

The Expression of Ependymin in *C. elegans* Roundworms - II

A Major Qualifying Project Report

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By

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Abstract

Ependymin is a neurotrophic factor that was first discovered in memory consolidation in goldfish. It has been since then found that partial portions of its gene sequence have been conserved across many species, including teleost fish and the horseshoe crab. Previous work here minimally confirmed ependymin expression in the invertebrate model organism *Caenorhabditis elegans*. Recently a new antibody has been produced against a bioreactive 8-aa ependymin fragment, which has successfully detected reactivity in developing horseshoe crabs' nervous systems. I have shown that ependymin is present along the nervous systems of *C. elegans* worms, during their early developmental stages. This project utilized immunostaining in conjunction with HRP light, fluorescence, and transmission electron microscopies to observe ependymin immunoreactivity in *C. elegans* from very early embryonic stage onwards to the L2 larval stage, most noticeably in developing embryos.

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Table of Contents

Abstract.....	ii
Acknowledgements.....	iii
Table of Contents.....	iv
Table of Figures.....	v
Introduction.....	1
<i>Caenorhabditis elegans</i>	1
Ependymin.....	4
Previous Studies of Ependymin.....	7
Project Goals.....	8
Materials and Methods	9
Preparing Specimens and Fixation.....	9
Serial Sections on the Microtome.....	12
Etching and Staining for Light and Fluorescence Microscopy.....	15
Staining for Transmission Electron Microscopy.....	17
Additional Pertinent Materials.....	19
Results.....	22
Discussion and Conclusions.....	33
Ependymin is Present as a Strong Neurotrophic Growth Factor in <i>C. elegans</i> Embryos.....	33
Ependymin is Present as a Neurotrophic Factor in the Nervous System in L1/L2 <i>C. elegans</i> Larval Stages.....	34
Further Notes on Ependymin Expression in <i>C. elegans</i>	35
Works Cited.....	37

Table of Figures

Figure 1: Illustration of body construct of typical <i>C. elegans</i> nematode worm.....	2
Figure 2: Illustration of typical <i>C. elegans</i> life cycle.....	3
Figure 3: Concentrated amounts of wild-type <i>C. elegans</i> settled in Eppendorf tubes.....	10
Figure 4: Schematic diagram of exposing <i>C. elegans</i> to resin mixtures.....	11
Figure 5: Assembly of resin-embedded specimens onto Sorvall Porter-Blum microtome machine.....	12
Figure 6: Photograph of manually prepared glass knives.....	13
Figure 7: Thick specimen sections fixed onto 8-well glass slide for light/fluorescence microscopy experiments.....	13
Figure 8: Cutting gold sections to place onto Ni-300 mesh grids.....	14
Figure 9: Photograph of hemocytometer device under light microscope.....	20
Figure 10: Viewing of section of <i>C. elegans</i> worms on hemocytometer under light microscope.....	21
Figure 11: Photograph of Epon-A Experimental <i>in utero C. elegans</i> embryos section, treated by HRP reaction mechanism.....	22
Figure 12: Second view of Epon-Araldite <i>C. elegans</i> developing embryos, treated by HRP mechanism.....	22
Figure 13: Photograph of experimental developing eggs and embryos, treated by HRP reaction mechanism.....	23
Figure 14: Photograph of Epon-A Experimental transverse section of L1 larval stage <i>C. elegans</i>	24
Figure 15: Photograph of Epon-A Experimental longitudinal L1 larval stage <i>C. elegans</i>	24
Figure 16: Photograph of L.R. White Experimental <i>in utero C. elegans</i> embryos section, treated by HRP reaction mechanism.....	25
Figure 17: L.R. White Experimental transverse section of L1 larval stage <i>C. elegans</i>	25

Figure 18: L.R. White Experimental longitudinal section of L1 larval stage <i>C. elegans</i>	25
Figure 19: 1:100 AlexaFluor 488 [®] experimental (A) and control (B) roundworm embryos in hermaphrodite sections from Epon-Araldite sections.....	26
Figure 20: 1:100 AlexaFluor 488 [®] experimental Epon-Araldite section with transverse sections of roundworms at different stages.....	27
Figure 21: Photograph of 1:100 AlexaFluor 488 [®] experimental L.R. White section of adult hermaphrodite with developing embryos.....	28
Figure 22: Photograph of 1:100 AlexaFluor 488 [®] experimental L.R. White section of worms with fluorescing neurons.....	28
Figure 23: Close-up of L2/L3 larval stage <i>C. elegans</i> depicting 1:10 mild anti-EPN detection at 27*1000 magnification.....	29
Figure 24: Transverse section of L1-stage <i>C. elegans</i> treated with 1:100 secondary antibody, at 8*1000 magnification.....	30
Figure 25: Transmission electron micrograph of anti-EPN reaction oddly presented on single <i>E. coli</i> bacterium.....	31
Figure 26: Results of NCBI Blast carried out to determine ependymin's presence in a strain of <i>E. coli</i>	31
Figure 27: Results of NCBI Blast carried out to determine ependymin's presence in the genome of <i>C. elegans</i>	32

Introduction

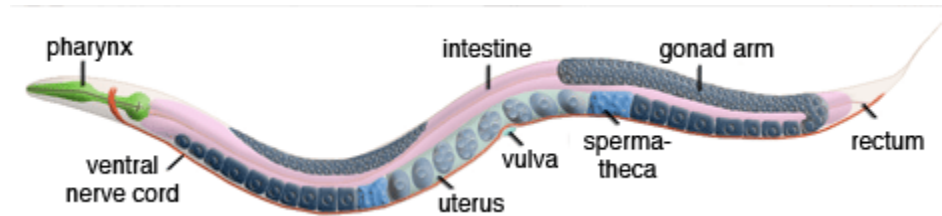
Caenorhabditis elegans

Caenorhabditis elegans, or *C. elegans* for short, is a small, simple, free-living nematode worm that has been used as a reliable model organism by most biologists up to today. Due to its simple construct, simple body plan, and reproductive characteristics, *C. elegans* has been used as a model organism in mainly the fields developmental genetics and study of the general nervous system (Blaxter, 2008). Dubbed also to some as round-worms, the *C. elegans* worm lives primarily in soil in terrestrial areas, and depend on bacteria as their major food source, mainly on *E. coli*. At certain points in its life cycle, the *C. elegans* worm sheds its outer cuticle; due to this property it is classified to fall under the subphylum Ecdysozoa (Freeman, 2005). In this sense, *C. elegans* is related to *Limulus polyphemus*, or the horseshoe crab, alongside the fact that they share some similarity on how their muscles and nerves are arranged (Sherman and Fourtner, 1972).

C. elegans has been recently introduced as a noteworthy organism to study since 1965, when the English biologist Sydney Brenner came across it and determined to rely on the nematode worm for studies in animal development and behavior (Riddle et al., 1997). *C. elegans*, within a population of nematode worms, exists mainly as a hermaphrodite worm, meaning it has both male as well as female organs and reproduces by self-fertilization. Male *C. elegans* form as well, due to the genetic condition non-disjunction, yet they constitute only 0.1% of a *C. elegans* population (Riddle et al., 1997). Its main anatomical features include an outer tubing overlaying a hypodermis; inner body

cavity; pharynx; lengthy digestive tract; muscular, nervous, and excretory systems; oral cavity; and openings for feeding, egg laying, and waste excretion (see Figure 1 below).

