University of Nevada, Reno

ATP Binding and Turnover in *Drosophila* Synapsin: Cloning and Characterization of the Synapsin 1 C-Domain

A thesis submitted in partial fulfillment
of the requirements for the degree of

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by

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i. Abstract

Synapsins are phosphoproteins in the brain located on synaptic vesicles and involved with regulating neurotransmitter release. The synapsins regulate the amount of synaptic vesicles available for release of neurotransmitters into the synapse through exocytosis and recycling mechanisms. Synapsin is evolutionarily conserved among species, especially in the large central C-domain. In mice lacking synapsin, neurons rapidly run out of synaptic vesicles, indicating that synapsin plays a key role in neuronal transmission. Synapsin mutations have been found in families with genetic forms of epilepsy.

Although the function of synapsin is unknown, it has been shown that the C-domain binds ATP. Experimental evidence indicates that the residue at position 373 has a key role in ATP binding. However, in *Drosophila,* there is a serine substituted for glutamic acid at this position. Therefore, synapsin may not bind ATP. The goal of this project is to study whether or not the *Drosophila* synapsin C-domain binds ATP. *Drosophila* synapsin will be cloned and expressed as a GST fusion protein, purified, and analyzed with stop-flow techniques. The results of these experiments will establish whether or not ATP binding is essential for synapsin function.

ii. Acknowledgements

I would like to thank Dr. Cynthia Corley Mastick and Irina Romenskaia for their continued support and guidance during my research project. I would also like to extend my acknowledgements to the Mastick lab for their assistance and support. Dr. Pai's (University of Nevada, Reno Department of Biology) generous contribution of the GM library used for PCR amplification is also greatly appreciated.

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vi. Introduction

Synapsins are abundant phosphoproteins that are important in regulating the release of neurotransmitters from the synaptic vesicles (Hosaka). The synapsins regulate the amount of synaptic vesicles available for release of neurotransmitters into the synapse through exocytosis and recycling mechanisms. This mechanism is important as it moderates the intensity of neuronal stimulation. Synapsin is evolutionarily conserved among species, especially in the large central C-domain (Baldelli). In mice lacking synapsin, neurons rapidly run out of synaptic vesicles. Synapsin mutations have been found in families with genetic forms of epilepsy.

The synapsin family consists of three homologous proteins which are each composed of common domains A, B, and C, with variable carboxy terminal domains. The C-domain is most evolutionarily conserved among vertebrate and invertebrate species. The conservation of the homologues emphasizes the importance and appropriateness of using *Drosophila* synapsin to study binding activity. Suggestions are that the residue at position 373 has a key role in ATP binding. *Drosophila* has a serine substituted for glutamic acid, therefore not suggested to bind ATP (Klagges). In *Drosophila* and honeybees, the conserved glutamic acid residue at position 373 is a serine for synapsin I. The glutamic acid residue is negatively charged, requiring a Ca2+ stabilization to bind with ATP. Lysine residues are positively charged, not requiring the Ca2+ stabilization. Serine, a neutral amino acid residue, has been suggested to neither require Ca2+ or ATP binding in the synapsin I C-domain of *Drosophila*. Thus, *Drosophila* may be a naturally occurring mutant which lacks ATP binding.

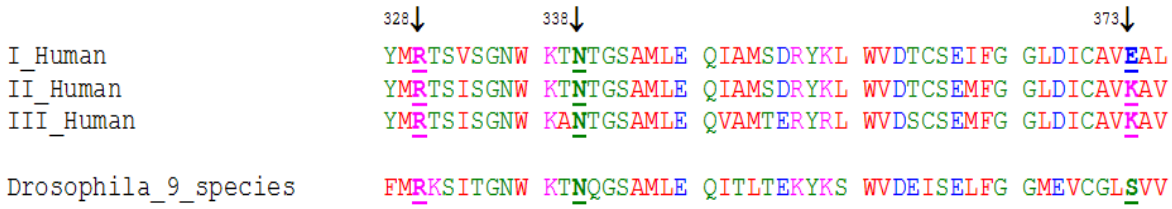

Figure 1. Serine Substitution for Glutamic Acid position 373 in *Drosophila* Synapsin

This figure shows the amino acid sequence of *Drosophila* synapsin in alignment with the three human synapsin isoforms. The marked positions indicate the residues of importance to this study. In mammalian synapsins, change from glutamic acid to serine eliminates ATP binding. The isoforms shown have either a positive or negative charge. Position 373 in *Drosophila* is a serine, raising the question of whether ATP binds to *Drosophila* synapsin.

