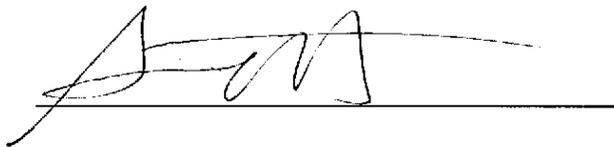Exploring GluR2-Interacting Proteins in *Caenorhabditis elegans*

Honors Thesis
Department of Life and Environmental Sciences,
Carroll College,
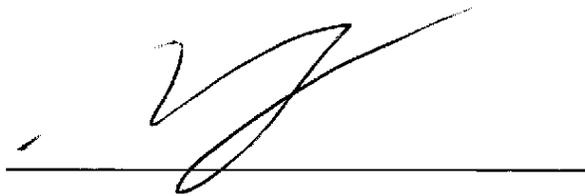Helena, Montana

John C. Brothers
April 27, 2016

Signature Page

This thesis for honors has been approved for the Department of Life and
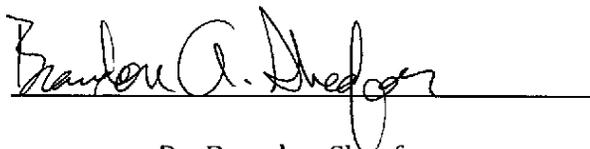
Environmental Sciences by:

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Director   Date

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Dr. William Parsons   Date

_____   _____

Dr. Brandon Sheafor   Date

Acknowledgements

I have been told it is a truly rare and wonderful thing to acknowledge the help of others, but I know it is an entirely different thing to do it justice with words. As a whole, this project was difficult and time-consuming, but no single aspect was more so than this one. Not because the people deserving acknowledgment are infinite, or because I will surely need their guidance and good recommendations again, but because the talents of my writing could never adequately express the level of appreciation they all so rightfully deserve. Nonetheless, what follows is without doubt the greatest I could do, and should it fall short of what everyone disserves, it is surely near what anyone could expect of me.

I must begin by extending my sincerest gratitude to my advisor and mentor, Dr. Stephanie Otto-Hitt. My time working in her lab can only be described as remarkable. And although the skills and lessons I learned were and are still invaluable, I cannot decide who is more obligated, whether I to her for hiring me when my summer was devoid of opportunity, or her to me for having not been hired, I would surely have napped more. There are those that say her reviews are critical and her classes are difficult, but they naively forget that as fire burns wood but purifies gold, so too does she.

In a particular order from here, I would like to express recognition of Dr. Gerald Shields. His unparalleled observance of even the most minute detail would buoy most any project. If one truly sees furthest from the shoulders of giants, Dr.

Shields, standing on unique age, tenure, and infinite publications, offers an unprecedented view to any student who survives the summit of his height.

There are times in research and in life when we all deceive the better angels of our nature, when confused they seek those persons who can tell us rather than those things that can teach us. I do not deny the appeal; listening is easier than learning, but it never resolves confusion to the same degree. For it appears one does infinitely better with eyes then with ears. It is thus necessary that I also acknowledge both those modern articles and those ancient books that taught me when I was confused.

It is a common practice to bury our professors with requests for thesis reviews. We need their time, their reviews, and their signatures, and we need them all by May. These requests are difficult and time-consuming, and they should not be fulfilled by anybody. They are accomplished best by those professors with extraordinary curiosity, astuteness, wisdom, and honesty; and so everyone should choose their reviewers in the same mode that I did: with a strict requirement for all the above qualities. It is altogether fitting and proper that it should be done this way because some reviewers are assigned and some are chosen, and we must choose the qualities we are never assigned. I hope my reviewers, Dr. William Parsons and Dr. Brandon Sheafor, will at some point see in me the same qualities I saw in them.

I have decided for the remainder of this to change both course and definition, for I must acknowledge the lacking completeness that plagues certain aspects of my project. Minor imperfections in the methods generate inconclusive results; but

never, never should one consider this the fault of any person mentioned above or below. These poverties of talent and time are mine and mine alone.

