significant decrease in the complexity of the dendritic branches. These results provide insight into the characterization of TWIN, POP2, and NOT3, and their roles within the development of *D. melanogaster*. Future experiments will examine the genetic and biochemical relationship between the deadenylation complex and FXS in the *D. melanogaster* model.

Keywords: Fragile X Syndrome, NOT3, POP2, TWIN, neuromuscular junction, deadenylation, *Drosophila*, synaptic development

1 INTRODUCTION

In the current human population, it is estimated that about 1.4 per 10,000 males and 0.9 per 10,000 females suffer from Fragile X Syndrome (FXS)¹. This neurodevelopmental disease is the most common form of inherited mental deficiency in humans and is one of the best characterized monogenetic causes of autism. FXS has been attributed to causing developmental delays in children, as well as hyperactivity, anxiety, attention deficits, and difficulty reaching early language milestones². A common method used to study this sort of disease is by using *Drosophila melanogaster* (the common fruit fly) as a genetic model organism. *D. melanogaster* offers the benefit of a relatively short regeneration time, a fully sequenced genome, and easy upkeep in a laboratory setting³.

In humans, glutamate is the primary neurotransmitter used to conduct signaling across the synaptic cleft in the brain. Similarly, in fruit flies, glutamate is the pri-

mary neurotransmitter used within the muscles, making the neuromuscular junction (NMJ) a good model for the conserved signaling pathways in humans. Another reason why *Drosophila* neurons are ideal models for mammalian neurons is due to their shared use of Fragile X mental retardation protein (FMRP) in controlling synapse development from the presynaptic side. This has been well-established and contributes to evidence suggesting that their functions may be related in both humans and *Drosophila*⁴. The strong similarity between these areas provides the foundation for using the *D. melanogaster* NMJ as a model of the human brain. The identification of the proteins and mechanisms involved in its pathogenesis presents significant potential for future research and the development of a treatment.

Of the potential causes behind FXS, FMRP has been well-characterized as a critical protein in the development of the brain and in synaptic regulation⁴. FMRP is an important neuronal protein required for the repression of specific mRNA translation, although its

exact mechanism and the genes it represses are still unknown⁴. The gene which codes for FMRP, Fragile X mental retardation 1 (Fmr1), is epigenetically silenced in FXS, thereby causing intellectual disability⁴. Originally, a CCG trinucleotide expansion in the 5' non-coding region of Fmr1 was found in organisms exhibiting FXS, mostly located in the promoter region of the gene^{2;5}. Therefore, previous studies have concluded that the molecular cause of FXS arises from a loss-of-function mutation in Fmr1⁴. There is a plethora of genes which help FMRP bind to mRNAs and regulate their translation. Prior research within the Barbee lab has identified genes that share a similar loss-of-function phenotype and may interact with and regulate the function of FMRP⁶. Preliminary data suggests that FMRP may bind to important mRNAs and regulate their translation by targeting them for deadenylation and 5' to 3' mRNA decay.

These mRNA molecules are critical to the functioning and survival of the cell. mRNA acts as an intermediate between DNA and ribosomes by working to convey genetic information between the two. In the ribosome, mRNA is translated into proteins which are integral to cell functioning and survival. It is highly regulated both individually and through mRNA processing and other protein interactions, such as with RNA-binding proteins. A key component of mRNAs is the addition of a poly-A tail⁷. The addition of a poly-A tail both promotes efficient translation and protects the molecule against non-specific degradation in the cytoplasm⁸. After that, poly(A)-binding protein nuclear I (PABNI) is attached to the string of adenosine molecules. PABNI acts to prevent degradation and preserve the length of the poly-A tail⁹. When a strand of mRNA is no longer needed, it is targeted for degradation. One of the first steps to its breakdown is to remove the poly-A tail (this process is known as deadenylation). Of the genes previously characterized as part of the functioning of FMRP, TWIN (also known as CCR4), POP2 (also known as CAF1), and NOT3 are of particular interest due to their role in the process of deadenylation of mRNA⁶. These three genes take part in the blueprint for the complex needed to perform deadenylation. Without them, mRNA potentially won't be degraded, leading to difficulties in overall cell function.

