Comparison of Different RNA-Binding Proteins in *Manduca sexta* Larva Memory Formation

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ABSTRACT

Multiple factors have been shown to affect how memories are formed and retained. In some studies, individual RNA-binding proteins that are found more commonly in the nervous system have been linked to the formation of short-term memory. Some of these RNA-binding proteins, Cytoplasmic Polyadenylation Element Binding protein (CPEB) and Pumilio, had been independently shown to affect memory formation. To see how these proteins affect memory formation, the protein concentrations are reduced by injecting double-stranded RNA molecules specific to the proteins into the larvae of *Manduca sexta.* The purpose of this study is to investigate how knockdowns of these proteins affect *M. sexta* memory formation and what happens when both proteins have a knockdown. The results of this study showed that when the larvae receive the knockdowns, the hornworm's memory improved when compared to the control group. There were multiple problems throughout the study, it needs to be repeated to demonstrate significance. One such problem being the non-statistically significant sample size of the experiment.

KEYWORDS: Cytoplasmic Polyadenylation Element Binding Protein, CPEB, Pumilio, Memory, RNA-binding, Small Interfering RNA, siRNA, Protein Knockdown

INTRODUCTION

This project is to determine the effect of knockdown Pumilio in *M. sexta* larva memory formation. Previous studies have examined the effects of a knockdown of another protein called cytoplasmic polyadenylation element binding protein 2 (CPEB2) on *M. sexta* larva memory formation. CPEB works by binding to a specific region of messenger RNA (mRNA) known as cytoplasmic polyadenylation element (CPE) on the 3' untranslated region[1]. When CPEB binds to the CPE it is responsible for both translational repression and translational activation by polyadenylation.

The Pumilio protein functions in a similar way as CPEB because it binds to a certain sequence on mRNA called the Pumilio Response Element (PRE)[2]. Like CPEB, Pumilio serves multiple functions within organisms and one of those functions is to help regulate memory formation and other neural processes[9]. There are multiple studies done that show that when

mice have a Pumilio knockdown or other problems related to proper Pumilio formation, the mice display problems making or retaining memories[7,8,10].

This research was done at San Juan College. The mentor, Dr. Evans, oversaw and managed the data collection in an ongoing investigation on the connection between CPEB2 and short-term memory in *M. sexta* larva and observed that by performing a knockdown for CPEB2 in *M. sexta* larva retain less memories than those that have not had a knockdown. Since both CPEB and Pumilio serve similar functions in memory formation, this study will investigate the relation between Pumilio in *M. sexta* larva and memory formation as well as comparing it to CPEB's relationship with memory formation. Finally, the study will investigate the effect of a knockdown of both RNA-Binding proteins. The expected result of the experiment is that the group of larvae that had a knockdown of both proteins would remember less than those that had only one protein knockdown or those that had no knockdowns. This project was funded by a grant through the Institutional Development Award (IDeA) from the National Institute of General Medical Sciences of the National Institutes of Health under grant number P20GM103451.

MATERIALS AND METHODS

siRNA Creation

Basic Local Alignment Search Tool (BLAST) Search. To conduct a BLAST search to determine if *M. sexta* had Pumilio protein, the researcher first searched the NCBI's protein database for Pumilio in an insect species closely related to *M. sexta*. For this blast search, the FASTA sequence from *Drosophila melanogaster* was used. This protein FASTA sequence was then used for the Insect Base BLAST search using blastp for *M. sexta*. This search pulled up five results for a Pumilio protein in *M. sexta*. From here the five FASTA sequences, with both protein and RNA sequences, were saved for other uses.

Clustal Omega Multiple Sequence Alignment System. Clustal Omega was used to determine sections of the five sequences found from the blast search had areas of high similarity. Firstly, Clustal Omega was used to find similarities in the protein FASTA sequence. All five protein sequences were put into the Clustal Omega program and all settings were left at their defaults. The program was run and produced a document with all the protein sequences aligned as seen in Figure 1.