Previous research has shown that synapsin mutations lead to epileptic symptoms in humans and impaired learning abilities in flies (Garcia). Mice with synapsin mutations have shown impaired learning as well (Baldelli). A nonsense mutation was found at position 356 of synapsin in families with epileptic symptoms, before the studied location of ATP binding in the C-domain (Garcia). With the relatively little that is known about synapsins, future research of synapsins will lead to advancements in academia and health implications caused by synapsin mutations (Klagges and Wallace). By understanding the binding of the synapsin C-domain, we can determine the regulation of synaptic vesicles and neurotransmission.

vii. Methods

Cloning and Transformation

Stock DNA was obtained from the *Drosophila* Genomics Resource Center (DGRC in Bloomington, IN) and eluted using their protocol for the Whatman FTA disc. The order stock came from RE44971 from Riken Embryo, stock 9299, with ampicillin resistance (received on 8/5/09) in the pFLC1 vector. To amplify the DNA, DH5α E. coli cells were transformed. However, no clones were obtained. Stock DNA was obtained from the DGRC again in November, 2009, and the previously used protocol was followed. The colonies were then transformed on LB + carbenicillin plates as suggested by the DGRC. Bacteria containing the DNA constructs were obtained in this second attempt.

Transformed bacteria were inoculated in LB broth with ampicillin overnight. DNA was prepared using a Bio-Rad Quantum Prep Plasmid Miniprep Kit (Bio-Rad; Hercules, CA) according to the manufacturer's suggested protocol. The samples were maintained in a -20°C freezer.

Restriction Digest to verify insert

Restriction digests were performed with the Promega samples in order to verify for the synapsin insert. The protocol for restriction digest involved 5µl DNA, EcoR1 NEB Buffer, 1/10 BSA, Enzyme (EcoR1, Not1, Xho1, and Kpn1), and distilled water. The samples were incubated at 1 hour in a 37°C water bath before verifying products on a 0.8% agarose gel.

Polymerase Chain Reaction (PCR) to amplify Drosophila synapsin C-domain

a. PCR Amplification

Primers were developed to amplify the *Drosophila* synapsin C-domain (dSynapsin). The forward primer sequence of: GAATTCAAGTACTTCACGCTGCTGG and reverse primer sequence of: CTCGAGTTAACGGCAGACGTTCTGCATC were used. The primers were used in the Polymerase Chain Reaction (PCR) to amplify the DNA and to allow for subcloning into pBluescript KS+.

b. PCR Conditions and Cycling Parameters

The PCR amplification was performed in a 50 µl reaction containing: 2 µl primer mix, 1 µl 1/1000 DNA, 4 µl 25µM $MgCl_2$, 1 µl DNTP, 5 µl PCR buffer, 36.5 µl dH_20, and 0.5 µl Taq polymerase.

The standard PCR cycling protocol was as follows: 94°C for 3 minutes; 5 cycles of 94°C for 1 minute, 65°C for 1 minute, 72°C for 2 minutes; 5 cycles of 94°C for 1 minute, 60°C for 1 minute, 72°C for 2 minutes; 30 cycles of 94°C for 1 minute, 55°C for 1 minute, 72°C for 2 minutes; 72°C for 7 minutes; and 4°C until samples were retrieved.

c. PCR Protocol for Subsequent Amplifications

Subsequent PCR amplifications varied in the DNA concentration, cycling parameters and techniques, and the use of a linear sample of the dSynapsin. Concentrations varied to using 1/100, 1/50, 1/25 and 1/10 DNA. Cycling parameters and techniques involved a hot start technique, waiting for the lid to heat up prior to use. The cycling procedure was altered to 94°C for 3 minutes; 5 cycles of 94°C for 1 minute, 65°C for 1 minute, 72°C for 2 minutes; 30 cycles of

94°C for 1 minute, 55°C for 1 minute, 72°C for 2 minutes; 72°C for 7 minutes; and 4°C until samples were retrieved.

d. Verification of PCR Products

Agarose Gel Electrophoresis: All PCR products were resolved on a 0.8% agarose gel and contained 3µl of Ethidium Bromide. The samples were loaded with BBXF loading dye.

Sequencing of dSynapsin samples

The samples obtained from the miniprep protocol were sequenced at the Nevada Genomics Center. Four sequencing primers were utilized in the sequencing of the dSynapsin. The machine used for sequencing at the genomics center was the Applied Biosystems Prism 3730 DNA Analyzer. A summary of the primers used is shown in Table 1. Sequencing was also performed with SynUp1, SynUp2, SynDown3, and SynDown4 to verify the homology of the sample to the DNA library in NCBI Blastx.

Table 1. Summary of Primers Used for PCR and Sequencing		
Name of Primer	**5' to 3' Sequence**	**Use**
forwardEcoR1dSyn	CGAATTCAAGTACTTCACGCTGCTGG	PCR
reverseXho1StopdSyn	GCTCGAGTTAACGGCAGACGTTCTGCATC	PCR
SynUp1	CGGCCCATCCAAGATCAGC	Sequencing
SynUp2	CTGCGGTTCAGAGCGCCACACAAC	Sequencing
SynDown3	CCGAGGAGCGGGAGGGCAACTT	Sequencing
SynDown4	CGCTGCTCGTCTCGCTGGCCG	Sequencing
SynPCR2/Forward	TCTTCCGAGGTAGACGATGTCGAT	PCR
SynPCR2/Reverse	TCTGCCGCGCTTCTTATTAGCGAT	PCR
forwardAMPr	TGACGCTCAGTGGAACGAAA	PCR
reverseAMPr	GAGTATTCAACATTTCCGTGTCGC	PCR

Linearization of PCR Template

A dSynapsin sample was digested with Not1 for 1 hour in a 37°C water bath. The samples were then run on a 0.8% low-melt agarose gel. The products were removed from the TAE agarose gel and purified using a Geneclean Kit (MP Biomedicals; Solon, OH) following the suggested protocol to purify DNA from TAE agarose gels. The purified product was then frozen in the -20°C freezer for future use.