Figure 1: Illustration of body construct of typical *C. elegans* nematode worm



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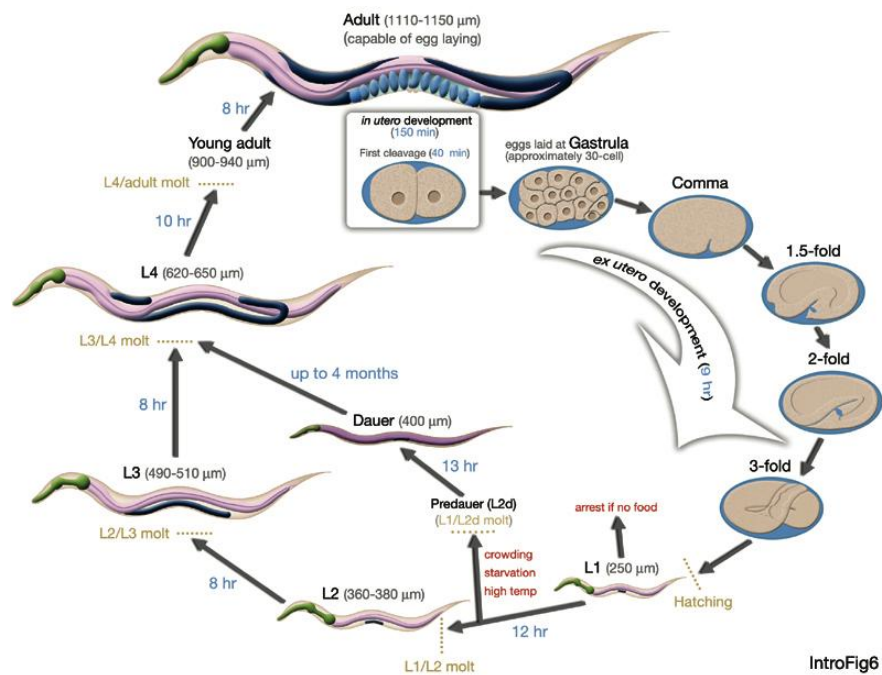
<http://www.sfu.ca/biology/faculty/hutter/hutterlab/research/Celegans.html>

Much incredible research has been over the past decades on *C. elegans*, including intense electron microscopic studies, genetic crosses, and analyses on the building of its complete neural circuitry to account for function and behavior. Only recently its entire genome has been completely sequenced, and this gives *C. elegans* scientists an opportunity to make analogies between its genome and that of other related species, as well as identify genes and proteins with close sequence homology to mammalian genes.

Compared to most other model organisms, *C. elegans* is a unique animal, especially in terms of its development and life cycle. As in the horseshoe crab, *C. elegans* undergoes a series of molts, but its only four molts bring it to maturity in just three to four days. The life cycle of *C. elegans* consists of a complex embryonic stage, followed by four larval stages, L1-L4, to follow with adulthood (see Figure 2 below). In a single mating, which may or may not involve the male nematode worm, a *C. elegans*

hermaphrodite can produce between 300-350 progeny. Once fertilization of the oocytes occurs, the developing embryos remain in the mother for about 150 min until at least thirty cells have formed; this is a beginning stage known as proliferation, where the embryos develop from zygote to having embryonic founder cells (Altun and Hall, 2006). The eggs are then laid, and until around 800 minutes, the embryos undergo further development, including the stages of gastrulation, organogenesis, and morphogenesis. This happens gradually in a series of substages: the 1-fold, 2-fold, and 3-fold stages (Altun and Hall, 2006). Eventually cells undergo differentiation to form bodily components and the young worms hatch to enter into the L1 larval stage.

Figure 2: Illustration of typical *C. elegans* life cycle, complete with embryonic development and main larval stages



IntroFig6

Obtained from website: <http://www.wormatlas.org/handbook/anatomyintro/anatomyintro.htm>

It is known that during advanced embryogenesis, the worm's nervous system has undergone advanced motor system development, as it can move around inside its confinement (Altun and Hall, 2006). Larvae in the proceeding L1 stage undergo further major nervous system development, primarily with ventral cord motor neurons. At the same time, the reproductive and coelomocyte systems begin to develop. The nervous system continues to develop through the L2 stage and synaptic growth tapers off, so that other parts must develop, especially the gonads. *C. elegans* growth in the L3 and L4 stages focuses on the complex reproductive system's gradual formation, as well as the endings and excretory openings of the body. Eventually in a matter of about fifty-six hours, or around three days, the L4 larvae reach up to complete adulthood.

Ependymin

Ependymin is a complex secretory glycoprotein that is found to serve as a neurotrophic growth factor in the developing central nervous system of animals, playing a significant active role in cellular events related to neuronal development and memory consolidation. Ependymin was discovered relatively recently in the 1970's decade, in the brains of goldfish, by the scientist Victor E. Shashoua and his colleague scientists. At the time, Shashoua and others postulated hypotheses on how learning, behavior, and neuroplasticity must be caused by molecular mechanisms of action.

Victor Shashoua carried out extensive work to isolate ependymin and determine its critical function through using goldfish as his models. He relied on classical conditioning as well as training mechanisms to visualize goldfish learning capability

(Shashoua, 1985). Certain control versus experimental goldfish were taken aside, and their brains were sliced and stained to view high levels of protein concentrated in mainly the zona endydyma of the optic tectum; hence this is how endydymin got its name. In a separate detection experiment, antisera was made against novel endydymin and injected into the fourth ventricle of other experimental goldfish after training; as a result the antibodies significantly blocked the experimental animals' recall of how to swim with the float. Separate conditioning experiments were done in laboratory mice as well, to note that endydymin-like protein changes, almost similar to those from trained goldfish brains, have been observed in mammalian brain (Shashoua, 1985).

Aside from this, Shashoua observed during his protein isolation techniques some properties of endydymin that dubbed it as being a neurotrophic factor. Upon the presence of calcium, EPN existed as a highly soluble protein; if little or no calcium was present and must exist in high intracellular concentrations, EPN quickly polymerized to form an insoluble matrix. Based on experimental study experiments by past researchers, Shashoua hypothesized that during learning and CNS plasticity moments, this calcium concentration change occurs, and endydymin polymerization takes place. In turn, the formation of the insoluble matrix encourages the growth and forthcoming of new synapses to form new neural connections (Shashoua, 1985).

Since then, endydymin, endydymin-related proteins, and endydymin precursors have been isolated and discovered from a limited variety of species, including teleost fish, such as the rainbow trout, Atlantic salmon, and pike; goldfish, zebrafish, and herring (Ebner, 1999). Intense research has been done on endydymin to find significant

homologous sequences conserved between diverse species, such as humans, horseshoe crabs (Cruikshank et al., 2003), piscine species (such as the fish above), and the Florida lancelet (NCBI eight-letter EPN protein blast, 2008). Recently, labs such as Dr. David Adam's laboratory have been able to molecularly clone and sequence some genes that encode EPN.

Ependymin is quite a unique protein compared to most others studied in the field of medicine, especially with it being a large, complex polypeptide. Ependymin exists as a hydrophilic, polar, mostly acid-amino-acid-based protein, in three major polymeric forms: α , β , and γ (Shashoua, 1985). The β and γ forms are found to predominate when the protein is in its active state (Shashoua, 1988). Precursor ependymin polypeptides isolated from piscine species have found to contain N-linked glycosylation sites, some of which are conserved between species (Ebner, 1999). In addition, ependymin's protein structure contains special sites which include active cysteine residues (pairs of which participate in disulfide linkages), asparagine-linked glycosylation sites, and unusually high antigenic regions. Protein sequence blasts that have been carried out on small active sequences of ependymin (including "KKETLQFR") have lead to question what exactly is ependymin's actual mechanism of action, as it is identified as being functional ependymin in some eukaryotic organisms, a hypothetical protein in some fish species, or a partial seven transmembrane cell-surface receptor membrane in few others (NCBI eight-letter EPN protein blast, 2008)

Previous Studies of Ependymin

So far, previous experiments carried out to isolate ependymin have involved the use of horseshoe crabs as the major model organism, with one attempt using *C. elegans*. Past experiments involved the usage of an anti-Ependymin polyclonal antibody dubbed as “SHEILA”, which was made in rabbits and worked against an 18-amino-acid peptide fragment at the carboxy terminus of the goldfish protein. This antibody did demonstrate immunoreactivity of ependymin in axons, ganglions, and neurophils in young developing juvenile horseshoe crabs (Barosso, 1999). It also was able to detect presence of ependymin in the blood of a wounded adult horseshoe crab (Costigan and Gallant, 2004). This same SHEILA polyclonal antibody was used in an attempt to detect ependymin immunoreactivity in *Caenorhabditis elegans* in a past study; however, it did not effectively work and only showed little/mild reactivity in the L2 stage of roundworms (Tworog-Dube, 2000).

Recently a new antibody has been commissioned, mainly involving reaction against an 8-amino-acid fragment called CMX-8933 with the ependymin sequence “KKETLQFR”. A recent study has determined that this specific short peptide has all the neurotrophic effects of the full-length protein when used in neuroblast cultures (Adams et al., 2003). The antibody to this was commissioned by New England Peptides and used in several immunological methods to successfully demonstrate evidence of ependymin expression in the central nervous systems and developing leg neurons of juvenile horseshoe crabs (Dionne & Krzyzewski, 2008).

Project Goals

The main purpose of this project was to go beyond the work of Erica Tworog-Dube and use the new anti-Ependymin (anti-KKETLQFR), along with improved laboratory techniques, in order to determine if ependymin is present in *C. elegans* during its developmental stages, particularly in larval stages other than the L2 stage. This project focused on detecting ependymin expression in major nerve cords along a typical worm's nervous system and, if possible, in the nerve rings of worms at any developmental stage. Objectives alongside the project included being extensively familiarized with *C. elegans* development and properties of ependymin. It was proposed that the presence of ependymin glycoprotein in the nematode worm at points during its developmental cycle would show that part of the ependymin gene is conserved in this taxa like in most others. This would also make *C. elegans* a worthwhile model, like the horseshoe crab, for research on ependymin's role in neuronal development and critical therapeutic actions.