Nevertheless, do not be too quick to condemn this little project of mine; for within it hides a solid foundation for a spectacular publication. It is not destined to gather dust on a basement shelf; for I know one day a student with more intellect and more diligence than me will come across what I have done. And having understood what I had wrote and knowing what I did not, they will surely write something worth reading.

"Although this enterprise may be difficult, nonetheless, aided by those who have encouraged me to accept this burden, I believe I can carry it far enough so that a short road will remain for another to bring it to the destined place."

Abstract

The expression of ϒ-3-hydroxy-5-Methlisoxaole-4-Propionic Acid Receptors (AMPARs) on the post-synaptic region of a neuron has direct impacts on many physiological functions of the brain, including learning and memory. Because AMPARs are first assembled in the endoplasmic reticulum and later transported to the post-synaptic region of a neuron, understanding the mechanisms involved in AMPAR-trafficking is crucial to understanding a variety of neuronal functions. The goal of this research was to investigate the role of the putative AMPAR-trafficking proteins DLI-1, VDAC-1, SVOP-2, and DCN-2 on AMPAR-mediated behavior in the nematode *Caenorhabditis elegans (C. elegans).* Unfortunately, several technical issues prevented development of the constructs and behavioral assays needed to fully accomplish the goals of this study. Therefore, I am unable to discourse on the roles of DLI-1, VDAC-1, SVOP-2, and DCN-2 in AMPAR-mediated behavior in *C. elegans*.

Introduction

The strength of signals moving between two neurons is proportional to the expression of ϒ-3-hydroxy-5-Methlisoxaole-4-Propionic Acid Receptors (AMPARs) on the post-synaptic neuron (Brown *et al*, 2007). Trafficking, or adjusting synaptic AMPAR-levels via directed transport, enable neurons to regulate neuronal-signal strength (Brown *et al*, 2007). Because such signals have direct impacts on many physiological functions such as learning, memory, and cognitive behavior (Brown *et al*, 2007), the identification of proteins involved in trafficking AMPARs to the synaptic surface became crucial.

AMPARs are tetrameric proteins formed from various combinations of four subunits: GluR1, GluR2, GluR3, and GluR4 (Rosenmund *et al*, 1998; Hollmann and Heinemann, 1994). AMPARs are first assembled in the endoplasmic reticulum and then trafficked to the post-synaptic region of neurons (Ziff, 2007). Transmembrane AMPAR Regulatory Proteins (TARPs) are believed to facilitate this trafficking (Ziff, 2007).

Assaying molecular interactions with the most abundant AMPAR subunit, GluR2, led Shanks *et al*. (2012) to suggest 421 proteins as potential GluR2 interactors. Thus far, only five of the candidates from this assay have been shown to affect the trafficking of AMPARs to the post-synaptic region (Esteban, 2008; Tomita *et al*, 2003; Kato *et al*, 2007). Two major questions persist: which of the remaining proteins from the Shanks *et al*, (2012) study have similar influences on AMPAR trafficking, and, what are their specific roles in physiological function.

The purpose of this research was to investigate these questions by identifying novel GluR2 interactors and characterizing their specific roles in AMPAR trafficking. Four genes were selected from the Shanks *et al*, (2012) list: DYNC1LI1, VDAC2, SV2A, and DCNT2. Human genes are inherently difficult to investigate, so model organisms were necessary. *Caenorhabditis elegans* (*C. elegans*) was selected as a model organism for four reasons: they are inexpensive and easy to maintain, their sequenced genome allows for quick identification of homologous genes, there exist well-established protocols for generating gene knockouts, and their large size allows observation with a dissection microscope rather than a compound microscope.

Homologous genes are genes from separate species that have conserved sequences and functions. Using Wormbase[1], the four homologous *C. elegans* genes were determined to be DLI-1, VDAC-1, SVOP-1, and DCN-2, respectively. This research hinges on the assumption that these four *C. elegans* genes and their human counterparts have similar physiological functions.