There are two complexes that work together to deadenylate cytoplasmic mRNA during degradation. The PAN2-PAN3 complex works first to shorten the poly-A tail to 110nt and is followed by a second complex, CNOT, which degrades the remaining portion of the poly-A tail¹⁰. It has been found that the 3' untranslated region (UTR) activates the CCR4-NOT complex via bound proteins, either directly or indirectly¹¹. The process of deadenylation is microRNA (miRNA) mediated. Within non-coding RNAs, miRNAs are believed to control translation of specific mRNAs by complement-

ing with antisense sequences in the 3' UTR of these messages, thereby repressing gene transcription¹². Within the CNOT complex, the NOT1 gene acts as the scaffold to which all other genes bind, including CAF1, CCR4, and NOT3. It has been hypothesized that the NOT3 and CAF1 complexes work to degrade the initial portion of the poly-A tail, while the CCR4 complex plays an important role in interacting with polyadenylate-binding protein 1 (PABP1) to be able to remove the last of the poly-A tail. Although there has been evidence that CNOT may have the capability to remove the entire poly-A tail, it has not been well studied up to this point¹³. Within a previous genetic screen in the Barbee

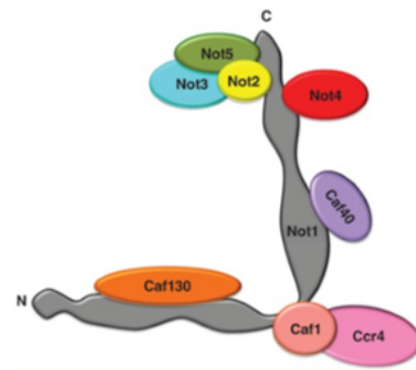


Figure 1. Sample image of the CCR4-NOT complex. Caf1 and Not3 are shown to directly interact with the Not1 scaffolding protein. Ccr4 is attached to the complex via its interaction with Caf1.

lab, results concluded that the pre-synaptic knockdown of NOT3, CCR4, and CAF1 all caused a significant increase in synaptic growth at the NMJ and are therefore important for regulating its development⁶. Synaptic growth has been previously measured by the number of boutons present at the synapse. Boutons are small swellings found at the terminal ends of axons and have been shown to be highly plastic in development. Although there are different classifications of boutons, I focused on counting type 1 boutons which are glutamatergic and can be either large or small¹⁴.

This study includes four different experiments which will address the different functionalities of NOT3, TWIN, and POP2 throughout various neurons of *D. melanogaster*. I first elucidated the pre-synaptic and post-synaptic effects of NOT3, POP2, and TWIN at the NMJ. There was a significant increase in number of synaptic boutons with both the pre-synaptic and post-synaptic knockdown of each of these genes. I next established the localization of TWIN and NOT3 at the NMJ. This experiment revealed that these genes localize in the pre-synaptic terminal but not in the post-synaptic terminal. Finally, this study determined the effects of POP2, TWIN, and NOT3 on sensory neuron dendrite morphogenesis. There was a demonstrated increase in dendritic

size with the knockdown of NOT3 and a demonstrated decrease in complexity with the knockdown of POP2. These results provide the foundation for future studies. It is with these results that I hope to further determine the role that TWIN, POP2, and NOT3 play in the function of FMRP, and ultimately in the molecular pathogenesis of FXS.

2 METHODS

2.1 *D. melanogaster* stocks

All fly lines and crosses were maintained at 25°C on standard Bloomington media. All strains used for each experiment were obtained from C380-Gal4, *sco/cyo*;24BGal4/Tm6b, BL CantonS, *ppkGal4*; UAS *cd4:tdTOMATO/cyo*, and UAS-TRiP LucIII, UAS-TRiP NOT3, UAS-TRiP/*cyo* POP2, UAS-TRiP TWIN.

2.2 Neuromuscular Junction Dissections

Larval body wall preps for NMJ and muscle analysis were performed on wandering third-instar larvae in JanJan buffer. Unless otherwise indicated, preps were stained as previously described⁶. The preps were fixed using a 4% paraformaldehyde solution followed by washes in 1X-phosphate buffered saline (1x-PBS) and overnight incubation in primary antibodies diluted with block. The following day, the preps washed with Triton X-100 (PBS-T) and incubated with secondary antibodies diluted in block for 1 hour. All preps were mounted in DAPI Fluoromount-G (Southern Biotech) and stored at -20°C until imaged via confocal microscopy.

2.3 Imaging

All imaging was performed on an Olympus Fluoview FV3000 scanning confocal microscope with 40X or 60X objectives (N.A. 1.30 and 1.42, respectively). A maximum Z projection for each synapse was assembled from 0.4 mm optical sections using the Olympus FV software. For all analysis, images were manually adjusted to threshold.