The sequences where the proteins were identical were shown by asterisks. Since the protein Clustal Omega showed a large section of identical sequences, a DNA Clustal Omega was run. This also showed a long DNA sequence where all five were the same as seen in Figure 2.

siRNA Ordering. Using a section found from the DNA Clustal Omega, a sequence from roughly in the middle of the identical area was found. This sequence was 30 nucleotides long and was used as the basis of the siRNA that was made for this project. The sequence included cytosines, guanines, and thymine. This sequence is CGTGTCGCCCGGCGGCGTGCTGGCGCCGCG and is seen in Figure 2.

Figure 1. The Clustal Omega results for the amino acid sequences found from the BLAST search. This is an area that is identical between the six sequences found.

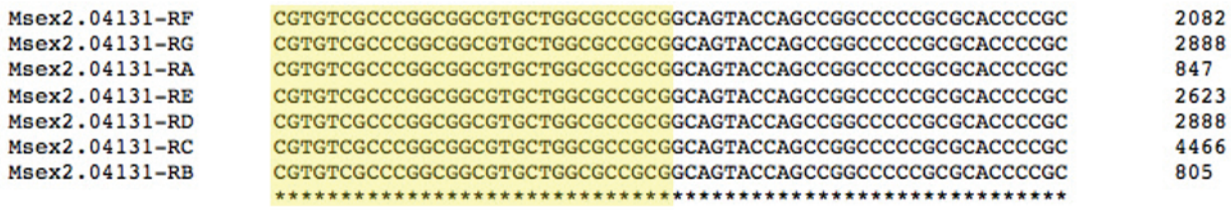


Figure 2. This Clustal Omega alignment is for the DNA sequence. The highlighted section shows the sequence used to create the siRNA.

Larva Maintenance

Larva Housing. The larvae were received shortly after hatching and were left in the food container they came in for four days in order to let them grow a little more. After four days, the larvae were moved into individual feeding tubes, that were also premade. Before they were moved into their new tubes, they were cleaned in a 1% bleach solution for a few seconds and rinsed in sterile water in hopes to alleviate a mold problem that was developing in the tube. The second shipment of the larva was received and was also left in the shipping container for four days. This set of larvae did not receive a bleach cleaning before being transferred to the individual feeding tubes. The second set of larvae also had a mold problem. Once the larvae were back in the tubes, they were put under a light to keep a constant temperature.

Larva Cleaning. About once a week, the tubes with any visible amount of mold were cleaned by sterilized a scoop with 95% ethanol between each use and the excrement and mold were removed. Once the mold was removed, the larvae were placed back into the tubes.

Larva Injection and Training

siRNA Injection Preparation. For the Pumilio knockdown group, the researcher added 20µl of the Pumilio siRNA (1µg/µl) to 990µl PBS (1X). For the Pumilio and CPEB knockdown group, the researcher added 20µl of the Pumilio siRNA (1µg/µl) and 20µl of the CPEB siRNA (1µg/µl) to 980µl PBS (1X). The main reason for the solution with both types of siRNA having an overall higher concentration of the siRNA's is that there was concern that using a half concentration of each individual siRNA wouldn't allow the individual siRNA to work as effectively.

Training Gel Setup. The gel was made with 7 g agarose powder and 35 ml of 2mM LiCl. This was combined and ddH_2O in order to reach 350 ml total. The LiCl was added to allow the electricity to pass through the gel. This was then poured into the bottom of the gel setup, with care to make sure the wires could be connected to the electricity. This can be seen in Figure 3.