The purified geneclean sample was to test for amplification of the synapsin C-domain. Primer sets were ordered to amplify outside of the anticipated area with forward

primer: SynPCR2/Forward and reverse primer: SynPCR2/Reverse. PCR amplification of the ampicillin resistant portion was performed as a positive control.

Preparation of pBluescript KS+ and pGex-6p-1

DH5α cells were transformed with pBluescript and pGex-6p-1. After plating on LB-amp plates, the colonies were selected for purification using a Bio-Rad Plasmid Midiprep Kit (Bio-Rad; Hercules, CA) according to the manufacturer's suggested protocol. To verify the DNA samples, a restriction digest was performed using EcoRI (NEB), XhoI, and both for a double digest. The samples were digested for 1 hour in a 37°C water bath. The vectors were frozen in a -20°C freezer for future use.

viii. Results

Cloning and Transformation

A clone of *Drosophila* Synapsin was ordered from the *Drosophila* Genomics Resource Center. The dSynapsin was transformed into the pFLC1 vector thus indicating a full size of about 8.8 kb.

The clones were verified by restriction digest. The expected cut sites are shown in Figure 2 with enzymes EcoR1 and Xho1. Figure 3 shows the Promega samples as they were digested with enzymes EcoRI, XhoI, and both to test for inserts in the pFLC1 vector. Three bands are expected to be shown by the digestion with EcoR1 at the sites shown in lane 2 with cut sites at 3.5kb, 4.9kb, and 5.3kb. Approximate sizes are 7kb, 1kb, and 404bp. Digestion with Xho1 should yield two cut sites at approximately 167bp and 5.3kb. Approximate sizes are 3kb and 5kb. Lane 4 corresponds to the double digestion performed with both EcoR1 and Xho1 yielding a cut site at 4kb. Lanes 1 and 5-12 represent the non-digested Promega samples.

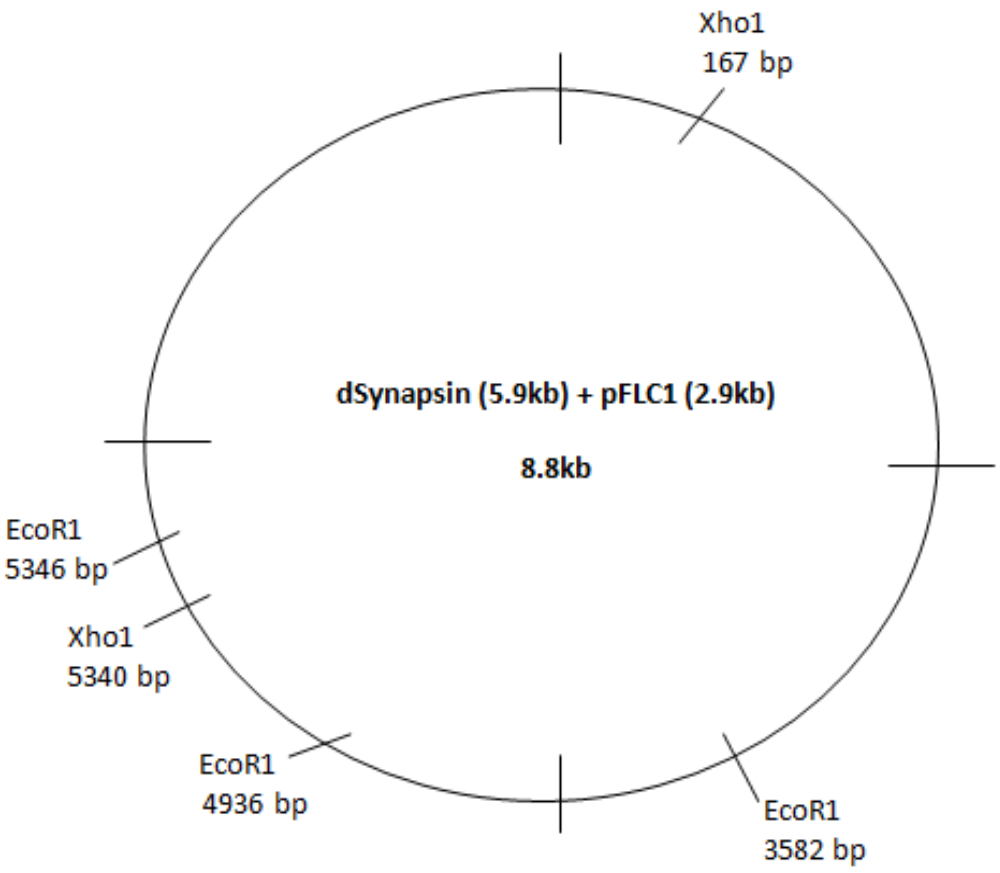

Figure 2. Restriction Map of dSynapsin and pFLC1 vector (8.8kb)

This figure shows the map of the dSynapsin in the pFLC1 vector showing the cuts sites of Xho1 and EcoR1.