Materials and Methods

To begin determining the expression and effects of ependymin in *C. elegans*, some basic skills were acquired and placed into practice. Stock serums of the newly commissioned 8-amino acid peptide anti-EPN (used last year by Dionne and Krzyzewski in their 2008 MQP), normal goat serum (NGS), and normal rabbit serum (NRS) were obtained to be ready for future use. They were aliquotted in 200-250 μ L amounts in 2-mL Eppendorf tubes and frozen in the -80°C freezer for later use. One of major basic skills practiced was plastic transfer pipette pulling. Worms harvested from stock cultures had to undergo intense fixation and post-fixation steps, as well as several dehydration steps and centrifugation, so pipettes with very narrowed diameters proved useful in sucking out excess solution without losing worms. Handling *C. elegans* worms with extreme care at every fixation step was another skill practiced and re-iterated before the actual experiment would officially begin.

C. elegans roundworms growing at random developmental stages were harvested at room temperature in five regular Petri dishes. The strain used was wild type, the roundworms had been growing in the WPI laboratory, and they were relying on *E. coli* bacteria as their food source.

Preparing Specimens, Fixation, and Embedding

To begin the fixation process, a 50:50 solution of paraformaldehyde and 200 mM phosphate buffer was prepared to add to the *C. elegans* stock cultures. In previous

practices on specimen preparation and resin curing, mixes of formaldehyde and sodium cacodylate in sucrose buffer were prepared and used, but worms ended up shriveled from the sucrose and no more sodium cacodylate was available in the lab. The paraformaldehyde and phosphate buffer mix was added to the Petri dishes of roundworms and swirled gently to then sit at room temperature for at least seven hours. This fixation process enabled the fixing of tissue proteins in the roundworms. A transfer pipette was then used to concentrate as many worms possible into at least two 1.5-mL Eppendorf tubes (See Figure 3 on the side). The tubes were shaken and inverted to distribute worms randomly, then centrifuged at 14 rpm (high speed) for at least four minutes. Supernatant was poured out carefully to be replaced with phosphate buffer for a rinse.

Figure 3: Concentrated amounts of wild-type *C. elegans* settled in four tubes



Thick pellets of roundworms at the bottom

The four total tubes of roundworms were then centrifuged for at least five minutes at high speed. 1% osmium tetroxide (OsO_4) for post-fixation of specimens was prepared in the fume hood by adding 4 mL 200 mM sodium cacodylate buffer to 2 mL 4% osmium tetroxide. Specimens from Tubes 1 and 2 were treated with the 1% OsO_4 for one hour, then rinsed twice, ten minutes each time, in dH_2O . Addition of 70% ethanol followed, and the tubes were stored away in the refrigerator for later treatments. Specimens from Tubes 3 and 4 were only treated with 70% ethanol and placed aside. Next, specimens from all tubes underwent dehydration by soaking three times, ten minutes each, in cold 100% ethanol.

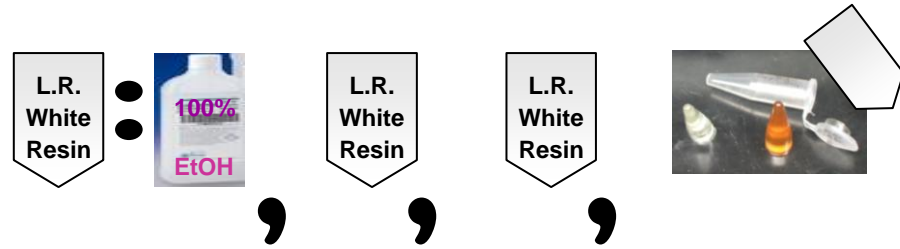
It was planned that for specimen embedding in this project, two types of resins would be separately used to preserve worms: Epon-Araldite resin and L.R. White resin.

The outcomes of anti-ependymin reactivity in *C. elegans* worms embedded by either resin would determine if each resin would prove useful for small specimen embedding in future projects. Propylene oxide was added to Tubes 1 and 3 as a necessary pre-embedding tool before adding Epon-Araldite resin, and allowed to soak in for at least fifteen minutes, then again for one hour. Roundworms in Tubes 1 and 3 were centrifuged vigorously and supernatant decanted carefully, to follow by two soaks, of at least one hour each, in 1:2 and 2:1 Epon-Araldite resin to propylene oxide. Meanwhile, Tubes 2 and 4 were treated only after being left in 100% ethanol with a 1-hour soak in a 1:1 mix of L.R. White resin to 100% ethanol.

Following these soaks, specimens in Tubes 1 and 3 were exposed to pure Epon-Araldite resin for an hour; those in Tubes 2 and 4 to pure L.R. White resin at the same time. Then the resins were decanted after an intense six-minute centrifugation, to follow with a new change of pure resins into the same Eppendorf tubes. The specimens were allowed to settle for a few minutes at room temperature, and next placed in the curing oven to harden overnight onwards at 70°C. It was suspected from previous studies that ependymin functionally exists as an intracellular glycoprotein, so we would mainly utilize sections that would be cut from Tubes 1 and 2.



Figure 4: Schematic diagram of exposing *C. elegans* to resin mixtures, to end with curing in pure resin.



Serial Sections on the Microtome

The specimens embedded in their resins were first taken out from their casings by striking their tips with a hammer. Extraneous plastic was cut off the outer rims of the resins using a jeweler's saw and the round ends shaved smooth using sandpaper. The specimens were glued with cyanoacrylate onto separate aluminum studs to then be screwed into circular clamps and mounted onto the Sorvall Porter-Blum Ultra-microtome provided in Dr. Gibson's lab (see Figure 5 below).

Figure 5: Assembly of resin-embedded specimens onto Sorvall Porter-Blum microtome machine



The Leica color scale provided was used in aid in cutting various-sized sections. Mainly about 0.45 μm -thick clear sections of *C. elegans* existing at varying developmental stages, positions, and angles were prepared onto 8-well colored water-testing glass slides for light and fluorescence microscopy experiments (see Figure 7 below). Diamond knives provided in the lab to be used with the microtome were initially used, but they did not have high-quality knife edges to effectively cut most any sections, so glass knives were made manually with the aid of a glass-cutter machine. Glass boats were then made using silver tape and ancient nail polish to be placed in the boat set-up in the microtome, and hold dH_2O and floating sections (see Figure 6 below).

Figure 6: Photograph of manually prepared glass knives

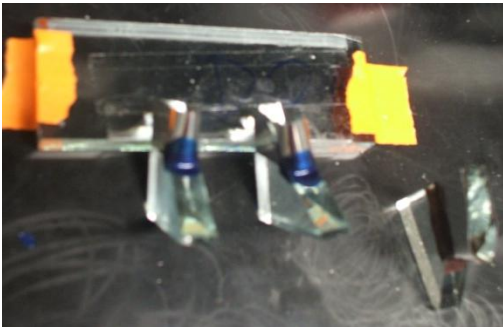
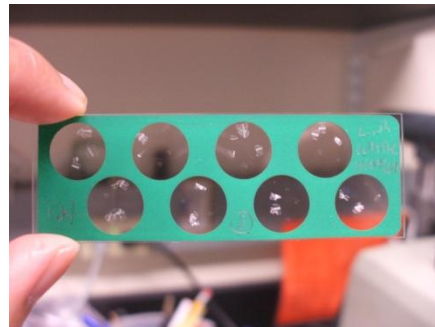


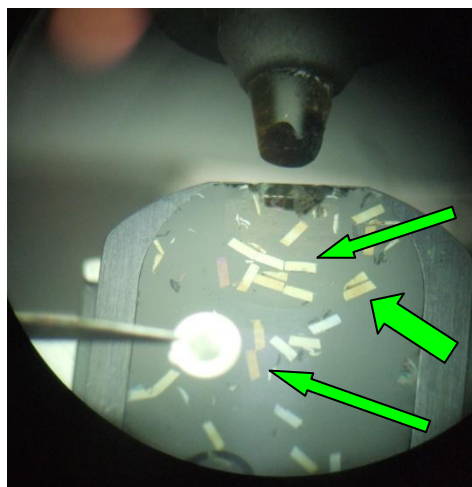
Figure 7: Thick specimen sections fixed onto 8-well glass slide for light/fluorescence microscopy experiments



Sections were placed on well-labeled slides by removing them from the boat with a flamed wire loop and hair, and placing down on the clear circles on the slides. The slides were placed on an 80°C hot plate to dry completely for five-seven minutes, so as to

fix the sections without ruining the quality of existing protein. Some unetched sections on each slide were stained with 1% Toluidine Blue/Sodium Borate Tetra in dH₂O and 1% Basic Fuchsin in 50% EtOH and dH₂O to observe different tissue structures. Separate ultra-thin gold sections about 60-90 nm (0.060-0.090 μm) were generated using mainly diamond knives for transmission electron microscopy experiments. They were placed in delicate fashion onto EMS wire Ni-300 mesh grids. The grids were held shiny-side up with non-magnetic steel jeweler's tweezers, submerged in the water and brought up underneath the sections, while they were guided as close possible to the center of the grid with a clean hair (See Figure 8 below). The grids were dried onto bibulous paper and then etched and treated with immunogold antibody and heavy metal staining. Wire mesh nickel variable grids and Copper-150 Formvar grids were initially tried as well, but did not end up with good clear results (i.e. the variable grids ended up torn along the process and the copper grids were unsatisfactory).