To investigate their physiological roles, genes can be inhibited and the phenotypic effects observed. A common gene-inhibition method for *C. elegans* is RNA interference (RNAi). The Central Dogma of biology asserts that DNA codes RNA which codes proteins. Typically, it does not matter whether the DNA or the RNA is inhibited since the ultimate goal of gene knockouts is the removal of gene-products encoded by a particular gene. RNAi targets mRNA molecules by introducing double-stranded RNA (dsRNA) into the *C. elegans* genome in order to induce

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[1] www.wormbase.org

PostTranscriptional Gene Silencing (PTGS) (Fire *et al*, 1998). Specifically for *C. elegans*, dsRNA is constructed to contain one strand of RNA complimentary to the target mRNA sequence. This dsRNA sequence can be transfected into certain *E. coli* strains and fed to populations of *C. elegans* (Timmons *et al*, 2001). The *E. coli* plasmid will be ingested and incorporated into the *C. elegans* genome via an unknown mechanism. Once the dsRNA is introduced, it can be recognized and bound by Dicer enzymes (Hannon, 2002). Dicer enzymes are a distinct family of dimeric RNases that cleave dsRNA into small strands of small-interfering RNA (siRNA), (Blaszczyk et al, 2001). The RNA-induced silencing complex (RISC) binds and unwinds these newly-formed siRNAs before using them as guides to find and bind to the complimentary strand of target mRNA (Hammond et al, 2000). RISC proceeds to degrade the target mRNA (Blaszczyk et al, 2001). Lacking the necessary mRNA, the cell cannot translate the corresponding proteins – an effectual gene knockout.

Individual worms can have their behavior assayed to gauge the specific phenotypic effects of the knockouts. Physiological functions of each gene can be explored by comparing learning, memory, and cognitive behavior between the knockout worm and a wildtype worm. I hypothesize these knockout worms will show inhibited neuronal function; therefore, suggesting DLI-1, VDAC-1, SVOP-1, and DCN-2 serve some role in learning, memory, or cognitive behavior. Because of known similarities between homologous genes, if DLI-1, VDAC-1, SVOP-1, and DCN-2 serve roles in *C. elegans* physiological function, it is likely DYNC1LI1, VDAC2, SV2A, and DCNT2 serve similar roles in human physiological function.

Materials and Methods

C. elegans Generation and Maintenance

A wildtype *C. elegans* strain (N2) was purchased from Carolina Biological[2]. All N2 vials, original stock and additional lab generations, were stored at -80° C. The vials were thawed as needed and placed on Nematode Growth Media (NGM) plates seeded with OP50 *E. coli*. Stocks of OP50 were also purchased from Carolina Biological[1].

NGM is created by dissolving 3.0 g NaCl, 2.5 g peptone, and 17.0 g agar (yields 1.7% plates) in 972 ml of reverse osmosis (RO) water. Once autoclaved, the following reagents were added in order: 1ml cholesterol in ethanol (5 mg/ml), 1 ml M $CaCl_2$, 1 ml M $MgSO_4$, and 25 ml M phosphate buffer (pH 6.0). The 1000 ml of NGM could be poured aseptically to form 35-40 NGM plates or stored in a sealed container at room temperature. All NGM plates were stored at 4° C.

NGM plates were seeded with OP50, an *E. coli* mutant that requires environmental uracil to survive (Brenner, 1973). This mutation is crucial because it limits the growth of the bacterial lawn. By limiting growth to the availability of uracil in the NGM plates, bacterial lawns were kept to a minimum size. This ensured visibility of the growing worms (Brenner, 1973). All OP50 vials, original and additional lab populations, were stored at -80° C. Vials were thawed as needed and mixed into culturing broth. Culturing broth was created by dissolving 1.0 g tryptone,

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[2] www.Carolina.com

0.5 g yeast extract, 0.5 g NaCl in 100 ml of RO water. This solution was autoclaved and stored at room temperature in two 50 ml screwcap tubes. Once the culturing broth was inoculated with OP50 stock, it was immediately incubated at 37° C for 24 hours. Inoculating the broth was completed under standard, aseptic technique.

To seed the NGM plates, 20 μl of OP50 was aseptically transferred to the center of a NGM plate. Such quantities of OP50 generated an ideal-sized bacterial lawn. Seeded plates were incubated at 37° C for 72 hours to instigate growth of the bacterial lawn and to ensure no contaminations occurred while seeding. After the 72-hour incubation, the seeded NGM plates were removed and either plated with *C. elegans* or stored at 4° C. Seeded NGM plates were stored at 4° C and not 37 ° C to limit the growth and extend the life of the bacterial lawn. Bacterial lawns left at 37° C would quickly deplete the available supply of uracil.