2.4 Pre-synaptic knockdown assay

To test whether a pre-synaptic knockdown of NOT3, POP2, and TWIN had any effect on the development of the synapse at the neuromuscular junction, 10-15 virgin females from C380-Gal4 line were crossed with 5 males from each UAS-TRiP line. The resulting wandering third-instar larvae from this cross were dissected using the protocol outlined above with the primary antibody anti-discs large (DLG) at a 3:300 dilution and the secondary antibodies anti-HRP-A647 and goat anti-

mouse A488, both at 1:500 dilutions. The muscles of their A3 segments were imaged using the protocol outlined above on a scanning confocal microscope. Images were randomized blindly and the total number of boutons at the synapse were manually counted and recorded for each image using the Cell Counting plugin for Image J v1.45. All image processing was done in ImageJ and Adobe Photoshop.

Statistical testing was performed in Graphpad Prism. A one-way ANOVA followed by Dunnett's multiple comparisons test was used to determine if there was any significance between the total number of boutons for each knockdown. A Brown-Forsythe test and a Bartlett's test were performed to ensure there was no significant variance between the samples.

2.5 Post-synaptic knockdown assay

To test whether a post-synaptic knockdown of NOT3, POP2, and TWIN had any effect on the development of the synapse at the neuromuscular junction, 10-15 virgin females from the muscle driver line *sco/cyo*;24BGal4/Tm6b were crossed with 5 adult males from each UAS-TRiP line. The same protocol as the pre-synaptic experiment was used for dissections, staining, and imaging with the same fluorescent antibodies.

Due to statistically significant variance being present between the samples, a Kruskal-Wallis test followed by a Dunn's multiple comparisons test was performed in Graphpad Prism on the resulting bouton counts.

2.6 Colocalization assay

To address the third aim of this paper, NMJ dissections were performed on wandering third-instar larvae from the control line, CantonS, according to the procedures outlined above. The primary antibodies used were rabbit anti-CCR4 in a 1:400 dilution and mouse anti-NOT1 in a 1:1000 dilution. The secondary antibodies used were anti-HRP A647, goat anti-rabbit A594, and goat anti-mouse A488, all at 1:500 dilutions. All imaging was performed according to the procedure above using laser scanning confocal microscopy. All image processing was done in ImageJ and Adobe Photoshop.

2.7 Sensory dendritic assay

In the final experiment, 10-15 virgin females from the *ppkGal4*; UAS *cd4:tdTOMATO/cyo* line were crossed with 5 males from each of the UAS-TRiP lines. A microscope slide was prepped for larvae imaging by placing two 1.5 Slip-Rite coverslips with a 1mm gap between them on the slide using clear nail polish. A solution of 15% chloroform in mineral oil was spread on the slide (to immobilize larvae) and the third-instar larvae

were placed in between the coverslips. A final 24x50 0.13-0.17mm thick cover glass was used to compress the larvae to the slide and minimize any movement.

The larvae were imaged through the cuticle to the underlying sensory neurons using scanning laser microscopy, as detailed above. All images were taken from A4-A6 segments of the larvae, with no more than 2 sensory neurons from each animal included in analysis.

Sensory neuron images were processed using ImageJ. Each image was manually adjusted to threshold and skeletonized using the ImageJ skeletonize plugin. The resulting skeleton image was assessed using the corresponding analyze function in ImageJ to generate branch information and results tables. From the results table, the total number of branches, average branch length, number of junctions and maximum branch length were recorded. Total branch length was then calculated for each sensory neuron by multiplying average branch length by total number of branches. For both the total branch length and the number of junctions, outliers statistical testing was done to determine any outliers using the ROUT method in Prism, which is similar to Grubb's method¹⁵. Once outliers were excluded from the data, a one-way ANOVA was performed followed by a Dunnett's multiple comparison test.

2.8 Statistical analysis

All data was recorded in Microsoft Excel and graphed and statistically analyzed in GraphPad Prism. Results were considered to be statistically significant at $p < 0.05$. Results shown throughout the study are mean \pm SEM. n.s. = not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

3 RESULTS

3.1 Pre-synaptic knockdown of NOT3, POP2, and TWIN significantly increased synaptic size

This experiment was confirming the results from the previous Barbee lab screen in determining whether NOT3, POP2, and TWIN play a pre-synaptic role in the development of the synapse. A motor neuron specific Gal4 driver (C380-Gal4) was used to drive expression of a short hairpin RNAi (shRNA) transgene targeting each of the genes. I analyzed synaptic bouton number at the neuromuscular junction of the A3 segment and statistically analyzed each data set for significance. All 3 of the genes demonstrated no significant differences in variance from the post-hoc tests and had significantly more boutons per synapse than the control sample (Table 1). A small-bouton phenotype was observed in the synapses from the knockdown of POP2, TWIN, and NOT3 that contributed to their significantly higher bouton counts (Figure 2, panels C-D). The greatest increase

in synaptic size was found in the knockdown of the TWIN gene. The smallest, yet still significant increase in synaptic size was found in the knockdown of the POP2 gene (Figure 2, panel A).