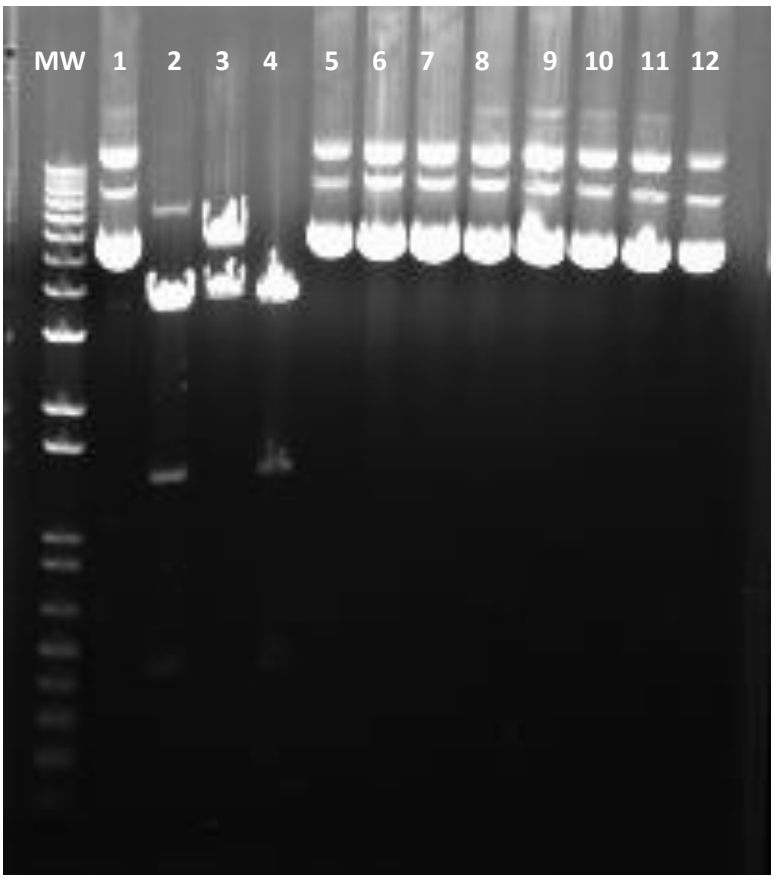

Figure 3. Restriction digests of dSynapsin and purified dSynapsin on a 0.8% agarose gel

This figure shows the miniprep samples from the dSynapsin as well as sample 1 digested with EcoR1, Xho1, and double digested with EcoR1 and Xho1. MW: molecular weight marker, 1kb ladder, lane 1 shows sample 1, lane 2 shows dSynapsin digested with EcoR1, lane 3 shows sample digested with Xho1, lane 4 shows sample double digested with EcoR1 and Xho1, lanes 5-12 show samples 2, 3, 4, 10, 6, 7, 8, 9, respectively.

A second digestion was done to verify the insert. Samples were digested with EcoRI, XhoI, NotI, and KpnI to test for inserts as an alternative to verifying the dSynapsin insert in pFLC1 vector. Figure 4 represents the results obtained from the digestion with these enzymes. Lane 1 shows the dSynapsin sample 2 used for this digestion. Lane 2 shows the digestion with Not1 which is not expected to make any cuts

compared to the original sample in lane 1. Lane 3 shows the digestion performed with Kpn1 with a single cut. Lane 4 shows digestion with EcoR1 with 3 cuts as previously performed. Lane 5 shows digestion with Xho1 and lane 6 shows dSynapsin sample number 10.