Figure 8: Cutting gold sections to place onto Ni-300 mesh grids



60-90 nm gold sections cut by good-quality diamond knife for placing on mesh grids for viewing anti-EPN immunoreactivity by TEM

Etching and Staining for Light and Fluorescence Microscopy

Upon having ready separate slides for light and fluorescence microscopies (i.e. Epon-A Experimental Sections, Epon-A Control Sections, L.R. White Experimental sections, and L.R. White Control Sections), sections were ready to undergo etching to expose any existing protein for anti-EPN reactivity. To start, the Epon-Araldite plastic was removed from its respective sections. First, NaOH-based dissolving solution and separately sodium periodate (NaIO_4) for OsO_4 removal was prepared. Stock solution of NaOH for dissolving was prepared by dissolving 2.0 grams of NaOH pellets in 20 mL EtOH mixed with 20 mL of propylene oxide in the fume hood, and placed on the stir plate for ten to fifteen minutes, or until a clear mixture resulted. Remaining solution could be capped tightly and stored away in the freezer for future uses. Meanwhile, 1% NaIO_4 was prepared by dissolving 0.1 grams NaIO_4 in 10 mL dH_2O . As in last year's MQP, it was decided that 1% sodium periodate would need to be prepared fresh each time (Dionne & Krzyzewski, 2008).

Sections embedded in Epon-Araldite were incubated in the dissolving solution for three minutes, then rinsed three times in 95% EtOH, two minutes per rinse. A three-minute rinse in 50% EtOH and then a five-minute rinse in dH_2O followed. Next, the slides fixed with L.R. White-embedded sections were obtained, and all sections were incubated with sodium periodate for five-seven minutes to remove OsO_4 bound to the worms' membranes. This was followed by two three-minute dH_2O rinses. Enough 0.1 mM PBS was finally applied to equilibrate slides, covered, for at least five to ten minutes, longer if needed.

It was decided that a 1:10 dilution of primary antibody would show sufficient immunoreactivity in roundworms, so experimental sections were stained with this dilution of rabbit anti-EPN antibody, while control sections received same dilutions of NRS. Solutions of both antibody serums were prepared either in 1.5-mL Eppendorf tubes or 15-mL conical centrifuge tubes, in 0.1 mM PBS and 1% NGS. The anti-EPN antibody, to make note, was a newly commissioned peptide made against a powerful 8-amino acid EPN fragment, from New England Peptide Co. Secondary antibodies to react against anti-EPN were used from the Invitrogen AlexaFluor 488[®] and Pierce ABC Peroxidase Staining Kits.

Slides designated for HRP reaction mechanism to detect anti-EPN reactivity underwent blocking to mask any extraneous/unreactive antibody for about thirty minutes. This was accomplished using a solution of 6% normal goat serum (NGS) mixed in PBS. Then, sections were rinsed with PBS two times, about ten minutes per rinse. Primary antibody solutions were applied to their respective sections and allowed to incubate overnight in the refrigerator. Next, the sections underwent three ten-minute rinses in PBS, followed by a thirty-minute incubation in goat anti-rabbit biotinylated antibody from the Pierce ABC kit. After this, sections were rinsed again twice, ten minutes each time, in PBS, and incubated in the ABC reagent for about fifteen minutes, up to thirty for thicker sections. Following this, the slides were rinsed again twice and sections incubated in a 1X nickel-enhanced Diamino-benzidine solution diluted 1:10 in hydrogen peroxide buffer (DAB substrate kit), for at least seven minutes, or until dark brown substance was seen developing in experimental sections, controls with dark background. Finally, carried out were two ten-minute rdH₂O rinses, and a light rinse in PBS.

Slides designated for fluorescence antibody solution treatment followed nearly the same protocol as above, except for using a different blocking solution and different secondary antibody. Sections underwent thirty-minute blocking under the Signal Enhancer Solution provided with the Alexafluor 488[®] staining kit, to follow with three PBS rinses. After primary antibody incubations and rinsing, sections were incubated only with a 1:100 dilution of goat anti-rabbit Alexafluor 488[®] secondary antibody for thirty minutes. This was followed by three ten-minute rinses in PBS and put away in the refrigerator to be viewed under fluorescence microscopy at a later time. Upon being ready for viewing, fluorescence slides were allowed to dry, and either glycerol or Fluoro-Gel Mounting Medium was applied, and a cover slip set on top. HRP slides were mounted with the Fluoro-Gel Mounting Medium, set with cover slips, and visualized under regular light microscopy at 40X, 100X, 400X, and 1000X (oil immersion) magnifications. Fluorescence slides were viewed under two separate fluorescence microscopes provided in the lab at 40X, 100X, and 400X magnifications.

Staining for Transmission Electron Microscopy

Ultra thin gold sections fixed on their nickel grids underwent first a brief etching process in a solution of 10% hydrogen peroxide in dH₂O. Drops of solution were placed onto a divided piece of rubber silicone and the sections soaked shiny-side down for five minutes. Next they were rinsed three times in dH₂O, the first two times for three minutes and the third time five minutes. The grids underwent similar blocking and antibody procedures as did the thick sections for light/fluorescence microscopies. A blocking solution of 6% normal goat serum in 0.1M PBS-Tween-80 (0.05%) was prepared and

homogenized in a 1.5 mL Eppendorf tube, and the gold sections were exposed to blocking solution for at least thirty minutes. Grids were then rinsed three times in a solution of 0.1M PBS-Tween-80 (0.05%)-NGS (0.25%). After this, the sections were incubated for two hours in 1:10 dilutions of primary antibody prepared in 0.1M PBS-Tween-80 (0.05%)-NGS (0.25%). Grids designated as experimental received diluted rabbit anti-ependymin serum as the primary antibody, while control grids were treated with normal rabbit serum (NRS).

Following the primary antibody incubation, the sections were rinsed three times with the newly formulated PBS solution. A dilution of no more than 1:100 goat anti-rabbit secondary antibody conjugated to 25-nm gold balls was prepared in an Eppendorf tube and drops were added to a clean divided silicone plate. The TEM sections were then incubated overnight in the freshly prepared secondary antibody solution, with the plate set up with wet paper towels and covered with a plastic dish to prevent any drying out. They next underwent two three-minute rinses in 0.1M PBS-Tween-80(0.05%)-NGS (0.25%) solution, followed by a five-minute rinse in PBS. Two thorough rinses in dH₂O followed, about five minutes per rinse. Before carrying out heavy metal staining on specimen sections, grids were allowed to soak in 2% gluteraldehyde for ten minutes. Exposing the sections to gluteraldehyde would aid in enhancement of the visualization of the colloidal gold balls to detect anti-EPN immunoreactivity under the transmission electron microscope.