Seeded NGM plates were populated with *C. elegans* in one of two modes: either directly from thawed stock populations or indirectly from already-populated plates. The former required aseptically transferring the entirety of one vial to a seeded NGM plate, avoiding placing the contents directly on the bacterial lawn. By placing the thawed worms on the perimeter of the plate, the worms were forced to travel across the plate toward the bacterial lawn, leaving behind the dead or weak worms. The latter mode required a method called "chunking." Chunking entailed transferring a populated chunk of agar from an existing plate population to an unpopulated seeded NGM plate. The chunks were not taken directly from or placed directly on a bacterial lawn. New and chunked populations were placed near the edge of the seeded NGM plate. This allowed healthy *C. elegans* to separate from the

weak or dead worms as they traversed the plate to the bacterial lawn (Brenner, 1973).

Populating new NGM plates, via chunking, occurred roughly every 10 days to avoid overpopulated plates. New populations from frozen stocks required an extended time frame for they required time to develop mature, egg-laying adults; storage at -80° C killed mature specimens but not eggs (Brenner, 1973). Both developing and newly populated NGM plates were stored in the dark at room temperature.

RNA Extraction and Purification

C. elegans populations were washed from four mature NGM plates into four 15 ml screw-cap tubes using 4 ml of M9 buffer. Each plate was washed a second time with a second 4 ml of M9 buffer to ensure a thorough removal of *C. elegans* populations. The most efficient method for washing NGM plates was excising a small triangle of agar on the edge of the plate. This created a well for pipetting the worms easily without pulling up any agar. The 15 ml centrifuge tubes were spun briefly at 1,000 RPM to pellet the *C. elegans*. In a fume hood, each of the pellets was re-suspended in 4 ml of TriZol. At room temperature, the tubes were vortexed until each pellet was completely dissolved. The entirety of the tubes was transferred to 16 Eppendorf tubes (1 ml per tube). These tubes were vortexed intermittently at room temperature for five minutes, alternating 30 seconds on, 30 seconds off. Two hundred and fifty microliters of chloroform were added to each micro-centrifuge

tube, and the contents were vortexed again for 15 seconds and left at room temperature for 2-3 minutes to facilitate lysis. The samples were centrifuged for 15 minutes at 14,000 RPM at 4° C separated phases. The top, aqueous layer from each tube was transferred to a new Eppendorf tube. The white interphase and the Trizol layers were avoided because Trizol contamination would have affected future experimentation. Five hundred microliters of isopropanol were added to each of the aqueous samples to precipitate the RNA. The tubes were briefly mixed and allowed to incubate at room temperature for 10 minutes.

After the 10 minute incubation, the samples were centrifuged at 14,000 RPM for 12 minutes at 4° C to pellet the RNA. The RNA pellets were clearly visible, and the supernatants were poured off. The pellets were washed with 100 μL of 75% ethanol in nuclease-free RO water and centrifuged at 7,500 RPM for five minutes at 4° C.

The ethanol supernatant was poured off, and the pellet was re-suspended in 100 μL of nuclease-free water. A small portion of the sample (8 μL) was removed for electrophoresis confirmation; the remaining samples were immediately stored at -80° C.

The samples were separated on a standard 1.7% agarose gel for nucleotide detection. A DNase I treatment was used to degrade any DNA remaining from the RNA extraction. DNase I was purchased from New England Biolabs. The protocol provided by New England Biolabs was followed. RNA (10μg) was mixed with 62 μl of 1X DNase Reaction Buffer for a final volume of 100 μl. Two units of DNase I was added, and the entire solution was quickly mixed and incubated at 37°C for 10

minutes. One μl of 0.5M EDTA was added to protect the RNA from the heat inactivation of the DNase I (10 minutes at 75°C). The post-DNase samples were analyzed on a standard 1.7% agarose gel.