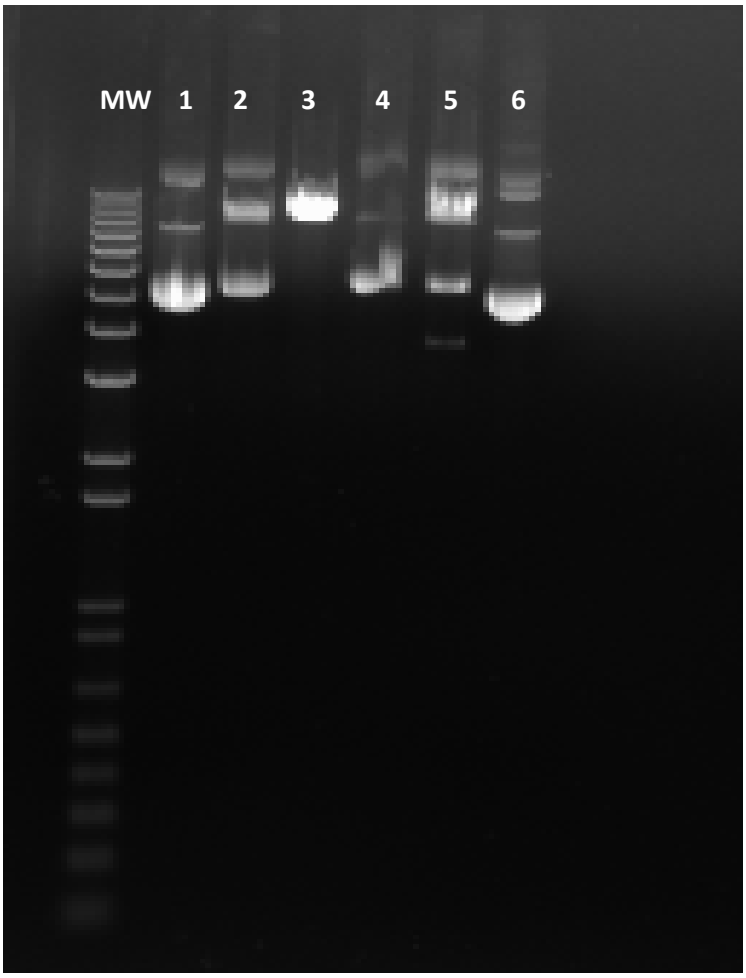

Figure 4. Restriction digests of dSynapsin and dSynapsin on a 0.8% agarose gel

This figure shows the miniprep samples from the dSynapsin as well as sample 2 digested with Not1, Kpn1, EcoR1 and Xho1. Lane 1 shows sample 2, lane 2 shows dSynapsin digested with Not1, lane 3 shows dSynapsin digested with Kpn1, lane 4 shows dSynapsin digested with EcoR1, lane 5 shows dSynapsin digested with Xho1 and lane 6 shows dSyapsin sample 10.

Polymerase Chain Reaction (PCR) to amplify Drosophila synapsin C-domain

PCR reactions were performed in an attempt to amplify the *Drosophila* synapsin 1 C-domain. Primers used are shown in Table 1. These were designed to yield a C-domain flanked by EcoR1 and Xho1 creating sites for cloning. There was also a stop codon inserted in-frame. This was designed to clone into pGex-6p-1 to create an in-frame fusion protein with GST. Conditions were changed for each new PCR reaction with varying concentrations of DNA, DNA samples, cycling parameters, primers and the use of a linear sample of the dSynapsin, as previously mentioned in the Methods section. No product was obtained by the methods used. As shown in figure 5, an expected band size would have been at about 950 kb. Instead, only primers were observed. The primers were used for multiple reactions to confirm its action in differing PCR conditions. However, the expected product was not recovered upon verification of the PCR products.

Figure 5 represents the general result of PCR reactions upon agarose gel electrophoresis for verification of the amplification of the C-domain. The boxed region shown in the figure represents the expected size of the PCR product at 950 kb. Lanes 1 and 2 represent the DNA samples as different conditions were varied. Lane 3 represents the negative control resulting in the primers, as shown.

PCR reactions were performed using the forward and reverse primers: forwardEcoR1dSyn and reverseXho1StopdSyn by varying the Promega samples' concentrations to 1/1000, 1/500, and 1/100. Concentrations of PCR mix for the protocol was also verified to confirm concentrations. The cycling parameters were altered to check for variation in its effect on yielding the expected PCR product.

An attempt was made to PCR amplify from another template. A GM library sample graciously donated by Dr. Chi-Yun Pai (Department of Biology at the University of Nevada, Reno) was used for another PCR reaction. The GM library was from the Berkeley *Drosophila* Genome Project. The sample was an ovary oligo-dT phage library. A 1/1000 dilution was made of the provided sample then run according to the standard PCR protocol. The expected product was not obtained. The recovery only showed primers as verified by the control shown in lane 3.

It was possible that the PCR primers ordered were not good for the reaction. Therefore, another PCR reaction was performed with primers that flanked the desired region: SynPCR2/Forward and SynPCR2/Reverse. However, this again did not result in the expected product.