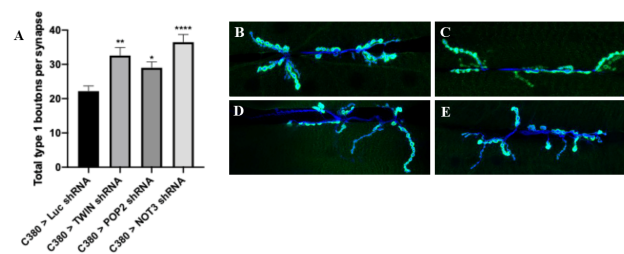


Figure 2. Green staining represents the staining of anti-DLG at the terminals of the post-synaptic type I boutons. Blue staining represents the anti-neuronal HRP pre-synaptic terminal staining. (A) Graphical representation of the total type 1 boutons per synapse for each genotype in the pre-synaptic knockdown. All 3 experimental knockdowns displayed a level of significance compared to the control. (B) Representative image of a synapse of C380 > LucIII shRNA. (C) Representative image of C380 > NOT3 shRNA synapse. (D) Representative image of C380 > POP2 shRNA synapse. (E) Representative image of C380 > TWIN shRNA synapse.

3.2 Post-synaptic knockdown of POP2, TWIN, and NOT3 significantly increased synaptic size

A muscle specific Gal4 driver (*sco/cyo*;24BGal4/Tm6b) was used to drive expression of a shRNA transgene targeting each of the genes. I analyzed synaptic bouton number at the neuromuscular junction of the A3 segment and statistically analyzed each data set for significance. Observations of the synapses from the post-synaptic knockdown of POP2, NOT3, and TWIN show a small-bouton phenotype that mirrors the one seen previously in the pre-synaptic knockdown experiment (Figure 3, panels D-E compared to panel B). The greatest increase in size of the synapse was seen with the knockdown of TWIN and NOT3, whereas POP2 had a smaller, but still significant increase (Figure 3, panel A).

3.3 TWIN and NOT3 are concentrated in the pre-synaptic terminal

The third aim of this experiment was to determine if NOT3 and TWIN localized to the NMJ. Third-instar larvae from the control CantonS line were dissected and stained using antibodies specific to CCR4 and NOT1 and subsequently analyzed for concentration of expression. Antibodies were available for NOT1 and TWIN. NOT1 was used as a substitute for the staining of NOT3 and there was no data collected on the localization of POP2 due to lack of an available antibody. Both anti-TWIN, anti-NOT1, and anti-HRP showed the exact

Table 1 Statistical values and mean bouton count for each genotype in the pre-synaptic knockdown.

Genotype	Mean # Total Boutons	Standard Deviation	P Value
C380 > LucIII shRNA	22.18	6.287	N/A
C380 > TWIN shRNA	32.59	9.612	0.0014
C380 > POP2 shRNA	29.00	7.357	0.0487
C380 > NOT3 shRNA	36.47	9.220	<0.0001

Table 2 Statistical values and mean bouton count for each genotype in the post-synaptic knockdown.

Genotype	Mean # Total Boutons	Standard Deviation	P Value
24bGal4 > LucIII shRNA	22.56	6.572	N/A
24bGal4 > TWIN shRNA	34.06	12.23	0.0065
24bGal4 > POP2 shRNA	31.31	6.519	0.0194
24bGal4 > NOT3 shRNA	34.75	12.77	0.0017

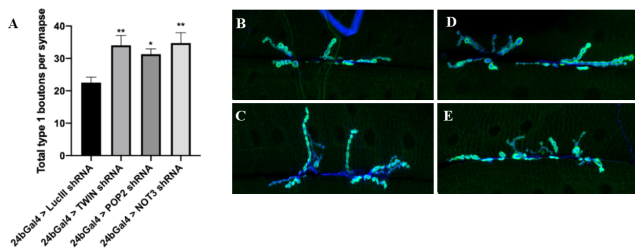


Figure 3. Green staining represents the staining of anti-DLG at the terminals of the post-synaptic type I boutons. Blue staining represents the anti-neuronal HRP pre-synaptic terminal staining. (A) Graphical representation of the total type 1 boutons per synapse for each genotype in the post-synaptic knockdown. All 3 experimental knockdowns displayed a level of significance compared to the control. (B) Representative image of a synapse of 24bGal4 > LucIII shRNA. (C) Representative image of 24bGal4 > NOT3 shRNA. (D) Representative image of 24bGal4 > POP2 shRNA. (E) Representative image of 24bGal4 > TWIN shRNA.