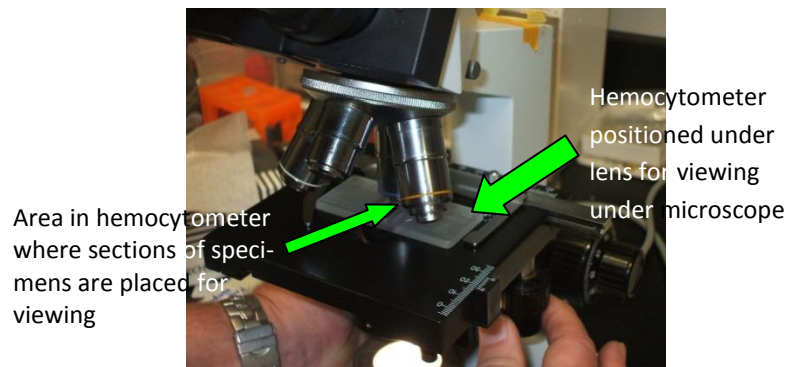
Sections were then rinsed again two times, five minutes each, in dH₂O, and dried on bibulous paper to prepare for heavy metal staining. Uranyl acetate and lead citrate

were separately prepared in centrifuge tubes, transferred in 1.25-1.80-mL quantities in Eppendorf tubes, and spun at high speed for at least 10-20 minutes in a centrifuge machine. Sections were next stained for five minutes, face down onto drops of uranyl acetate for five minutes on a clean silicone plate, and rinsed three times in dH₂O. After that, the grids were dried and stained with lead citrate, shiny side up, briefly for only between 30 seconds-1 minute. Finally, the sections were rinsed three times again in dH₂O and dried to be safely placed away for being visualized under TEM. Viewing was with a JEOL 100CX transmission electron microscope, and images were captured on Kodak 4489 film. Film negatives were scanned with a backlit digital scanner to produce the final figures.

Additional Pertinent Materials

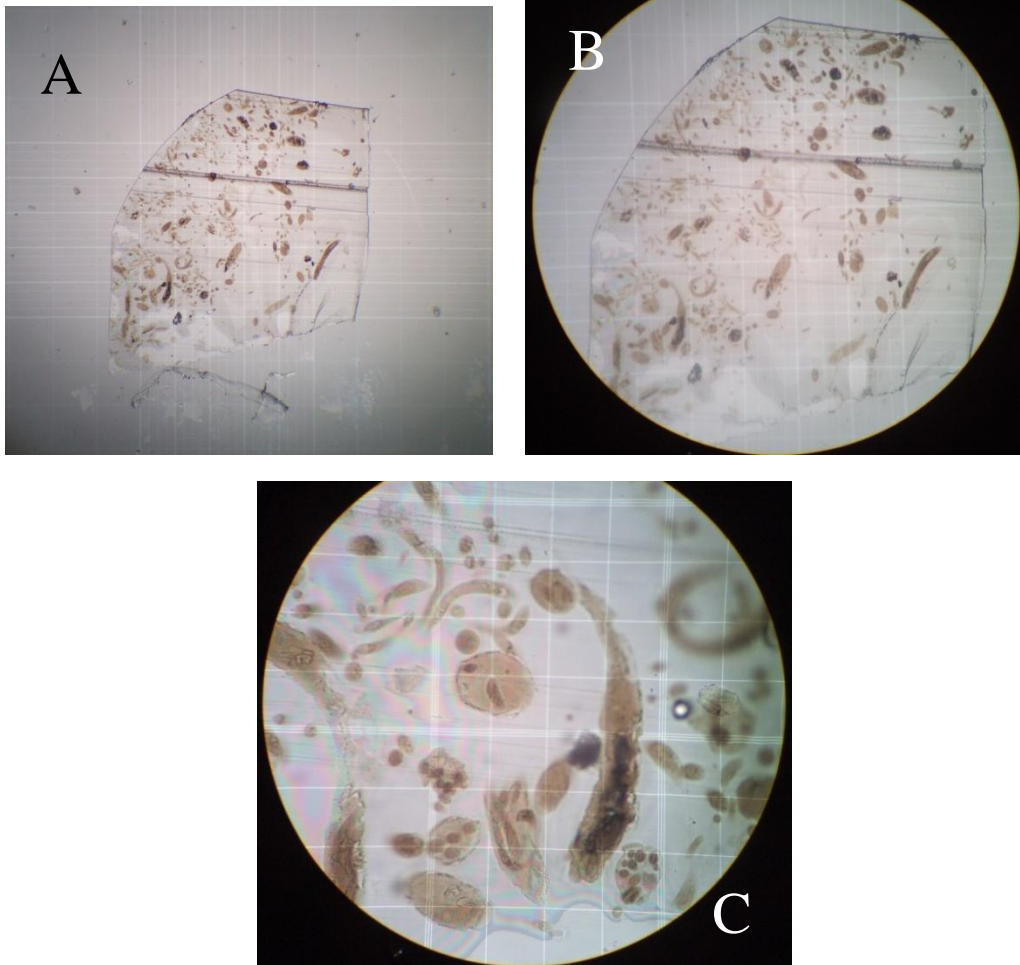
One major objective of this project was to be familiar with *C. elegans* and its developmental process and associated major stages. It was necessary to have extensive knowledge on each of *C. elegans*' larval stages, and in turn, have an idea of the dimensions of the roundworms at these stages so it would be easy to determine approximately at what points in roundworm development ependymin would be present. One technique that proved useful was the use of a hemocytometer to visualize small samples or thin sections under a light microscope. Such a small rectangular device was available in the laboratory, and it was set up so that a large grid and several small grids were seen under mainly 100X and 400X magnifications (See Figure 9).

Figure 9: Photograph of hemocytometer device under light microscope



Grids were seen as perfect square shapes, the smallest being 50 μm x 50 μm , and larger ones being 200 μm x 200 μm . A section of roundworms present at all different stages and cut at different planes was made by the microtome and positioned on the hemocytometer to be observed under 100X and 400X magnifications (See Figure 10). At the same time, approximate visual notes were made on sizes of worms at their developmental stages.

Figure 10: Cut section of *C. elegans* set up on hemocytometer device and viewed at (A) 100X magnification, (B) closer shot at 100X magnification, and at (C) 400x magnification



Aside from this, information was looked up to note the literature values of approximate length and diameter dimensions of *C. elegans* at their larval stages. These values were then incorporated onto photographs taken of the *C. elegans* sections at 100X and 400X magnifications, to have clear sense as to approximately what stages specific worms with significant anti-EPN immunoreactivity would be at in experimental sections.

Results

Figures 11 and 12 below show transverse slices of early-stage *C. elegans* embryos developing inside an adult hermaphrodite worm, once embedded in Epon-Araldite, and stained with Pierce ABC Peroxidase Kit for HRP. Anti-ependymin immunoreactivity is observed as distinct dark brown spots present around the inside circumference of the embryos. This suggests ependymin is present to strongly serve as a nerve growth factor in the embryos as they develop.

Figure 11: Photograph of Epon-A Experimental *in utero* *C. elegans* embryos section, treated by HRP reaction mechanism

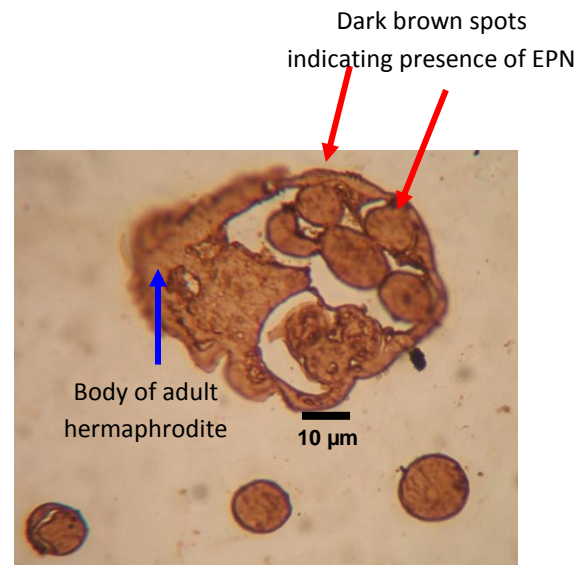
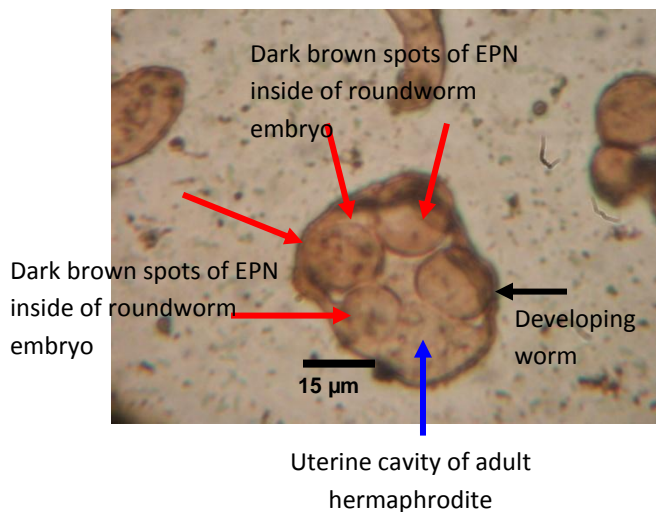
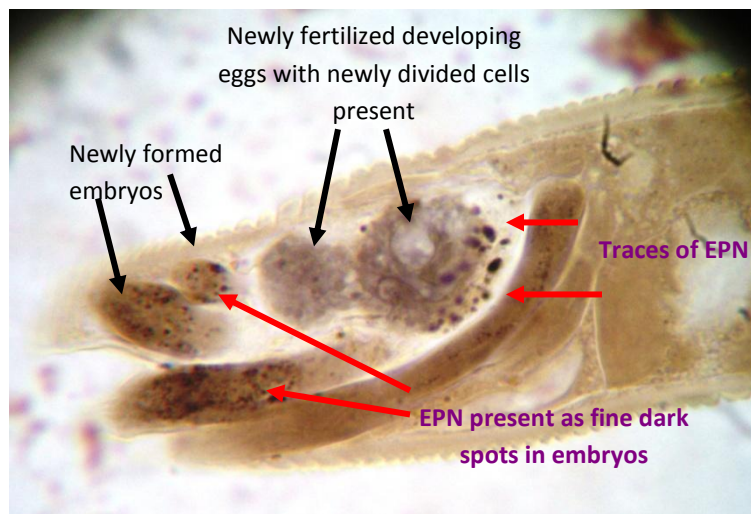