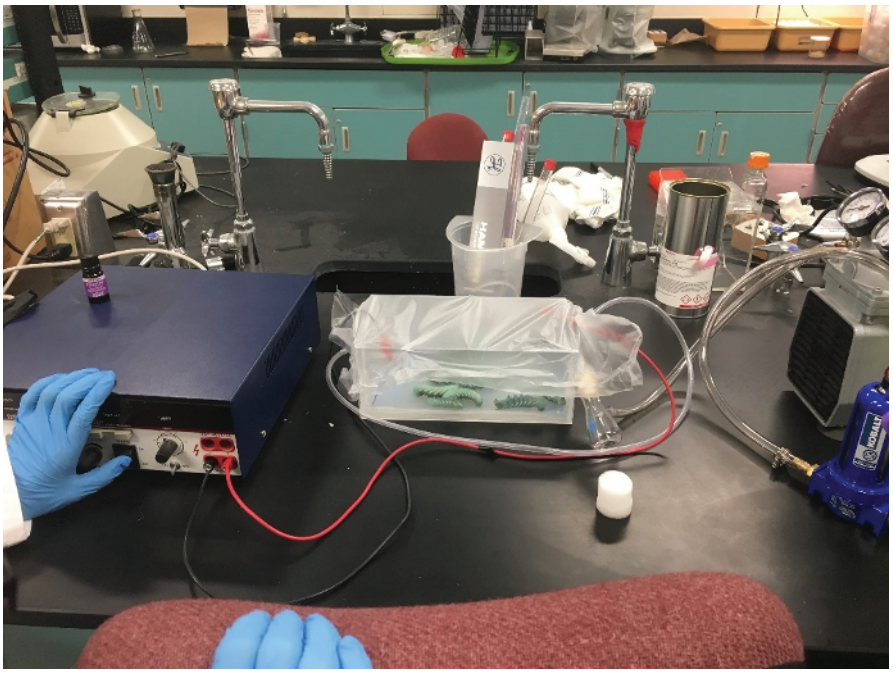


Figure 3. This photo shows the larvae in the gel box right before they would receive the shock.

Larva Injection. The larvae were removed from the feeding tubes and set in a container filled with ice, separated by different types of injections. Three hundred microliters of the siRNA mix for the group (Pumilio, CPEB, and Both) were pulled up into the syringes. Once the larvae have been on ice for at least five minutes to slow their metabolism, remove them from the ice and swab the area behind the third abdominal prolegs with ethanol to sterilize the area. The needle was put in at less than a 45º angle in the area that was swabbed and 25 µl of the corresponding siRNA was injected.

Larva Training. Lavender essential oil is placed in an Erlenmeyer flask that is connected to the gel box and air compressor. One group is placed on the gel and the gel box is covered in plastic wrap as seen in Figure 4.

Figure 4. A close-up view of the larvae inside the gel box.

Once larvae are in the box, turn the air compressor on for ten seconds. Immediately after the air compressor is turned off, turn the electricity on for ten seconds at 90 V. Remove that group of larvae and repeat for the rest of the larvae. Once all groups are done, wait thirty minutes and repeat. This was done a total of six times over a period of two hours thirty minutes. Once all training is done, place larvae in the corresponding food tube and wait until the next day.

Olfactometer Test. Place the same lavender oil in the Erlenmeyer flask attached to the Olfactometer Y-Tube. Setup the Y-Tube for the test as seen in Figure 5. Check airflow at the olfactometer's lowest setting, so that the lavender smell is making its way through the setup. Place the larva at the intersection of the tube and cover the end with mesh. Cover the setup with tin foil to make a dark environment. Turn on the air compressor for five minutes and record the larva's position. Once the data was recorded, the larvae were placed in labeled bags and frozen before disposal.

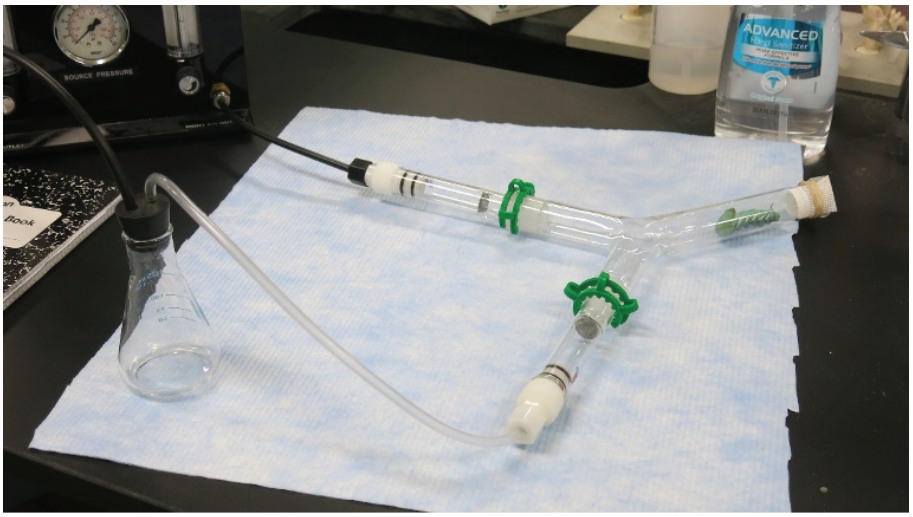


Figure 5. This is a photo of the y-tube setup before the foil was placed on top, showing the larva location.