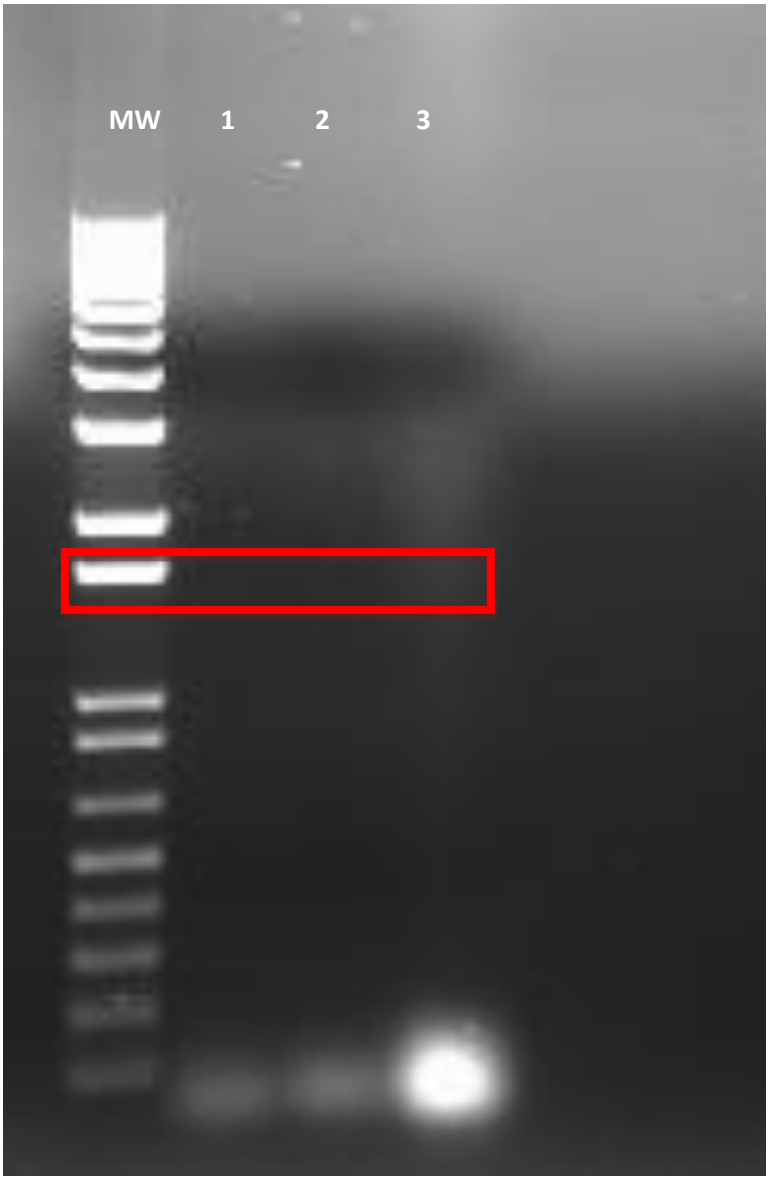

Figure 5. PCR products and negative control on a 0.8% agarose gel
This figure shows the products of a PCR reaction with 1/1000 DNA and 1/100 DNA. A negative no template control was also used for this reaction. Lane 1 shows a molecular marker, lanes 2 and 3 show the DNA sample with no product yielded, and lane 4 shows the negative control.

Sequencing of dSynapsin samples

Another possible explanation for the failure to PCR amplify the clone was that the PCR primers did not match the sequence exactly. To verify the DNA sequence of the

template, sequencing was performed with the sequencing primers mentioned in Table 1. Sequencing results from the Promega sample 7 with sequencing primer SynUp2 yielded the result shown in figure 6 as an NCBI Blast. The result yielded approximately 240 base pairs. There was a 99% alignment with *Drosophila* synapsin and the query and subject lines indicate the match between the samples used in the lab against that in the NCBI library for confirmation of homology. The highlighted portion indicates the homology of primer sequence previously used upstream of the AAG site in the C-domain. Other sequences verified the presence of the other primers.