same staining pattern, indicating that there is enrichment in the pre-synaptic terminal of both CCR4 and NOT3 (Figure 4, panels B-D). There was much more robust staining observed with NOT1 compared to CCR4 (Figure 4, panel C compared to panel D). Both proteins are also expressed in muscle, but the staining was shown to be much weaker in this location (Figure 4, panel A).

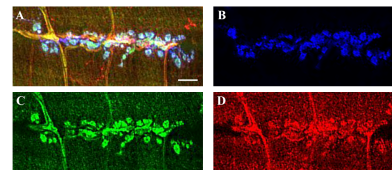


Figure 4. Colocalization of deadenylation proteins in CantonS at the NMJ. (A) Merged channels of staining on synapse. (B-D) Larvae were stained with antibodies targeting HRP (blue), NOT1 (green), and CCR4 (red).

3.4 TWIN increases sensory dendrite growth and POP2 decreases sensory dendrite complexity

The final goal of this paper was to determine if NOT3, TWIN, or POP2 had any effect in the development of neuron dendrites in *D. melanogaster* larvae. Previous research indicated that *Fmr1* has an effect in dendritic arborization neurons of the body wall¹⁶. A sensory neuron specific Gal4 driver (*ppkGal4; UAS cd4:tdTOMATO/cyo*) was used to drive expression of a shRNA transgene targeting each of the genes. Virgin females from *ppkGal4* were crossed with adult males from each *UAS-TRiP* line and the resulting wandering third-instar larvae were imaged. Sensory dendrites were analyzed for total branch length and number of junctions. Interestingly, there was a significant increase in total branch length in the knockdown of TWIN but not with the knockdown of POP2 (Figure 5, panel A).

There was also a significant decrease in number of junctions with the knockdown of POP2 but not with the knockdown of TWIN (Figure 5, panel B). It should be noted that the cross between ppkGal4 and NOT3 produced no third-instar larvae and was therefore excluded from analysis, suggesting that the knockdown of NOT3 in sensory neurons is second-instar larval lethal.

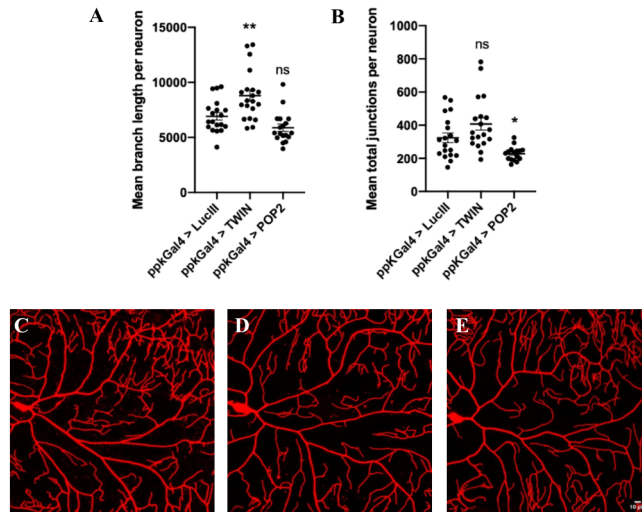


Figure 5. (A) Graphical representation of the mean branch length per neuron of each genotype. (B) Graphical representation of the mean total number of junctions per neuron for each genotype. (C) Representative image of a sensory neuron for ppkGal4 > LucIII shRNA. (D) Representative image of a sensory neuron for ppkGal4 > POP2 shRNA. (E) Representative image of a sensory neuron for ppkGal4 > TWIN shRNA.

4 DISCUSSION

Although Fragile X Syndrome is an overall well-characterized disease, its underlying molecular mechanisms are still unknown. The present study aimed to further characterize some of the suggested genes that could be involved in FXS using *D. melanogaster* as a model.