Figure 12: Second view of Epon-Araldite *C. elegans* developing embryos, treated by HRP mechanism

Figure 13 below shows an experimental section of the uterine portion of a fully grown hermaphrodite worm, stained using the Pierce ABC Peroxidase Kit. The distinct

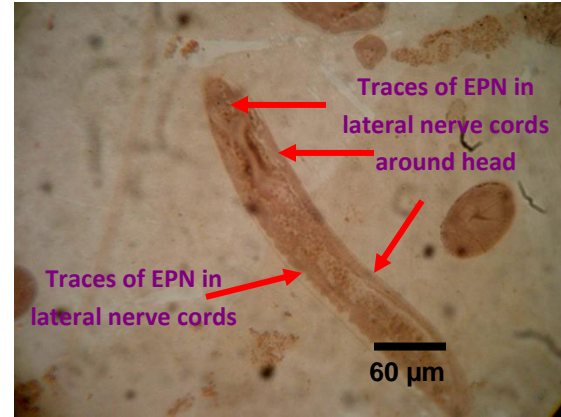
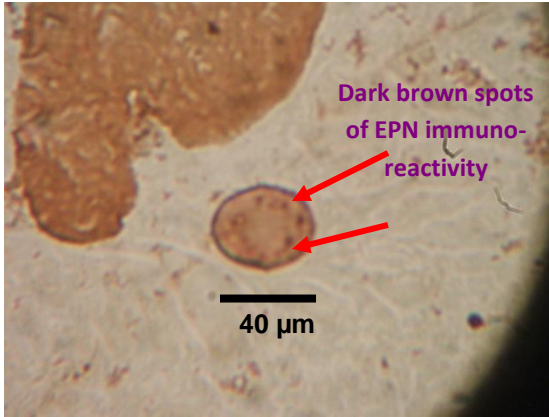
dark brown spots indicate strong ependymin immunoreactivity in fertilized eggs developing into embryos, as well as early-stage embryos undergoing proliferation inside their hermaphrodite mother. This section was present in an HRP experimental slide containing sections once embedded in Epon-Araldite resin.

Figure 13: Photograph of experimental developing eggs and embryos, treated by HRP reaction mechanism



Figures 14 and 15 show experimental Epon-Araldite-associated sections of L1-stage *C. elegans* undergone the same staining treatment as the above sections. Here, ependymin immunoreactivity is seen present directly in spaces where major nerve cords are structured in a typical developing worm.

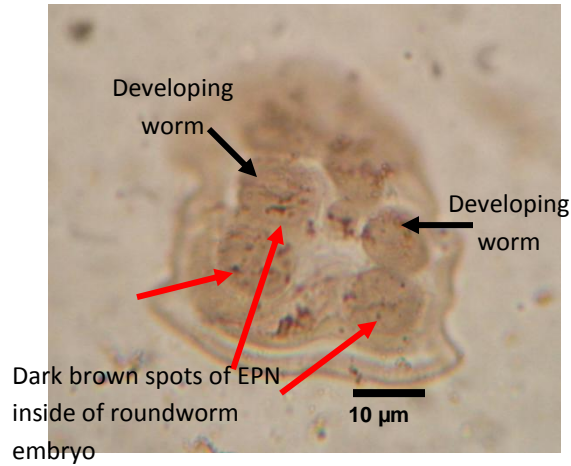
**Figure 14: Photograph of Epon-A
Experimental transverse section of
L1 larval stage *C. elegans***



**Figure 15: Photograph of Epon-A
Experimental longitudinal L1
larval stage *C. elegans***

Sections of worms that were embedded in L.R. White resin showed similar results. Figure 16 on the next page illustrates an experimental section of *C. elegans* embryos developing inside an adult worm, with ependymin immunoreactivity visible in areas where nerves are expected to proliferate.

Figure 16: Photograph of L.R. White Experimental *in utero* *C. elegans* embryos section, treated by HRP reaction mechanism.



Figures 17 and 18 show experimental L.R. White sections of L1-stage *C. elegans* undergone HRP staining for light microscopy as the above sections. Here, it is evident that ependymin immunoreactivity appears much clearer, due to the higher antigenicity property of L.R. White resin.

Figure 17: L.R. White Experimental transverse section of L1 larval stage *C. elegans*

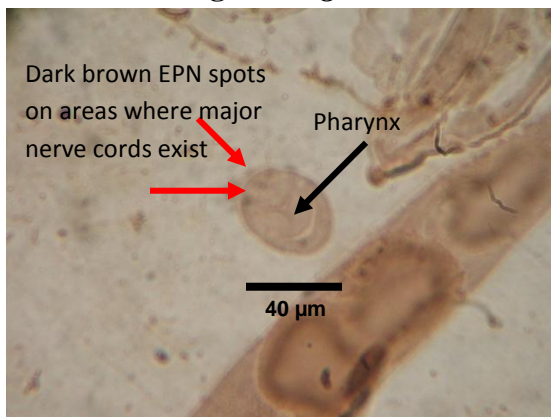


Figure 18: L.R. White Experimental longitudinal section of L1 larval stage *C. elegans*

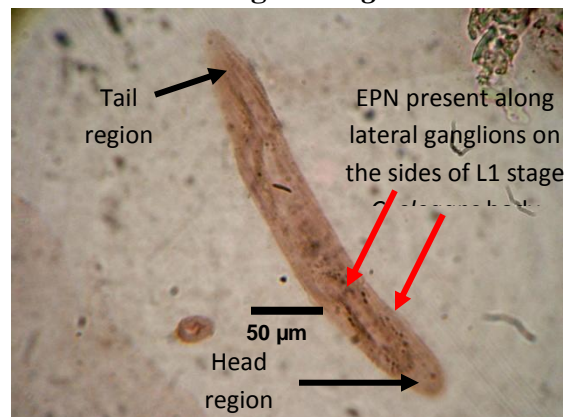


Figure 19 below shows the immunoreactivity of ependymin in an Epon-Araldite experimental section of *C. elegans in utero* embryos stained with the AlexaFluor 488[®] kit, as compared to control section of the exact same worms. To note, for both HRP reaction stained sections, and sections treated with fluorescing secondary antibody, controls were visualized to note only background staining present on worms. This indicated no presence of anti-EPN immunoreactivity in control worms as compared to what was observed in experimental sections. From Figure 19, it is evident that bright green fluorescence in forming embryos and nerve cords in embryos of the experimental section indicate areas of ependymin immunoreactivity.