RESULTS

The results indicate that the control group remembered less about the training test than both the Pumilio Knockdown group and the Pumilio/CPEB Knockdown group (Shown in Table 3 and 4 and Graph 1 and 2). For the purpose of this research, the "turned left" and the "no motion" observations indicate that the larva did not remember the training, while the other two observations show they remember the training. Additionally, the combination of both Pumilio and CPEB knockdowns did not seem to effect memory any more than just the Pumilio knockdown. The CPEB knockdown group showed that the hornworms remember more than the control group. Of all the groups, the control group had less larvae that remembered the training than any of the other groups that had the proteins knocked down.

Control				
Larva	Turned Left (Towards Smell)	Turned Right (Away from Smell)	Turned Around	No Motion
1				X
2			X	
3				X
4				X
5	X			
6		X		
7				X
8				X
9	X			
10			X	
Total	2	1	2	5

Table 1. Results from the control trial.

Pumilio Knockdown				
Larva	Turned Left (Towards Smell)	Turned Right (Away from Smell)	Turned Around	No Motion
1			X	
2	X			
3			X	
4		X		
5	X			
6			X	
7	X			
8			X	
9	X			
10			X	
Total	4	1	5	0

Table 2. Results from the Pumilio knockdown trial.

CPEB Knockdown				
Larva	Turned Left (Towards Smell)	Turned Right (Away from Smell)	Turned Around	No Motion
1		X		
2			X	
3			X	
4				X
5	X			
6		X		
7		X		
Total	1	3	2	1

Table 3. Results from the CPEB Knockdown trial.

Pumilio and CPEB Knockdown				
Larva	Turned Left (Towards Smell)	Turned Right (Away from Smell)	Turned Around	No Motion
1			X	X
2			X	
3	X			X
4		X		X
5	X			
6	X			
7			X	X

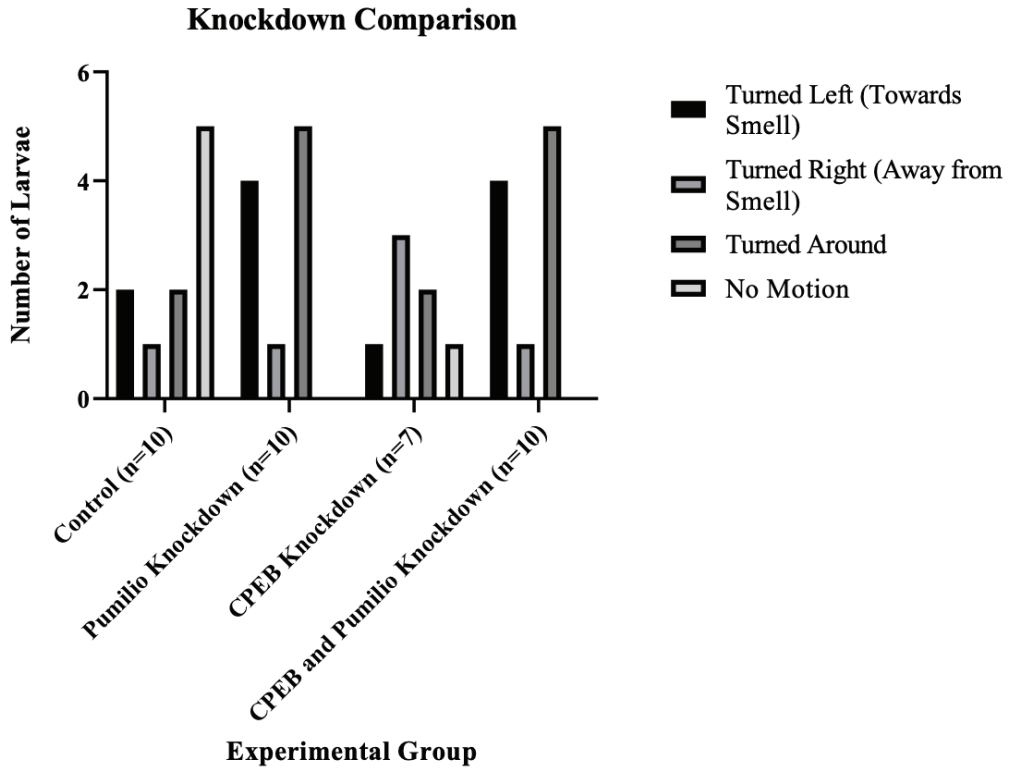
8			X	X
9			X	
10	X			
Total	4	1	5	5

Table 4. Results from the Pumilio and CPEB knockdown trial.