This research first concentrated on the potential effects of POP2, TWIN, and NOT3 at the neuromuscular junction. This is based on results from a genetic screen conducted in the Barbee lab. The results from the pre-synaptic knockdown of each of these genes demonstrated a significant increase in synapse size, as measured by the total number of boutons present. A small-bouton phenotype was also observed with the synapses resulting from the knockdown of these genes, confirming the previous work's conclusions. Within this significant result, there was variability in how much larger the synapse became with the knockdown of each gene. For example, C380 > NOT3 shRNA showed the most significant effect on the synaptic size at $p < 0.0001$. On the other hand, C380 > POP2 shRNA showed a smaller,

yet still significant effect on synapse size at $p < 0.0487$. This finding supports previous literature that demonstrates NOT3 having the largest influence on synaptic size compared to the other genes involved⁶. One explanation for the variation observed in strength of phenotype between these genes could be their location and role within the CNOT complex. For example, NOT3 is closely associated with the NOT1 scaffolding proteins whereas TWIN is not directly associated with NOT1 and therefore is further from the center of the complex (see Figure 1). Another explanation could be the different roles that TWIN and POP2 have in the CNOT complex compared to NOT3. NOT3 plays a much more integral role in the functioning of the deadenylation complex, whereas TWIN and POP2 act more as accessory proteins which help with the deadenylation process¹⁷. These findings in the variation of how these proteins function indicate that there is still much more to be learned about the details within the functioning of CNOT on the poly-A tail.

As a primary deadenylation complex, CNOT is comprised of many proteins, each of which performs a distinct role. Within the CNOT complex, TWIN and POP2 are catalytic subunits part of the core complex¹⁸ NOT1 is the scaffold protein to which they attach, and therefore without NOT1 neither of the catalytic subunits can function. CCR4 is believed to interact with PABP1 to remove it from the poly-A tail so that all adenosine molecules are completely removed, whereas CAF1 is believed to aid in the overall functioning in the complex without one specific role¹⁹. The close association of NOT3 with the scaffolding protein NOT1 helps to explain why the knockdown of NOT3 resulted in a much more significant increase in synaptic size compared to TWIN and POP2. It has also been found that POP2 is dispensable for the deadenylase activity of TWIN, as shown in previous studies that CCR4 is the principal deadenylase in the CCR4-NOT complex²⁰. This insight gives a better understanding as to why the knockdown of POP2 had the least significant change in synapse size – although still important, it is less critical to the functioning of the complex as opposed to NOT3 and TWIN. These findings reinforce the research that has been done previously on this topic and supports the first aim's hypothesis that there would be a significant increase in synaptic size with the pre-synaptic knockdown of TWIN, POP2, and NOT3.

In order to address the second aim of this experiment, each of the 3 genes examined were knocked down in the muscle of *D. melanogaster*. The post-synaptic knockdown of POP2, TWIN, and NOT3 demonstrated a significant increase in synapse size for each case. Similar to the results from the pre-synaptic knockdown of these genes, the greatest significant increase in synapse size was observed in the absence of NOT3. Likewise, POP2 demonstrated the smallest significant increase

Table 3 Comparison of total branch length of each genotype.

Genotype	Mean Total Branch Length	Standard Deviation	P Value
ppkGal4 > LucIII shRNA	6917	1471	N/A
ppkGal4 > TWIN shRNA	8793	2256	0.0032
ppkGal4 > POP2 shRNA	5887	1405	0.1458

Table 4 Comparison of mean total number of junctions of each genotype.

Genotype	Mean Total Number of Junctions	Standard Deviation	P Value
ppkGal4 > LucIII shRNA	324.2	126.5	N/A
ppkGal4 > TWIN shRNA	407.2	160.1	0.0789
ppkGal4 > POP2 shRNA	228.2	42.64	0.0484

in synapse size. One implication from the findings in experiments 1 and 2 is that NOT3, POP2, and TWIN are all important to the regulation of mRNA on both sides of the synapse. The observed overgrowth at the synapse can be attributed to the upregulation of translation with the knockdown of these genes. It can also be suggested that local translation is occurring on both sides of the synapse, and therefore a dysregulation is causing synaptic overgrowth. It is unsurprising, then, to conclude that translation needs to be regulated on both sides of the synapse in order to control synaptic growth. This finding supports the hypotheses of aims 1 and 2.

Based on a role for the deadenylation complex in NMJ development, I next wanted to ask if any of these proteins were enriched locally at the synapse. The performed immunohistochemistry procedure addressed the third aim of this study. It was concluded from the resulting images that that TWIN and NOT3 colocalize at the pre-synaptic terminal of the NMJ. The NOT1 antibody was used instead of NOT3 due to ready availability. However, the staining of NOT1 can be used as a representative of NOT3 localization because both genes are highly correlated with each other as part of the scaffolding portion of the deadenylation complex.