Figure 19: 1:100 AlexaFluor 488[®] experimental (A) and control (B) roundworm embryos in hermaphrodite sections from Epon-Araldite sections

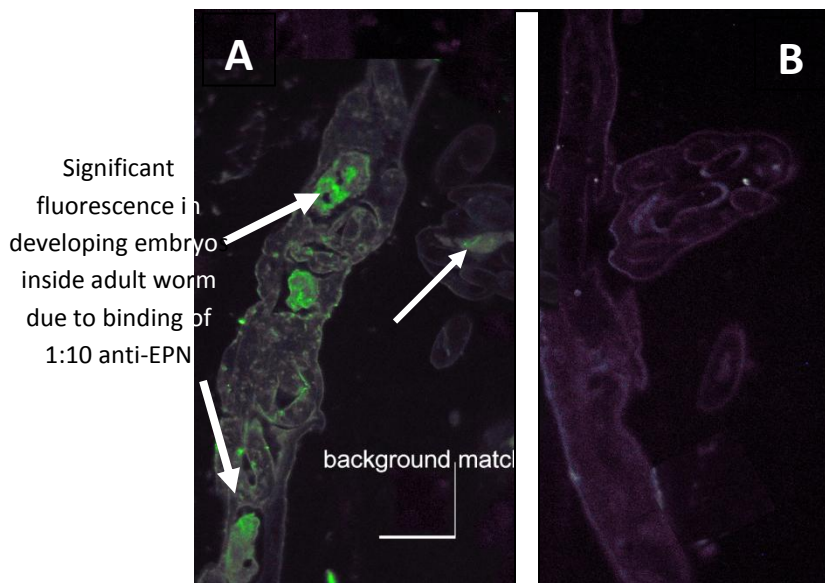
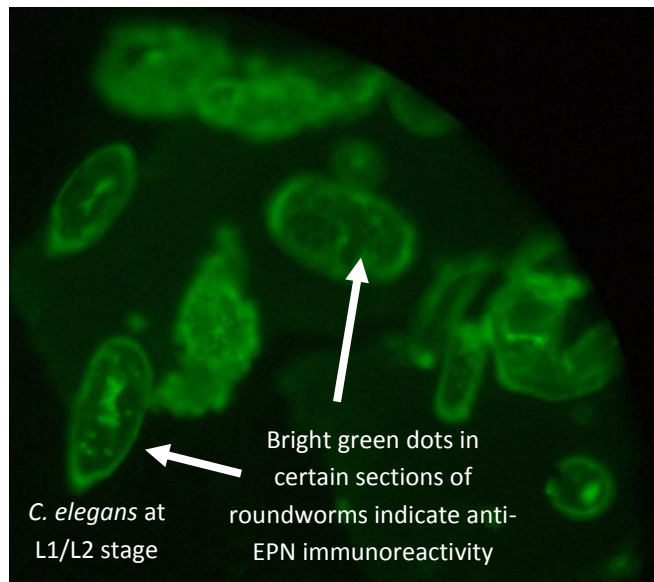


Figure 20 below illustrates ependymin immunoreactivity in areas of nerve growth in experimental sections of mainly L1-L2 larval stage *C. elegans*. This section, as the previous (Figure 19 above) was embedded in Epon-Araldite resin.

Figure 20: 1:100 AlexaFluor 488[®] experimental Epon-Araldite section with transverse sections of roundworms at different stages



Sections of worms that were embedded in L.R. White resin and treated with AlexaFluor 488[®] secondary antibody showed similar results. Figure 21 on the next page below illustrates an experimental section of *C. elegans* embryos developing inside an adult worm, with bright green fluorescence indicating ependymin immunoreactivity. Anti-EPN fluorescence was observed in the head region of full-bodied embryos developing to L1 larval stage, as well as parts of the bodies of L1 larval stage roundworms. Bright green fluorescence was also observed as spots in localized areas where major nerve cords exist, in L2 larval stage worms, as shown in Figure 22 below.

Figure 21: Photograph of 1:100 AlexaFluor 488[®] experimental L.R. White section of adult hermaphrodite with developing embryos

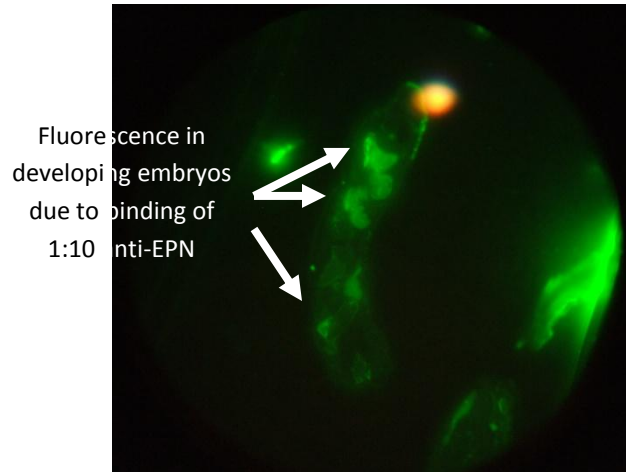


Figure 22: Photograph of 1:100 AlexaFluor 488[®] experimental L.R. White section of worms with fluorescing neurons

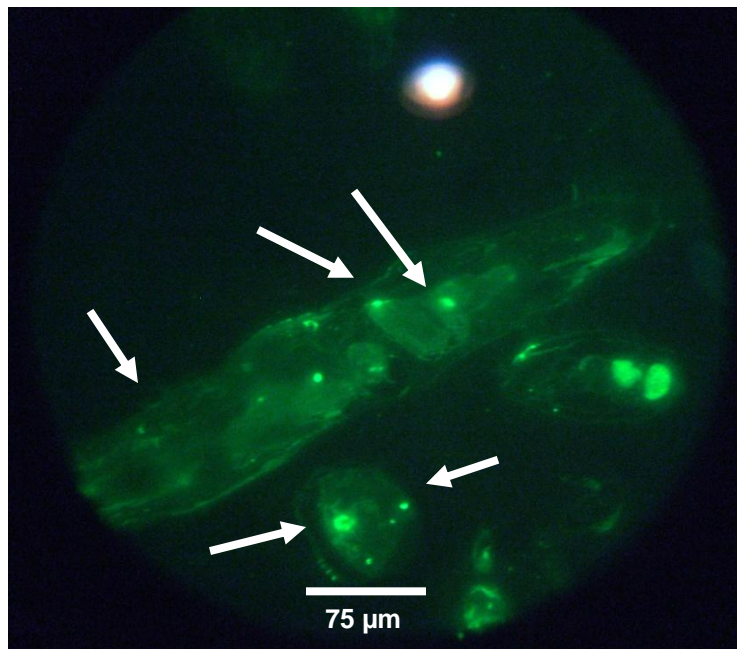


Figure 23 shows a transmission electron microscopy photograph of inner body cavity tissue of a *C. elegans* worm at L2/L3 larval stage from a gold section of worms once embedded in L.R. White resin. The section has been stained with 1:10 dilution of anti-KKETLQFR-EPN as the primary antibody, followed by 1:10 dilution of 25-nm gold balls conjugated to goat anti-rabbit serum as the secondary antibody. As is indicated by the red arrows, 25 nm black balls are seen binding between axons in muscle tissue, indicating presence of ependymin immunoreactivity here. Although immunoreactivity is sparse, it is present in expected locations.

Figure 23: Close-up of L2/L3 larval stage *C. elegans* depicting 1:10 mild anti-EPN detection at 27*1000 magnification

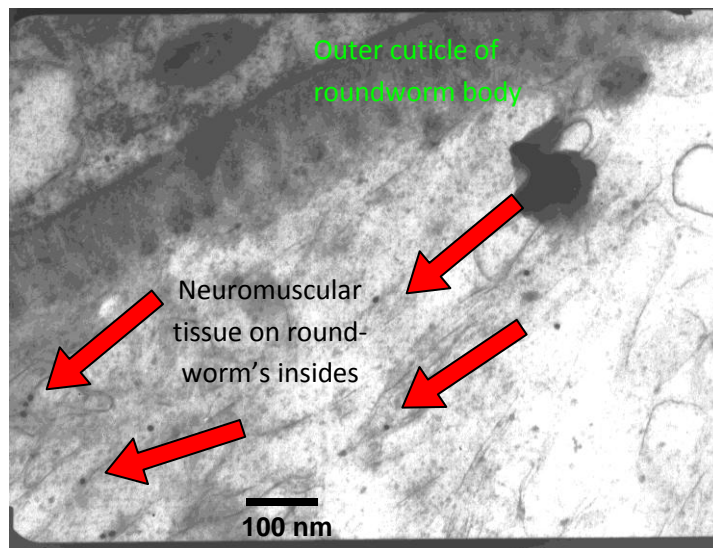
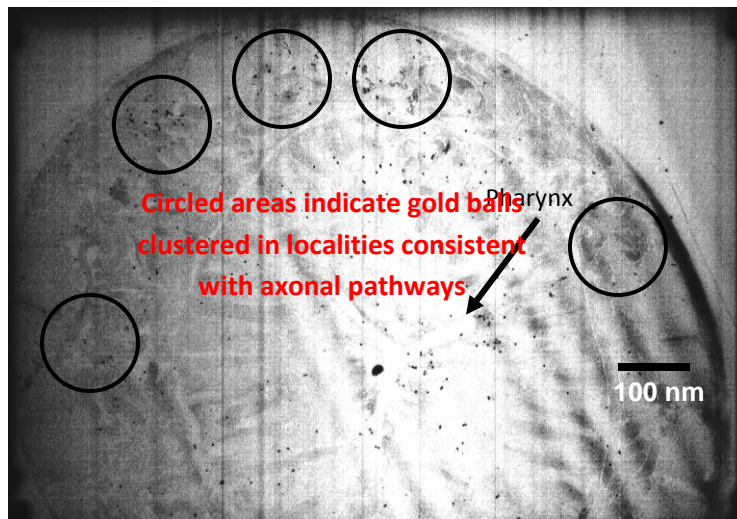


Figure 24 shows a transmission electron micrograph of a transverse section of a roundworm at L1 larval stage from a gold section of worms once embedded in Epon-Araldite resin. The section has been stained with 1:10 dilution of anti-KKETLQFR-EPN as the primary antibody, followed by 1:100 dilution of 25-nm gold balls conjugated to goat anti-rabbit serum as the secondary antibody. It is evident from this that EPN is present as a neurotrophic growth factor in major axons in young worms, especially in the L1 larval stage.