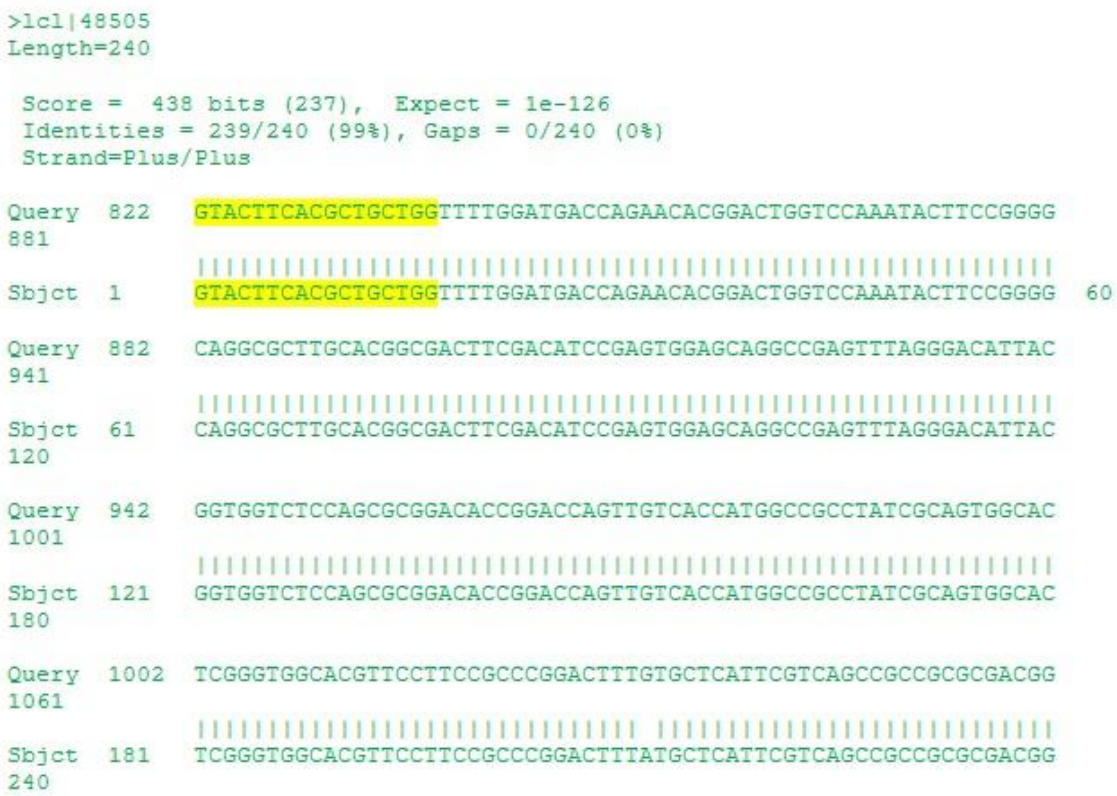

Figure 6. Sequence alignment of dSynapsin with NCBI library *Drosophila* synapsin

This figure shows the NCBI library of *Drosophila* synapsin with the sequencing results of dSynapsin sample 7 sequenced with primer SynUp2. The sequences aligned at 99% confirming that the dSynapsin samples are correct and valid as verification of the primer site.

Geneclean: to linearize plasmid DNA

A final possibility was that the DNA had a conformation that would not allow amplification. Therefore, linearization of the plasmid DNA was performed in hopes of being able to amplify the synapsin C-domain. Figure 7 shows the results of the agarose gel to confirm that the samples were prepared properly and as anticipated with a band at about 5kb shown in the boxed region of the gel.

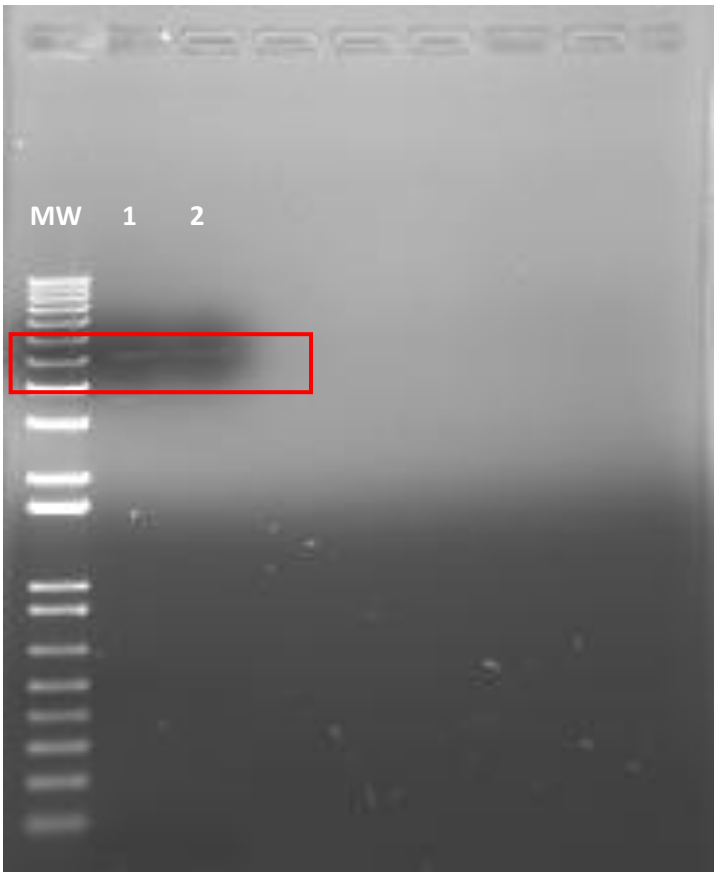

Figure 7. Geneclean samples of dSynapsin on a 0.8% agarose gel
This figure shows the samples as a result of the geneclean procedure of the dSynapsin. Lane 1 shows the molecular weight and lanes 2-3 show the Not1 digested samples indicating a band at 5kb.

The geneclean product was then run in a PCR reaction. An additional control reaction was done using primers to amplify the AMP resistance gene from the vector for

a positive control to verify accuracy of the PCR protocol. Lanes 1-4 show the sample with previously used primer sets forwardEcoR1, reverseXho1, SynPCR2/Forward, and SynPCR2/Reverse. Lanes 5 and 6 represent the negative controls for each primer set. Lanes 1-6 did not yield the expected product size of 950kb. Lane 7 represents the primer set for ampicillin resistance indicating that the positive control was successful as shown in the boxed region on the agarose gel below at about 800bp.