Primer Design

Primers were designed for the PCR amplification of the target sequences and the subsequent production of the dsRNA. Primers were designed for each of the four experimental *C. elegans* genes (DLI-1, VDAC-1, SVOP-1, and DCN-2) as well as for one control *C. elegans* gene (ACT-1). ACT-1 is a known housekeeping gene in *C. elegans,* so it was reasonable to assume ACT-1 mRNA would be in high concentrations and serve as a positive control.

Wormbase[3] was used to identify the sequences of the experimental and control genes. From this data, the introns were separated from the exons to identify coding sequences. NEBcutter[4] from New England Biolabs was used to identify restriction enzymes that would not cut the gene's coding sequence. The restriction sites for two of these enzymes were in the forward and reverse primers to facilitate cloning into the expression vector. Double Digest[5], another tool from New England Biolabs, was used to ensure buffer compatibility for all combinations of the selected restriction enzymes.

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[3] wormbase.org
[4] nc2.neb.com/NEBcutter2
[5] neb.com/tools-and-resources/interactive-tools/double-digest-finder

The forward primers all started with the same three components: a two base-pair overhand (typically AT), the selected restriction enzyme site, and a start codon (ATG). The fourth component varied with each primer, as it was approximately the first 20 nucleotides from the 5' end of each gene, omitting the start codon. The exact number of nucleotides selected depended on the calculated melting temperature of the whole sequence of all parts; the equation used for calculating the melting temperature was $°C = 2(A+T)+4(G+C)$. Nucleotides were added or subtracted to ensure a melting temperature near 60°C. The final nucleotide was kept as either cytosine or guanine because the extra set of hydrogen bonds strengthens the terminus of the strand.

The protocol for designing the reverse primers varied slightly. The reverse primers all started with the same first two components as the forward primers: two base pair overhang and restriction enzyme site. Reverse primers included a stop codon instead of a start codon and approximately the first 20 nucleotides complimentary to the 3' end not the 5' end. The equation mentioned above was used to calculate the melting temperature, and the tendency to terminate on a cytosine or guanine was upheld.

First Strand Synthesis with Reverse Transcriptase

A reverse transcriptase kit was purchased from New England Biolabs, and its instructions were followed. The following reagents were mixed in a sterile PCR tube for a final volume of 16 µl: 5 µl RNA, 2 µl reaction primers, 4 µl reaction dNTPs, 5 µl

of nuclease-free water. After being heated for 4 minutes at 72° C, the PCR tube was immediately placed on ice. 2 μl 10X RT buffer, 1 μl RNase Inhibitor, 1 μl Reverse Transcriptase were added to the solution. All 20 μl of solution was incubated for 1 hour at 42° C. Enzymes were inactivated with a 10-minute exposure at 90° C. The entire tube was stored at -80° C.

PCR Amplification

The following reagents were mixed in a sterile PCR tube for a final volume of 50 μl: 25 μl 2X Reaction Mix, 2 μl 25X Enzyme Mix, 2 μl 10 mM Forward Primer, 2 μl 10 mM Reverse Primer, 14 μl nuclease-free water, and 5 μl RNA. The RNA was added just before the PCR was started. The PCR conditions were as follows: 30 minutes at 50°C, 15 minutes at 95°C, 32 cycles of (0.5 minutes at 94°C, 0.5 minutes at 60°C, 1 minute at 72°C), 10 minutes at 72°C, and hold at 4°C. For positive control, ACT-1 primers were used first. PCR products were analyzed on a standard 1.7% agarose gel. The procedure was never performed for experimental *C. elegans* genes.

Results

RNA Extraction and Purification

The RNA extraction protocol consistently produced broad-spectrum RNA smearing when analyzed on a 1.7 % electrophoresis gel (Figure 1). Lanes two through seven represent the six RNA samples. Lane one is the RNA ladder, which consisted of RNA fragments of known sizes. All six samples were analyzed to ensure an adequate amount of RNA was extracted in each sample. The isolated band at approximately 1,000 base pairs is consistent size with that of the OP50 genomic plasmid. This was further confirmed when treating the RNA samples with DNase I erased the band (Figure 2). Even-numbered lanes were left untreated as negative controls for the DNase I treatment. Residual DNA in the wells was