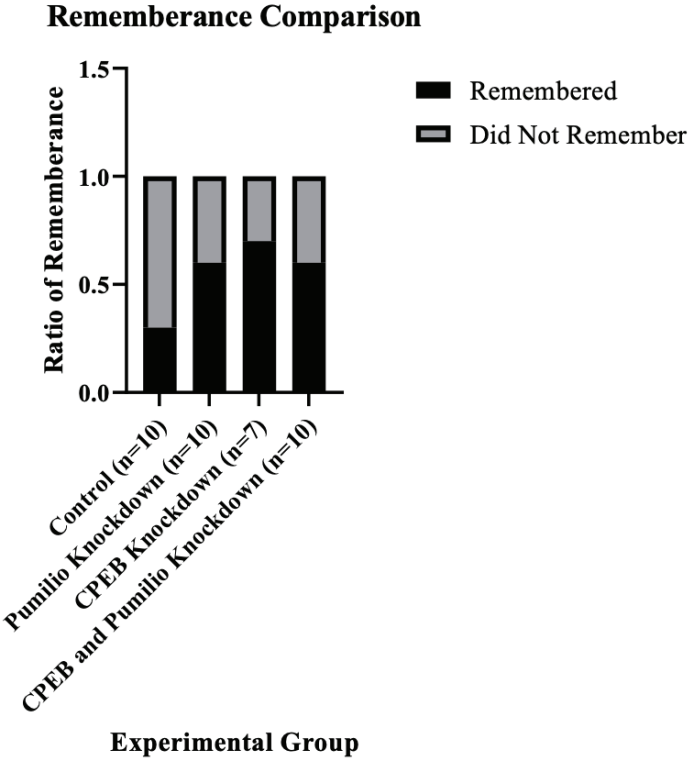
CONCLUSIONS

This study asked if disrupting the function of the RNA-binding proteins CPEB and Pumilio using RNA interference would have an effect on memory retention in *M. sexta* larvae. There were multiple novel outcomes of this work. One thing shown through this study is that *M. sexta* does have homologs of Pumilio proteins within the organism. Then, the results of the y-tube tests indicate that the larva that experienced the siRNA interference of the RNA binding proteins remembered more than the control group that had no siRNA interference. Also, the data indicates that the larvae with the CPEB siRNA injection remembered more when compared to the Pumilio and Pumilio/CPEB knockdown groups. Based on these results, the presented hypothesis should be rejected because the larvae with both protein knockdowns demonstrated greater avoidance of the negative stimulus than the control group. However, previous work in this lab has demonstrated that a higher percentage of untreated *M. sexta* larvae avoid the odor associated with the negative stimulus than shown in these experiments, suggesting that a larger sample size and more experiments may be needed. However, these results still suggest that CPEB and Pumilio do play some role in memory retention.

Due to the surprising results, different potential confounding factors have been identified that suggest that the results of this study should be interpreted with caution. One, the sample sizes for all four groups had different numbers of larvae. Two, there was difficulty matching the developmental stage of the larvae for the experiment. The Pumilio and the CPEB/Pumilio groups larvae at the beginning of the 5th instar while the control and the CPEB larvae were closer to the end of the 5th instar. Three, the larvae ordered for this experiment had a persistent problem with mold, which may have affected their behavior. In these experiments, lavender essential oil was used as the odor associated with the negative stimulus instead of the ethyl acetate that had been used previously in other studies and in this lab. This change in procedure was decided on due to ethyl acetate's negative health effects on humans. This study was also unable to verify that a knockdown affect from the siRNA injections. The experiment needed to be verified by looking for reduced RNA expression using RT-PCR or reduced protein expression by Western Blotting. For the next phase of this project, the sample sizes would need to be increased as well as keeping them consistent. Also, ways to test memory other than an olfactometer would be investigated



Graph 1. A comparison between all four groups, showing the four categories shown in the tables.