Figure 24: Transverse section of L1-stage *C. elegans* treated with 1:100 secondary antibody, at 8*1000 magnification



In addition to our findings of gold balls indicating ependymin immunoreactivity in roundworm eggs and up until the L3 stage, we happened to discover signs of immunoreactivity in *E. coli* bacteria that was present in the sample because it is used as food for the growing worms. Figure 25 on the next page shows a photograph of *E. coli* single

bacteria cut at various sectional planes with 25 nm gold balls bound to their interiors. This was a bizarre finding that made us question whether the KKETLQFR ependymin epitope, could be expressed by the genome of this microbe. Therefore, an online NCBI protein blast was conducted to determine if this is so. Figure 26 on the bottom depicts a graphic of the results of the blast.

Figure 25: Transmission electron micrograph of anti-EPN reaction oddly presented on single *E. coli* bacterium

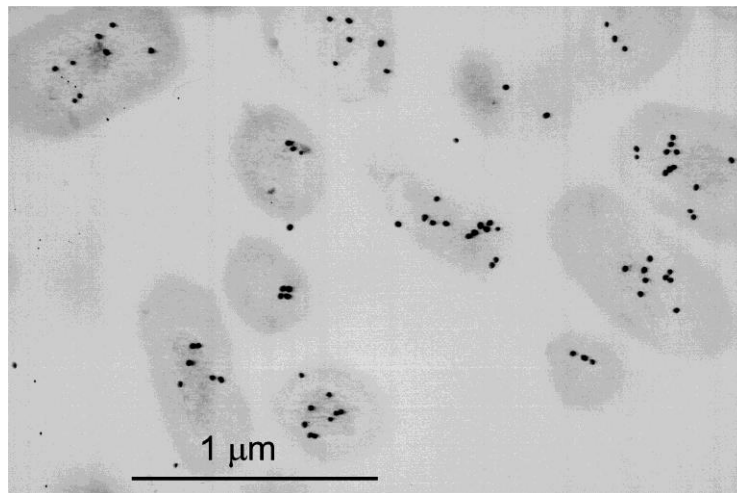


Figure 26: Results of NCBI Blast carried out to determine ependymin's presence in a strain of *E. coli*


```
> \[M\]ref|AP\_004864.1| hypothetical protein [Escherichia coli str. K-12 substr. W3110]
ref|YP\_001733082.1| \[G\] hypothetical protein ECDH10B_4534 [Escherichia coli str. K-12
substr. DH10B]
Length=59

Score = 16.9 bits (32), Expect = 288, Method: Composition-based stats.
Identities = 6/7 (85%), Positives = 6/7 (85%), Gaps = 0/7 (0%)

Query 2  KETLQFR 8
      KETL FR
Sbjct 3  KETLMFR 9
```

As anti-KKETLQFR antibody was the main antibody used to determine ependymin expression during any of the development stages of *C. elegans*, it was questionable if this 8-amino acid was really conserved in the organism's genome. This, a protein blast similar to the one conducted on *E. coli* was performed online to indicated whether or not the entire peptide sequence, or a huge part of it, is present as part of *C. elegans'* genome. Figure 26 below summarizes the results of the blast.

Figure 27: Results of NCBI Blast carried out to determine ependymin's presence in the genome of *C. elegans*

```
>ref|NP_872211.1|  Seven TM Receptor family member (str-12) [Caenorhabditis elegans]
Length=346

GENE ID: 353481 str-12 | Seven TM Receptor [Caenorhabditis elegans]
(10 or fewer PubMed links)

Score = 17.3 bits (33), Expect = 511
Identities = 6/7 (85%), Positives = 7/7 (100%), Gaps = 0/7 (0%)

Query 2      KETLQFR 8
      KET+QFR
Sbjct 327    KETIQFR 333
```

Discussion and Conclusions

The purpose of this project was to determine if ependymin is present in *C. elegans* during any of its developmental stages, mainly in the nervous system in major nerve cords, and if possible, in the nerve ring. This was done through immunostaining with the newly commissioned antibody anti-KKETLQFR and improved laboratory techniques, and three different types of secondary antibody staining: the Pierce ABC Peroxidase staining kit for light microscopy, AlexaFluor 488[®] staining kit for fluorescence microscopy, and 25 nm Immunogold for transmission electron microscopy. These stains were used on sections of *C. elegans* embedded in either Epon-Araldite or L.R. White resins and present in all developmental stages at all different kinds of orientations and angles at the same time.

Ependymin is Present as a Strong Neurotrophic Growth Factor in *C. elegans* Embryos

Figures 11, 12, 13, 16, 19, and 21 show clear staining of ependymin immunoreactivity in concentrated regions of *in utero* *C. elegans* embryos treated with material from either the Pierce ABC Peroxidase kit or the Alexa Fluor 488[®] kit. This is evident that ependymin is expressed and therefore present in *C. elegans* embryos when they merely develop for 150 minutes in their mother and continue to develop when laid outside the worm.

Ependymin is Present as a Neurotrophic Factor in the Nervous System in L1/L2 *C. elegans* Larval Stages

Figures 14, 15, 17, 18, 20, and 22 depict ependymin staining, especially in the form of distinct dots in patterns, in areas where major nerve cords/axons are located in the roundworm, when the worm is continuing development in the L1 and L2 larval stages. It is noticeable from these photographs that ependymin glycoprotein must exist amidst the proliferating and maturing neurons and synapses as the worm grows during these times during its development. These results do support the past discovery that ependymin is present in *C. elegans* during its L2 larval stage, as well as it is involved in synaptic changes that occur during nerve growth.

It is noticeable from Figure 23 that when *C. elegans* approaches development between L2 and L3 stages, the expression and actions of ependymin gradually diminish, as it is evident from the sparsity of the gold balls in axons in body tissue. Referring especially to the adult hermaphrodite seen on the left hand side of Figure 22, it can be noted that very minimal amounts of ependymin is present when the roundworm undergoes the third and fourth larval molts and completes maturation until past adult stage. We infer from this that ependymin is functionally expressed in *C. elegans* in the L1 and L2 larval stages as well, and its expression tapers off when the worm approaches completion of its development.

Further Notes on Ependymin Expression in *C. elegans*

It is evident that from the above conclusions that ependymin is expressed and functional mainly as a nerve growth factor during the first few larval stages of its overall development cycle. When the embryos grow into the L1 stage and follow through the L2 stage and beyond, the glycoprotein's function is probably to concentrate along the pathways that the nerves proliferate and grow to become nerve cords. Referring back to Figure 26, one can note that nearly the entire 8-a.a. peptide sequence is conserved in the roundworm's genome, with at least one difference in an amino acid. It is also depicted in the blast results that the protein is characterized in the roundworm as being a seven trans-membrane family member, implying that it may not be free in extracellular fluid but has partially extracellular character, serving in cell communication and the process of synaptic growth and change during neural development.

Referring to Figures 24 and 25 above, one might find it bizarre to see that ependymin is expressed in simple *E. coli* bacteria at some point during their proliferation. *C. elegans* growing in Petri dish colonies rely on specialized strains of *E. coli* bacteria as their food source. Some TEM photographs of *C. elegans* viewed under the transmission electron microscope have been taken to find that some anti-EPN reactivity is seen in the gastrointestinal area. This finding is less puzzling now that it has been revealed that the inner contents of the bacteria include the ependymin epitope, but a larger question remains: what might this conserved sequence be used for in the bacterium itself?

This project overall went beyond the work of Erica Tworog-Dube and successfully attempted to demonstrate the presence of ependymin as a neurotrophic growth factor in the first few developmental stages of *C. elegans*. It has concluded that a significant portion of the ependymin gene has been conserved in the *C. elegans* genome throughout the course of its evolution. Ependymin will likely be found in most any animal group where it is looked for. Perhaps a nervous system without ependymin is an impossibility!

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