Figure 1. Electrophoresis gel of 6 RNA extraction samples (lane 1 ladder). The broad-spectrum RNA can be seen the whole length of the column. *E. coli* plasmid can be seen near the top.
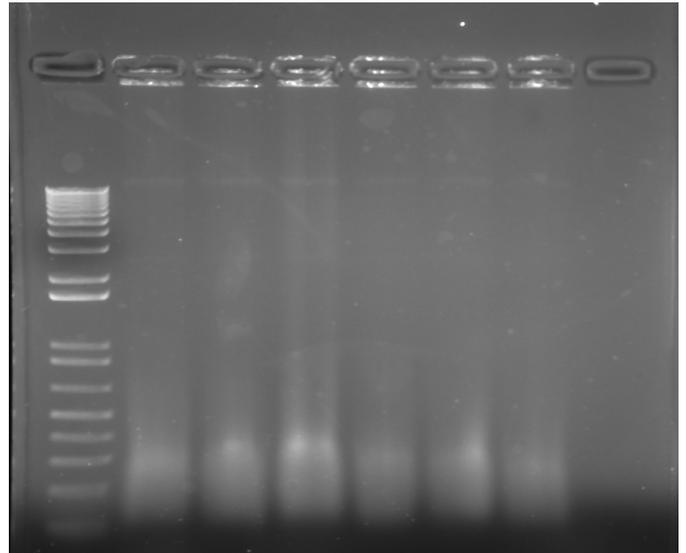
Figure 2. Electrophoresis gel of the same 6 RNA extraction samples (lane 1 ladder) with DNase treatments in lanes 3, 5, and 7 to remove *E. coli* plasmid near the top of the lanes.
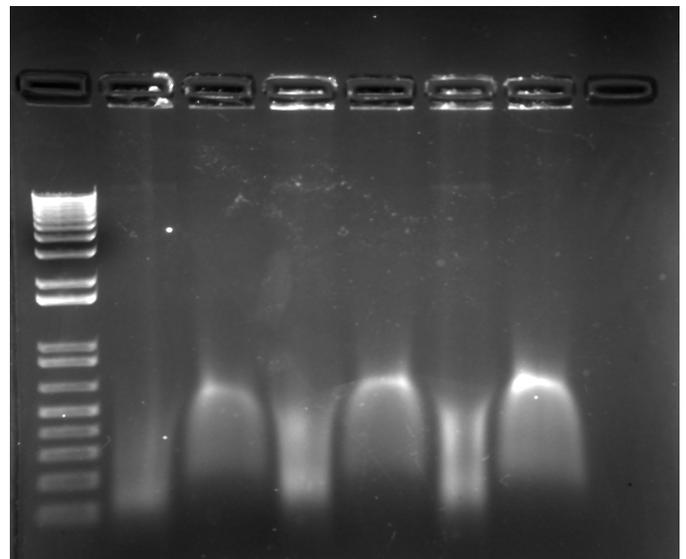
also degraded (Fig. 1 & 2).

The concentrations and UV ratios of each sample are displayed in Table 1. The concentration calculations were performed in triplicate and averaged to counter the varying analysis returned by the spec. The same procedure was performed for the UV-ratios despite the consistent nature of the returned analysis.

Table 1. 6 RNA extraction and purification levels alone and averaged (done in triplicate). UV ratios are presented and averaged.

Sample	[μg/ml]	Average	260:280	Average
1	6913.		1.117	
1	6952.	6986.	1.130	1.127
1	7093.		1.135	
2	3494.		1.148	
2	3645.	3568.	1.139	1.142
2	3564.		1.139	
3	1630.		1.197	
3	1766.	1682.	1.159	1.163
3	1650.		1.134	
4	4825.		1.118	
4	4780.	4792.	1.111	1.117
4	4770.		1.123	
5	4460.		1.132	
5	4536.	4443.	1.143	1.136
5	4334.		1.132	
6	2950.		1.134	
6	2855.	2899.	1.145	1.130
6	2891.		1.112	

Primer Design

The primer constructs necessary for the creation of dsRNA, both experimental and control, are displayed in Table 2. In addition to the primer sequences, restriction enzymes and melting temperatures are also displayed. All primers were constructed to have melting temperatures between 60°C and 62°C.