Unlike the previous two experiments, this colocalization assay does not lend information on the molecular interactions of these genes. Rather, the focus of this assay was simply to observe where these proteins are strongly localized. The above data suggests that the presynaptic function is local in axon terminals. It is not only important to examine the functionality of these

genes; it is also important to look at their patterns of expression. Although it is difficult to definitively analyze the resulting images shown in Figure 4, the clear staining of NOT1 demonstrates its colocalization at the pre-synaptic terminal. In the merged image it becomes apparent that its staining matches that of anti-HRP on the axon (Figure 4, panel A). The staining with CCR4 was not as clear, making it more difficult to draw a strong conclusion on its colocalization specifically in the pre-synaptic terminal. While it is shown to match the staining of anti-HRP, unlike NOT1 it does not demonstrate a robust expression at the synaptic boutons. Both proteins are also expressed in muscle, but at lower levels (Figure 4, panel A). The information gathered from this experiment indicates that there is specific and significant localization of TWIN and NOT1 at the NMJ which helps pave the way for future studies.

There have been no previous studies performed examining the effect of these genes on sensory neurons in *D. melanogaster*. Sensory neurons were imaged through the cuticle of third-instar larvae. Sensory dendrites were analyzed for both total branch length as a measure of the size of the neuron, and number of junctions in the dendrites as a measure of complexity. Interestingly enough, the results showed a significant increase in total area of the neuron only with the knockdown of TWIN, suggestive that TWIN is a negative regulator of dendrite morphogenesis. Conversely, only a significant decrease in total number of junctions per synapse was observed in the knockdown of POP2. This difference in results suggests that TWIN may be more important for the regulation of mRNA in sensory neuron development,

whereas POP2 does not play as integral a role in the deadenylase complex. It is worth mentioning that there were no results from the cross between *ppkGal4* and *NOT3*. No third-instar larvae developed from that cross, although first- and second-instar larvae were observed. Therefore, it can be concluded that the knockdown of *NOT3* in sensory neurons is second-instar larval lethal.

There are many implications within the result of the sensory neuron experiment, including what it could mean for the role of these genes in the overall development of the animal. The conclusions from this experiment support the idea that the most significant change in larvae development occurred with the knockdown of *NOT3*. This means that *NOT3* can be inferred as a critical protein to the deadenylation complex and that the complex cannot function without it. There is still much more to be discovered about how these genes take exhibit such an effect on the development of sensory neurons.

5 FUTURE DIRECTIONS

There are a few areas of improvement within the experiments outlined above. When imaging the pre- and post-synaptic NMJs, it was difficult to ensure consistency in counting each bouton. There is no reliable, automated system to count boutons at the synapse, and discovering one in the next few years would help to eliminate any human error with this process. Within the process of choosing a muscle driver to use for this experiment, there were some issues to finding one that worked well with each of the *UAS-TRiP* lines. For example, one muscle driver originally chosen for this experiment was *MHC-Gal4*. Once the crosses were set up, however, it was found that it was third-instar larval lethal. The muscle driver that was eventually chosen for this experiment is not ideal due to the declined health of the flies from the mutations on chromosomes 2 and 3. Future studies from this experiment should consider attempting the use of other muscle driver lines to increase the health and accuracy of the results. Another caveat with these studies is that there was no way to be certain that the knockdown of the target genes was successful. In a repeat of this experiment, it would be worthwhile to employ a similar procedure to the immunohistochemistry procedure used in experiment 3 to ensure that there was no significant concentration of the targeted gene at the neuromuscular junction after knockdown.

Lastly, within the sensory neuron assay, there have been suggestions of other programs that may have the capability to better quantify dendrite area with a more accurate reading of total branch length. In the future, studies could try different techniques for quantifying total branch length and number of junctions to test which one is the most efficient and accurate. For example,

one considered method was to try outlining the dendrites by hand and then analyzing the resulting skeleton. Although it may not be conducive for efficiency, this method may lend knowledge to a more accurate technique to quantify total branch area and number of junctions.

In conjunction with the results of the sensory dendrite experiment, one potential future study could be on the colocalization of *TWIN*, *POP2*, and *NOT3* within sensory neurons. This could provide information not only on how these genes are affecting the dendritic area and complexity, but also how they are expressing in these areas. It would be worthwhile in future studies to perform staining of all 3 genes in a control NMJ. It is also apparent that better antibodies need to be determined to better characterize the localization of *CCR4*.