Graph 2. A comparison between the four groups separated by whether the data indicated the larvae remembered or if they did not remember, as defined in the results section.

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ABBREVIATIONS

BLAST, Basic Local Alignment Search Tool; CPE, cytoplasmic polyadenylation element; CPEB, cytoplasmic polyadenylation element binding protein; ddH2O, double-distilled water; DNA, deoxyribonucleic acid; IDeA, Institutional Development Award: LiCl, Lithium Chloride; mRNA, messenger ribonucleic acid; NCBI, National Center for Biotechnology Information; PBS, Phosphate-buffered Saline; PRE, Pumilio Response Element; RNA, ribonucleic acid; RT-PCR, reverse transcription polymerase chain reaction; siRNA, small interfering ribonucleic acid.

REFERENCES

Charlesworth, A.; Meijer, H. A.; Moor, C. H.. Specificity factors in cytoplasmic polyadenylation. *Wiley Interdisciplinary Reviews: RNA* **2013,** *4*(4), 437-461. doi:10.1002/wrna.1171

Chen, G.; Li, W., Zhang; Q., Regulski; M., Sinha, N.; Barditch, J.; . . . Dubnau, J. Identification of synaptic targets of Drosophila Pumilio. *PLoS Computational Biology,Preprint* (2008) **2005**. doi:10.1371/journal.pcbi.0040040.eor

Dong, H.; Zhu, M.; Meng, L.; Ding, Y.; Yang, D.; Zhang, S.; . . . Xu, E. Y. *Pumilio2*regulates synaptic plasticity via translational repression of synaptic receptors in mice. *Oncotarget* ***2018,*** *9*(63). doi:10.18632/oncotarget.24345

Dong, S.; Wang, Y.; Cassidy-Amstutz, C.; Lu, G.; Bigler, R.; Jezyk, M. R.; . . . Wang, Z. Specific and Modular Binding Code for Cytosine Recognition in Pumilio/FBF (PUF)RNA-binding Domains. *The Journal of Biological Chemistry* **2011***, 286*(30), 26732-26742. doi:10.1074/jbc.M111.244889

Dubnau, J.; Chiang, A.; Grady, L.; Barditch, J.; Gossweiler, S.; Mcneil, J.; . . . Tully, T. The staufen/pumilio Pathway Is Involved in Drosophila Long-Term Memory. *Current Biology* **2003,** *13*(4), 286-296. doi:10.1016/s0960-9822(03)00064-2

Gennarino, V. A.; Palmer, E. E.; Mcdonell, L. M.; Wang, L.; Adamski, C. J.; Koire, A.; . . . Zoghbi, H. Y. A Mild PUM1 Mutation Is Associated with Adult-Onset Ataxia, whereas Haploinsufficiency Causes Developmental Delay and Seizures. *Cell* **2018,** *172*(5). doi:10.1016/j.cell.2018.02.006

Gennarino, V.; Singh, R.; White, J.; De Maio, A.; Han, K.; Kim, J.; . . . Zoghbi, H. . Pumilio1 Haploinsufficiency Leads to SCA1-like Neurodegeneration by Increasing Wild-Type Ataxin1 Levels. *Cell* **2015***, 160*(6), 1087-1098. doi:10.1016/j.cell.2015.02.012

Lu, G.; Hall, T. Alternate Modes of Cognate RNA Recognition by Human PUMILIO Proteins. *Structure* **2011***,19*(3), 361-367. doi:10.1016/j.str.2010.12.019

Siemen, H.; Colas, D.; Heller, H. C.; Brüstle, O.; Pera, R. A. . Pumilio-2 Function in the Mouse Nervous System. *PLoS ONE* **2011***,6*(10). doi:10.1371/journal.pone.0025932

Vessey, J. P.; Schoderboeck, L.; Gingl, E.; Luzi, E.; Riefler, J.; Leva, F. D.; . . . Macchi, P. Mammalian Pumilio 2 regulates dendrite morphogenesis and synaptic function. *Proceedings of the National Academy of Sciences* **2010***,107*(7), 3222-3227. doi:10.1073/pnas.0907128107