Table 2. Primer Constructs

	Direction	Sequence [5'-3']	Melt Temp.
DLI-1	Forward	AT-TCTAGA-ATG-CCACCAACTGCGCAAC	60° C
	Reverse	TA-GGTACC-TTATGCATCACTGTCCCGGG	62° C
VDAC-1	Forward	AT-TCTAGA-ATG-GCCCCACCAACCTTCG	62° C
	Reverse	TA-GGTACC-GTTGGATGGATCGAATTC	62° C
SVOP-1	Forward	AT-GCGGCCGC-ATG-GGAGATAAAGCAATTCTAAC	61° C
	Reverse	TA-GGTACC-TTA-ATGGGAGTCCATCATTTGA	60° C
DNC-2	Forward	AT-GCGGCCGC-ATG-TCATCTATTGGAAAAGAAGAT	62° C
	Reverse	TA-GGTACC-TCA-CTTCAAATCTGCCACTTTT	60° C
ACT-1	Forward	AT-TCTAGA-ATG-TGTGACGACGAGGTTGCCG	62°C
	Reverse	TA-GGTACC-TTA-GAAGCACTTGCGGTGAACG	60°C

First Strand Synthesis and PCR Amplification

The product of the first strand cDNA synthesis was used as the template for a PCR reaction in order to try and generate double stranded DNAs of the target genes. Instead, the procedures moved directly to PCR amplification because successful PCR amplification would suggest the first-strand synthesis was successful. The control trials did not successfully amplify ACT-1. When the gene-specific PCR products were analyzed on a 1.7% electrophoresis gel, there were no corresponding bands

present; aside from the DNA ladder bands, the gel was blank, and therefore omitted from this document.

<u>Discussion</u>

C. elegans Generation and Maintenance

The protocols designed in 1973 by Sydney Brenner for generating and maintaining *C. elegans* worked flawlessly throughout the entirety of this research project; however, to combat destructive contaminations it was beneficial, but in no way necessary, to allow new unseeded NGM plates to incubate for several days at 37°C. Typically, contaminations did not arise until the NGM plates were seeded and incubating at 37°C. This unseeded incubation allows contaminated plates to be identified and discarded earlier in the process.

RNA Extraction and Purification

The broad-spectrum RNA smearing on the electrophoresis gels is a good indication the extraction was successful and the RNA samples contained widely varying and intact strands of RNA. Despite its high-temperature procedure, the DNase I treatment showed no apparent degradation of the RNA in sequential analysis of both concentration and UV-ratio. The failure of the ACT-1 amplification could be the result of inappropriate concentrations of RNA. Either decreasing or

increasing the concentration of the RNA may alter the effectiveness of the cDNA synthesis protocol.

PCR Amplification

Attempts to amplify ACT-1 by polymerase chain reaction (PCR) were not successful. It is more likely that the PCR conditions were inappropriate than the ACT-1 template was absent. ACT-1 is a popular control for *C. elegans* experimentation because of its high concentration. Running these same procedures with other popular control-genes (ACT-2 or ACT-3) would confirm this. If all three control-genes fail to yield PCR products, it is less likely to be the availability of template strands and more likely to be the conditions of the PCR reaction. Future experimentation must involve alterations of the PCR conditions. Given the archaic methods used to generate melting temperatures for PCR protocol, I would suggest a recalculation of the melting temperatures, and following any necessary nucleotide adjustments, the conditions should be altered accordingly.

Future Experimentation

At this point in experimentations, it would be inappropriate to conclude or even discourse on the physiological roles of the experimental GluR2-interacting genes. Future experimentation must entail the above-mentioned PCR alterations. Using ACT-2 and ACT-3 to confirm whether ACT-1 is an appropriate control may

also prove to be a useful endeavor. It is also possible that certain reagents were ineffective due to age, primarily the chloroform used for the RNA extractions. Aqueous chloroform could over time generate an acidic solution, which in turn could degrade the RNA samples in the chloroform-separation protocols. Partially degraded RNA samples could register on the electrophoresis assay but fail in the RT-PCR procedures.

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