Finally, a proposed follow-up study could test other components of the deadenylation and decay pathways play similar roles in the development of *D. melanogaster*. For example, some of the genes involved in the de-capping process of mRNA may also influence synapse size or sensory neuron complexity. Each of these potential studies would contribute to our knowledge of genes involved in *FXS* and could go so far as to lead to therapeutic possibilities.

The work outlined above only begins to characterize *NOT3*, *POP2*, and *TWIN* as critical proteins in animal development relating to deadenylation. There is still much to be done regarding the characterization of these genes in the context of the *FXS* model, including many potential future studies. As mentioned previously, one such study could draw on the results from the immunostaining experiment and not only repeat the procedure but include antibodies to better stain *CCR4* and an antibody to stain *CAF1* as well. This would contribute to an understanding of the localization of these genes at the NMJ. Overall, this work continues to support and add to the growing research being done on the molecular basis of *Fragile X Syndrome* which will hopefully eventually lead to better treatments for this genetic disease.

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7 EDITOR'S NOTES

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REFERENCES

- [1] Hunter, J. *et al.* Epidemiology of fragile X syndrome: A systematic review and meta-analysis. *American Journal of Medical Genetics, Part A* (2014).
- [2] Drozd, M., Bardoni, B. & Capovilla, M. Modeling fragile X syndrome in drosophila (2018).
- [3] Adams, M. D. *et al.* The genome sequence of *Drosophila melanogaster* (2000).
- [4] Sidorov, M. S., Auerbach, B. D. & Bear, M. F. Fragile X mental retardation protein and synaptic plasticity (2013).
- [5] Pfeiffer, B. E. & Huber, K. M. The state of synapses in fragile X syndrome (2009).
- [6] Pradhan, S. J. *et al.* The conserved p body component HPat/Pat1 negatively regulates synaptic terminal growth at the larval *Drosophila* neuromuscular junction. *Journal of Cell Science* (2012).
- [7] Zhang, X., Virtanen, A. & Kleiman, F. E. To polyadenylate or to deadenylate. *Cell Cycle* (2010).
- [8] Wahle, E. & Winkler, G. S. RNA decay machines: Deadenylation by the Ccr4-Not and Pan2-Pan3 complexes (2013).
- [9] Kühn, U. *et al.* Poly(A) tail length is controlled by the nuclear Poly(A)-binding protein regulating the interaction between Poly(A) polymerase and the cleavage and polyadenylation specificity factor. *Journal of Biological Chemistry* (2009).
- [10] Yamashita, A. *et al.* Concerted action of poly(A) nucleases and decapping enzyme in mammalian mRNA turnover. *Nature Structural and Molecular Biology* (2005).
- [11] Temme, C., Zaessinger, S., Meyer, S., Simonelig, M. & Wahle, E. A complex containing the CCR4 and CAF1 proteins is involved in mRNA deadenylation in *Drosophila*. *EMBO Journal* (2004).
- [12] Shivdasani, R. A. MicroRNAs: Regulators of gene expression and cell differentiation (2006).
- [13] Nousch, M., Techritz, N., Hampel, D., Millionigg, S. & Eckmann, C. R. The Ccr4-Not deadenylase complex constitutes the main poly(A) removal activity in *C. elegans*. *Journal of Cell Science* (2013).
- [14] Hoang, B. & Chiba, A. Single-cell analysis of *Drosophila* larval neuromuscular synapses. *Developmental Biology* (2001).
- [15] Motulsky, H. J. & Brown, R. E. Detecting outliers when fitting data with nonlinear regression - A new method based on robust nonlinear regression and the false discovery rate. *BMC Bioinformatics* (2006).
- [16] Lee, A. *et al.* Control of dendritic development by the *Drosophila* fragile X-related gene involves the small GTPase Rac1. *Development* (2003).
- [17] Viswanathan, P., Ohn, T., Chiang, Y. C., Chen, J. & Denis, C. L. Mouse CAF1 can function as a processive deadenylase/3-5'-exonuclease in vitro but in yeast the deadenylase function of CAF1 is not required for mRNA poly(A) removal. *Journal of Biological Chemistry* (2004).
- [18] Xu, K., Bai, Y., Zhang, A., Zhang, Q. & Bartlam, M. G. Insights into the structure and architecture of the CCR4-NOT complex (2014).
- [19] Yi, H. *et al.* PABP Cooperates with the CCR4-NOT Complex to Promote mRNA Deadenylation and Block Precocious Decay. *Molecular Cell* (2018).