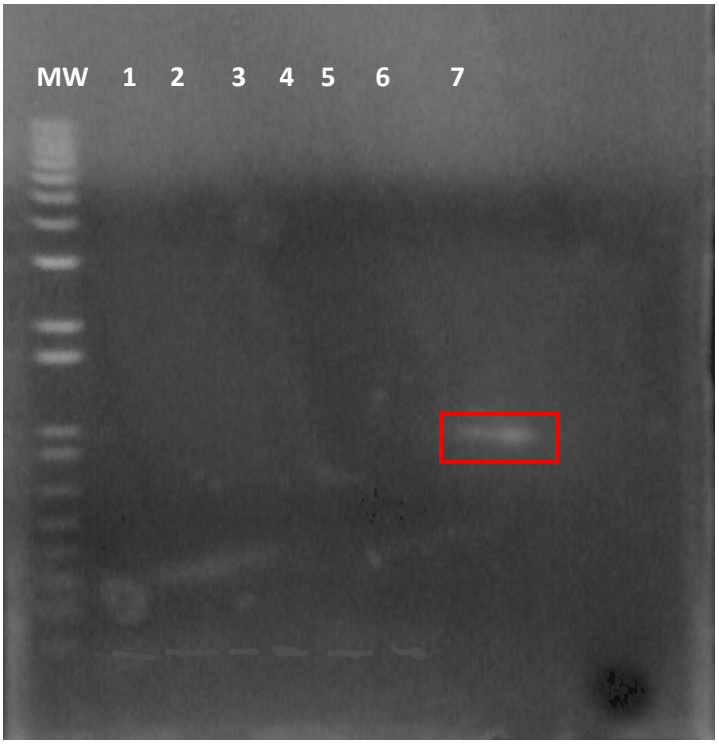

Figure 8. PCR products, negative control, and positive AMPr control on a 0.8% agarose gel

This figure shows the molecular weight, the geneclean samples run with the primer sets in Table 1 for forward and reverse on lanes 1-4, lanes 5-6 represent the negative control for the primer sets, and lane 7 represents the primer set for ampicillin resistance indicating a successful positive control for verification of the PCR protocol. However, no expected product was recovered.

ix. Discussion

The overall goal of the project was to analyze the ATP binding and turnover in *Drosophila* synapsin. However, the amplification of the synapsin C-domain has been a big obstacle in proceeding with the anticipated goals of the project. Therefore, the question raised by information involving figure 1 was inconclusive in this study. The first clones received from the *Drosophila* Genomics Resource Center did not yield enough cells. Few colonies had grown; this indicated that the cells used to transform the clones may not have been competent enough. Clones were reordered from the *Drosophila* Genomics Resource Center. Clones were successfully grown and the samples were purified using a miniprep protocol. The use of LB + carbenicillin plates instead of LB + ampicillin plates in transformation aided in yielding more colonies than the original transformation as the carbenicillin is less toxic to the bacteria resulting in a better yield. Figure 2 shows the confirmation of the samples for further use in the amplification of the synapsin C-domain. The dSynapsin samples from the DGRC were inserted into the pFLC1.

PCR amplification was a difficult process in attempting to obtain the synapsin C-domain. A sample of cDNA library of the synapsin from an ovary oligo-dT sample was graciously sent by Dr. Pai (Department of Biology; University of Nevada, Reno) for more PCR amplification.

The PCR protocol was subsequently performed using the dSynapsin miniprep samples. Various conditions were altered each time in an attempt for amplification. The changes involved those mentioned in the methods. The alternations did not successfully yield a product. Primers were ordered outside of the originally ordered primer set in

order to amplify a larger segment in hopes of using that piece to amplify the smaller segment. Although the sequencing primers confirmed a 100% homology to *Drosophila* synapsin, the PCR amplification was again unsuccessful.

A Geneclean protocol was employed to linearize the plasmid DNA. This was done to overcome the circular structure or any super-secondary structures that may have been inhibiting the PCR amplification. Figure 7 shows the successful result of the Geneclean protocol of the dSynapsin. Again, the procedure was not an effective change in accordance to the reactions performed thus far. However, as shown in figure 8, the positive control with the ampicillin portion yielded positive results indicating that the PCR protocol conditions were able to yield a product. The expected band at 950kb was not recovered through this final process using the Geneclean product.

Previous research on synapsin has had difficulty involving the cloning of the synapsin protein as well. In Klagges' research on invertebrate synapsins, their study findings enabled them to clone a 50% identity gene with 309 amino acids. However, none of the cDNAs isolated were full-length (Klagges). Research by Hosaka has suggested that the molecular structure of synapsin is complex, enhancing the difficulties of PCR amplification of specific domains (Hosaka). Past research in the Mastick lab has also indicated that the synapsin protein is difficult to PCR amplify of specific regions (Mastick).

Future directions of this project would be to finish amplifying the synapsin C-domain. Upon doing so, the methods should continue with expression into GST fusion proteins for analysis of the ATP on and off rates to better understand its activity. The completion of this experiment will greatly benefit the characterization of the *Drosophila*

synapsin 1 C-domain. This will be of great benefit to the scientific research and clinical community as it brings forth beneficial information regarding synapsin's activity and how it can affect patients with epileptic symptoms. A better understanding of the synapsin C-domain can help draw relations to effects in humans and can enable studies of related enzymes and biological functioning.

x. References

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